Dysregulation of Angiopoietin-Tie-2 axis in Ugandan children hospitalized with pneumonia

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Abstract

Objective: Pneumonia is the leading cause of death in children under 5, with the highest burden in resource-limited countries. Endothelial activation occurs in pneumonia and can be assessed using quantitative levels of biomarkers angiopoietin (Ang)-1 and Ang-2. We examined admission levels of Ang-1 and Ang-2 in pediatric pneumonia and their association with disease severity and outcome.

Methods: Prospective cohort study of children with hypoxemic pneumonia admitted to two hospitals in Uganda. Clinical, radiographic, and microbiologic characteristics were measured at admission. Disease severity was assessed using the Respiratory Index of Severity in Children (RISC). Plasma levels of Ang-1 and Ang-2 were quantified by enzyme-linked immunosorbent assay. Vital signs, oxygen supplementation, and mortality were assessed prospectively.

Results: We included 65 patients (43% female) with median age 19 months (IQR 8-24). Admission Ang-2/Ang-1 ratio directly correlated with RISC (ρ=0.32, p=0.008) and lactate level (ρ = 0.48, p<0.001). Ang-2/Ang-1 ratio was higher in pneumococcal pneumonia than viral RTI (0.19 [IQR: 0.076-0.54] vs. 0.078 [IQR: 0.027-0.11]; p=0.03). Elevated Ang-2/Ang-1 ratio (>0.084) was associated with prolonged tachypnea (HR 0.50 (95%CI 0.29-0.87), p=0.02), fever (HR 0.56 (95%CI 0.33 to 0.96), p=0.02), longer duration of oxygen therapy (HR 0.59 (95%CI

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Abbreviations: Ang-1: Angiopoietin-1, Ang-2: Angiopoietin-2, CRP: C-reactive protein, RISC: Respiratory Index of Severity in Children, RTI: respiratory tract infection, sTie-2: soluble tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2

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0.35-0.99), p=0.04), and hospital stay (HR 0.43 (95%CI 0.25-0.74), p=0.001). The Ang-2/Ang-1 ratio at admission was higher in fatal cases relative to survivors (0.36 [IQR: 0.17-0.58] vs. 0.077 [IQR: 0.025-0.19]; p= 0.05)

Conclusion: Endothelial activation in hypoxemic pediatric pneumonia, reflected by high plasma Ang-2/Ang-1 ratio, is associated with disease severity, prolonged recovery time, and mortality.

Keywords: Ang-1; Ang-2; Hypoxemic Pneumonia; Biomarker; Endothelial activation

1. Introduction

Globally, pneumonia is the leading cause of death among children under five years of age. In 2015, there were an estimated 138 million clinical cases of childhood pneumonia, and an estimated 921,000 deaths.[1] The burden of childhood pneumonia is highest in resource-limited countries of South Asia and sub-Saharan Africa.

Pneumonia, like other systemic inflammatory processes, is associated with endothelial activation. Widespread endothelial activation with capillary leak leads to multi-system organ failure in severe sepsis[2,3] and malaria.[4] Endothelial activation in the pulmonary vasculature may account, at least in part, for alveolar fluid accumulation, radiographic consolidation, impaired gas exchange, and hypoxemia in pediatric pneumonia.[5-7] Following activation by pro-inflammatory stimuli, soluble endothelial cell receptors are released into circulation via ectodomain shedding or alternative splicing, such that peripheral blood levels of key regulatory proteins reflect the state of whole-body endothelial activation.[8,9]

Angiopoietins represent a distinct family of vascular growth factors that are increasingly recognized for their role in infectious disease pathogenesis.[10-13] Four molecules have been characterized, Ang-1, Ang-2, Ang-3, and Ang-4, of which Ang-1 and Ang-2 signal through the tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (Tie-2), on endothelial cells. Ang-1 is secreted from pericytes within the vascular intima, whereas Ang-2 is stored, along with von Willebrand factor, in Wiebel-Palade bodies.[14,15] Ang-1 and Ang-2 act antagonistically: Ang-1 promotes stability of the vascular endothelium and Ang-2 promotes permeability of the blood-tissue barrier.[16,17] By inhibiting vascular endothelial growth factor-induced internalization of adherens junction protein VE-cadherin, Ang-1 prevents paracellular

leakage across the endothelium.[43] Conversely, Ang-2 antagonizes Ang-1 at the Tie-2 receptor. Independent of Tie-2, Ang-2 causes endothelial destabilization by promoting redistribution of β1-integrin, a regulator of cell adhesion, away from cell-cell junctions.[17] Ang-2 also functions as an autocrine regulator by sensitizing the endothelium to tumor necrosis factor, resulting in increased adhesion receptor expression for tethering and diapedesis of leukocytes across the endothelium.[18] Like other endothelial surface molecules, the Tie-2 receptor is released into circulation in severe illness. Our group has previously described the levels of peripheral blood angiopoietins and soluble Tie-2 (sTie-2) for prognosis in sepsis[3], malaria[8,10,19,20] and chronic HIV.[21] The Ang-Tie pathway has also been implicated in acute lung injury, which is associated with pulmonary vascular leak.[22] In vitro, Ang-2 increases permeability of lung endothelial monolayers whereas Ang-1 reduces permeability.[23] In a murine model of pneumococcal pneumonia, lung tissue expression of Ang-2 is increased and Ang-1 is decreased.[23] Ang-2 knockdown results in reduced permeability on pneumolysin stimulation.[23] In adult patients with pneumonia, decreased serum Ang-1 and increased Ang-2 levels were observed as compared with healthy subjects.[23] Ang-1 and Ang-2 levels have not previously been reported in pediatric pneumonia, to our knowledge.

To examine whether endothelial activation plays a pathogenic role in clinically-defined hypoxemic pediatric pneumonia in low-income settings, we investigated endothelial biomarkers in children hospitalized in Uganda. We hypothesized that circulating Ang-1, Ang-2 and sTie-2 levels, would be associated with clinical severity, prolonged recovery times, and mortality in hypoxemic pediatric pneumonia.

2. Material & Methods

2.1. Study design

Prospective cohort study, examining the relationship between endothelial biomarkers, measured at hospital admission, and clinical outcomes.

This study was reviewed and approved by the Makerere University School of Biomedical Sciences Research Ethics Committee (REC Protocol # SBS 139), the Uganda National Council on Science and Technology (Ref SS 3331), and the University Health Network Research Ethics Committee, Toronto, Canada (UHN REB Number 13-6168-AE). All participating children had a parent or caregiver that provided written informed consent.

2.2. Study setting and population

Uganda has an under-five mortality rate of 49/1000 live births.[24] Pneumonia is one of the leading causes of mortality in children under five years, accounting for 15% of total deaths.[25] This study was conducted at two inpatient pediatric facilities: Jinja Regional Referral Hospital in Eastern Uganda and Kambuga District Hospital in Western Uganda. Inclusion criteria were: (1) age<13 years; (2) admitted to hospital; and (3) peripheral oxygen saturation (SpO_2) <90%. The sole exclusion criterion was clinical suspicion of tuberculosis.

2.3.Study procedures

Information was collected on the child's demographics, presenting signs and symptoms, physical examination findings, laboratory investigations, treatments during hospitalization, and outcome (survival or death). Chest radiograph was performed within 24 hours of admission and was

interpreted by a Canadian board-certified radiologist, using standardized WHO-endorsed definition of primary endpoint pneumonia.[26] Vital signs were recorded every four hours during hospitalization. Tachypnea and tachycardia were defined as respiratory rate (RR) and heart rate (HR) above the 99th percentile for age, respectively, based on WHO norms.[27] Time of resolution of tachypnea, tachycardia, and fever was defined as the last documented time that the vital sign was elevated, followed by normal measurements for at least 24 hours. Weaning oxygen followed a standardized objective procedure. A validated composite clinical severity score, Respiratory Index of Severity in Children (RISC) was computed as previously described.[28] Clinicians recording vital signs, making treatment decisions, and weaning oxygen were blind to admission biomarker levels (performed in batch, off site, at the end of the study).

2.4.Measurement of Ang-1, Ang-2, sTie-2, CRP, and lactate

Venipuncture blood was collected in EDTA coated tubes, centrifuged on site, and plasma was isolated and stored at -80°C until shipment to Canada. Enzyme-linked immunosorbent assays (ELISAs) for Ang-1, Ang-2, sTie-2, and CRP (R&D Systems, Minneapolis, MN) were performed according to the manufacturer's instructions, blinded to clinical data. Background signal was determined from blank wells on each plate and subtracted from all samples and standards prior to analysis. A 4-parameter logistic regression curve fitted to data was used to determine biomarker concentrations from the ELISA optical density. Lactate was measured from fresh venous blood using a point-of-care device, the Lactate Scout Analyzer (Sports Resource Group, Inc., Minneapolis, MN).

2.5.Pathogen detection

A nasopharyngeal (NP) swab was collected and stored at -80°C prior to shipment on dry ice for analysis. Nucleic acid extraction was performed using KingFisher™ mL Purification System (Thermo Fisher Scientific Inc, Waltham, MA) and the MagaZorb® Total RNA Mini-Prep Kit (Promega, Madison, WI). Quantitative real-time PCR (qPCR) with FTDResp33 (Fast-Track Diagnostics, Esch-sur-Alzet, Luxembourg) and the 7500 Real-Time PCR System (Applied Biosystems®, Foster City, CA) was used to identify common respiratory pathogens: *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, *Staphylococcus aureus*, rhinovirus, human adenovirus, human metapneumovirus, human bocavirus, and *Pneumocystis jiroveci.* To quantify the number of genome copies of *S. pneumoniae* present in each sample, we used manufacturer-published standard curves relating the rtPCR cycle threshold (Ct) to plasmid DNA concentrations ranging from 10^9 to 10^2 copies/ml, in a tenfold dilution series. For the purpose of this analysis, pneumococcal pneumonia was defined as detection of *S. pneumoniae* at a genomic load > $\log_{10}6.9$ in the nasopharynx plus primary endpoint pneumonia (alveolar consolidation) on the chest radiograph.[26,29] Viral respiratory tract infection (RTI) was defined as detection of rhinovirus, human adenovirus, human metapneumovirus, or human bocavirus nucleic acid in the nasopharynx in the absence of radiographic consolidation.

2.6.Statistical analysis

Data analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA, 2012), and R. To examine associations between variables; non-parametric methods (Mann–Whitney U test) were used for continuous data, and the two-tailed Pearson Chi-Square or Fisher's exact test were used for categorical data, as appropriate. Correlations between continuous variables were assessed using Spearman's rank correlation coefficient (ρ). Recovery

times (time to resolution of tachypnea, tachycardia, fever, time to wean oxygen, and time to discharge), stratified by admission Ang-2/Ang-1 ratio, were studied using Kaplan-Meier analysis. Cox proportional hazard models were fit to the data to estimate the hazard ratio (HR) and 95% confidence interval (CI).

3. Results

We enrolled 65 children admitted with hypoxemia from September 2013 to July 2015. Clinical characteristics of the cohort are shown in Table 1. The median age of patients was 19 months (interquartile range [IQR]: 8-24) and 28 (43%) were female. General danger signs[30] were present in 38 (58%) and 40 (62%) were classified as severe pneumonia or very severe disease.[30] All were hypoxemic at admission and 19 (29%) had lobar consolidation on chest radiograph. 31 patients (48%) had *S. pneumoniae* detected from nasopharyngeal swab. 62 (95%) were treated with ceftriaxone. Overall, the in-hospital mortality was 4/65 (6.2%). Levels of Ang-1, Ang-2, and sTie-2 are shown in Table 2. Biomarker levels were similar in patients with clinical signs and symptoms associated with dehydration, including vomiting, diarrhea, inability to feed/drink, and requirement for intravenous fluid therapy ($p>0.05$ for all comparisons).

Table 1. Patient Characteristics, stratified by admission Ang-2/Ang-1 ratio

	Entire Cohort (N=65)	**Ang-2/Ang-1 >0.084 (N=32)**	**Ang-2/Ang-1 ≤0.084 (N=33)**	**P-value**
Demographics				
Female sex	28 (43)	16 (50)	12 (36)	0.39
Age (months), median (IQR)	19 (8-24)	12 (3.7-19)	11 (5.0-25)	0.52
History				
Cough	57 (88)	27 (84)	30 (91)	0.48
Difficulty breathing	62 (95)	30 (94)	32 (97)	0.61
Lethargy	11 (17)	8 (25)	3 (9)	0.11
Convulsions	14 (22)	7 (22)	7 (21)	>0.99
Vomiting	19 (29)	10 (31)	9 (27)	0.94
Diarrhea	10 (15)	4 (12)	6 (19)	0.51
Unable to feed/drink	26 (40)	12 (38)	14 (42)	0.88
Physical examination findings				
Weight (kg), median (IQR) Underweight[1]	8 (6-11) 9 (14)	7 (5.45-10) 7 (22)	9 (7-12) 2 (6)	0.08 0.08
Temperature (°C), median (IQR) Fever[2]	37.4 (36.6 – 38.3) 30 (46)	37.5 (36.5-38.3) 15 (47)	37.4 (36.8-38.3) 15 (33)	0.47 >0.99
Blood pressure (mmHg)[3]				

Systolic, median (IQR)	100 (90-102.5)	100 (90-100)	100 (90-110)	0.95
Diastolic, median (IQR)	50 (50-56.25)	50 (50-50)	50 (50-60)	0.31
Heart Rate (bpm), median (IQR)	163 (144-175)	157 (144-172)	165 (146-176)	0.36
Tachycardia[4]	39 (60)	16 (50)	23 (70)	0.17
Respiratory rate (bpm), median (IQR)	66 (50-76)	69 (58-78)	60 (48-72)	0.02
Tachypnea[4]	53 (82)	29 (91)	24 (72)	0.11
Hypoxemia (SaO_2<90%)	65 (100)	32 (100)	33 (100)	>0.99
Altered level of consciousness[5]	13 (20)	9 (28)	4 (12)	0.12
Deep breathing	55 (85)	23 (72)	32 (97)	0.01
Nasal flaring	49 (75)	25 (78)	24 (72)	0.83
Intercostal retractions	50 (77)	25 (78)	25 (76)	>0.99
Subcostal retractions	46 (71)	24 (75)	22 (67)	0.64
Wheeze	13 (20)	3 (9)	10 (30)	0.06
Stridor	4 (6)	3 (9)	1 (3)	0.36
Crackles on auscultation	31 (48)	16 (50)	15 (45)	0.91
General danger sign[6]	38 (58)	18 (56)	20 (60)	0.92
Radiographic findings				
CXR[7]				0.64
Normal	21 (32)	10 (31)	11 (33)	
Lobar consolidation	19 (29)	11 (34)	8 (24)	
Other	23 (35)	10 (31)	13 (39)	
Laboratory findings				
Hypoglycemia[8]	2 (3)	2 (6)	0 (0)	0.24
Lactate (mmol/L), median (IQR)	2.7 (2.1-4.5)	3.4 (2.7-7.4)	2.2 (1.5-2.9)	<0.0001
<2.0	14 (22)	2 (6)	12 (38)	
2.0-4.0	30 (46)	15 (45)	15 (47)	
>4.0	17 (26)	14 (42)	3 (9)	
C-reactive protein (mg/L)	28 (5.0-57)	39 (5.5-63)	20 (5.0-49)	0.36
Pathogens detected, n (%)				
RSV	18 (28)	7 (22)	11 (33)	0.45
Adenovirus	4 (6)	0 (0)	4 (12)	0.11
Human metapneumovirus	4 (6)	1 (3)	3 (9)	0.61
Human bocavirus	7 (11)	2 (6)	5 (15)	0.43
Streptococcus pneumoniae[9]	31 (48)	14 (44)	17 (52)	0.71
Haemophilus influenzae type b	4 (6)	1 (3)	3 (9)	0.61
Staphylococcus aureus	11 (17)	6 (19)	5 (15)	0.96
Pneumocystis jiroveci	3 (5)	0 (0)	3 (9)	0.24
Malaria with respiratory distress[10]	6 (9)	4 (13)	2 (6)	0.43
HIV seropositive with pneumonia[11]	2 (3)	2 (6)	0 (0)	0.49
Diagnosis				
Cough or cold	1 (2)	0 (0)	1 (3)	>0.99
Pneumonia	24 (37)	13 (41)	11 (33)	0.72
Severe pneumonia or very severe disease	40 (62)	19 (59)	21 (64)	0.92
Treatment[12]				

Antibiotics[13]				
Ceftriaxone	62 (95)	30 (94)	32 (97)	0.61
Gentamicin	32 (50)	18 (56)	14 (44)	0.45
Ampicillin	10 (16)	5 (16)	5 (16)	>0.99
Azithromycin	9 (14)	8 (25)	1 (3)	0.01
Antipyretic	42 (66)	21 (66)	21 (66)	>0.99
Antimalarial	19 (30)	11 (34)	8 (25)	0.58
Salbutamol	16 (25)	7 (22)	9 (28)	0.77
Corticosteroid	14 (22)	4 (13)	10 (31)	0.13
Intravenous fluids	14 (22)	8 (25)	6 (19)	0.76

Data represent n (%) unless otherwise specified. IQR, Interquartile Range

[1]Weight-for-age below -3SD [58]

[2]Axillary temperature >37.5°C

[3]Blood pressure was recorded for 32/65 (49%) of patients.

[4]Vital sign >99 percentile for age [59]

[5]Any one of the following: fails to watch or follow with eyes, fails to localize to painful stimulus, fails to cry or verbalize appropriately with pain

[6]Presence of any general danger sign (vomiting, convulsions, unable to feed/drink, altered consciousness) [30]

[7]CXR was performed on 63/65 (97%) of patients.

[8]Random blood glucose < 3 mmol/L

[9]*Streptococcus pneumoniae* detected in the blood or at high genomic load (>6.9 $\log_{10}$ copies/mL) in the nasopharynx.

[10]Malaria diagnosed by positive rapid diagnostic test, and positive blood smear

[11]HIV testing was performed on 61/65 (94%) of patients.

[12]Patients may have received multiple treatments, such that percentages do not sum to 100%

[13]Additional antibiotics included penicillin (n=7), cloxacillin (n=7), amoxicillin (n=3), metronidazole (n=2), ciprofloxacin (n=2), co-trimoxazole (n=1), linezolid (n=1), and tetracycline (n=1).

Table 2: Biomarker levels at admission

Biomarker	Concentration (ng/mL)
Ang-1	18 (10-27)
Ang-2	1.5 (0.92-3.1)
Ang-2/Ang-1*	0.084 (0.028-0.26)
sTie2	46 (27-63)

Data represent median (IQR)

*Dimensionless ratio

3.1. Angiopoietins correlate with composite clinical severity score at hospital admission
The clinical severity was measured using the RISC score.[28] A significant correlation between Ang-2 levels (ρ=0.28; p=0.02) and RISC score was found (Fig 1). Correlation was strongest (ρ=0.32; p=0.008) between the Ang-2/Ang-1 ratio and RISC scores.

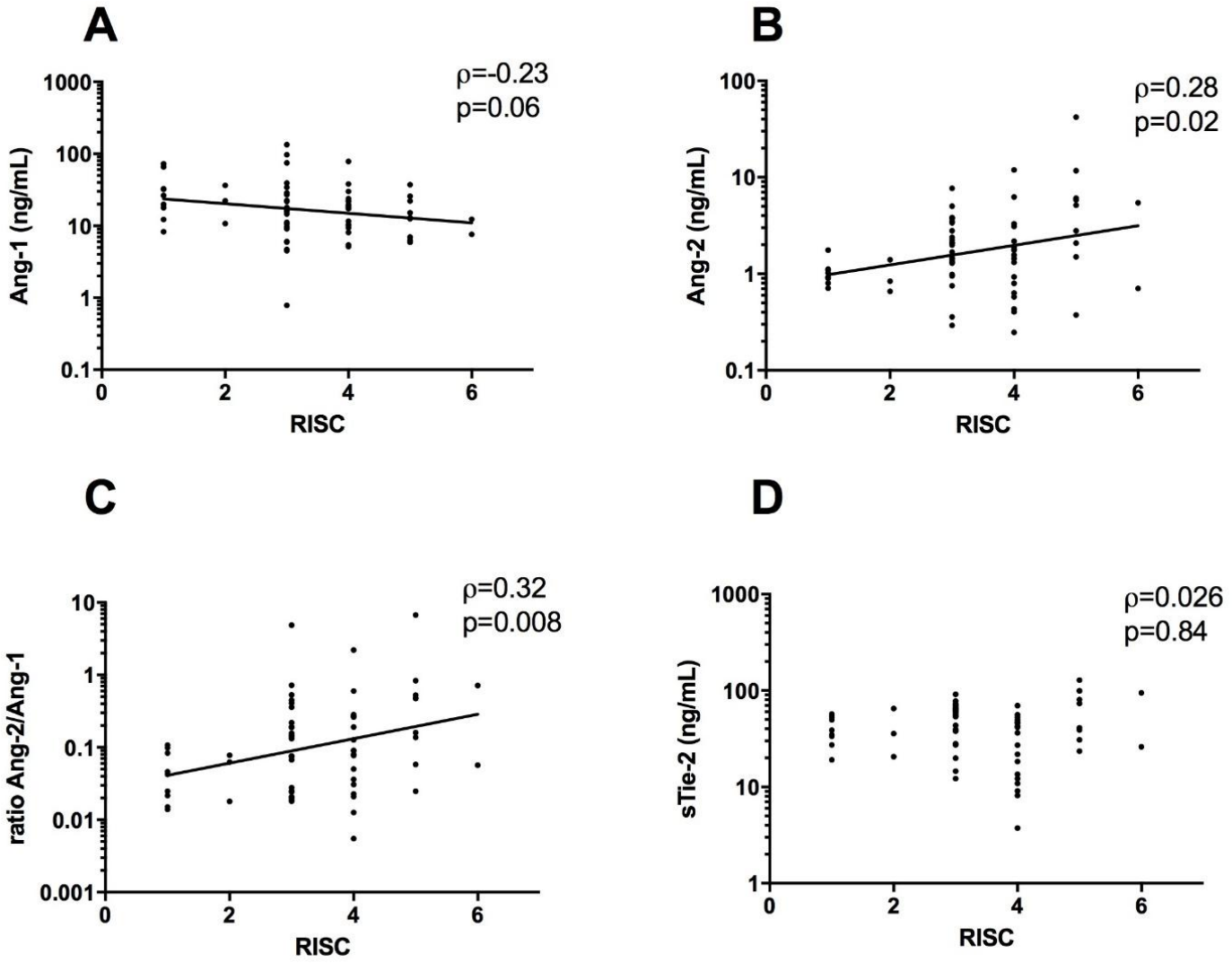

Figure 1. Correlation between Ang levels and RISC score. **A.** Ang-1 and RISC (ρ=-0.23; p=0.06). **B**. Positive correlation between Ang-2 and RISC (ρ=0.28; p=0.02). **C.** Positive correlation between Ang-2/Ang-1 ratio and RISC (ρ=0.32; p=0.008).

3.2. Angiopoietins are dysregulated in pneumococcal pneumonia compared to viral RTI

Pneumonia may be caused by a broad spectrum of etiological agents such as bacteria, viruses and fungi.[30] The median Ang-2 serum level in patients with pneumococcal pneumonia was significantly higher (2.80 ng/ml [IQR: 1.80-3.56] vs. 1.22 ng/ml [IQR: 0.91-1.76]; p= 0.01; Fig 2) compared to the viral RTI group. Similarly, the ratio of Ang-2/Ang-1 was significant between the two groups (p=0.03).

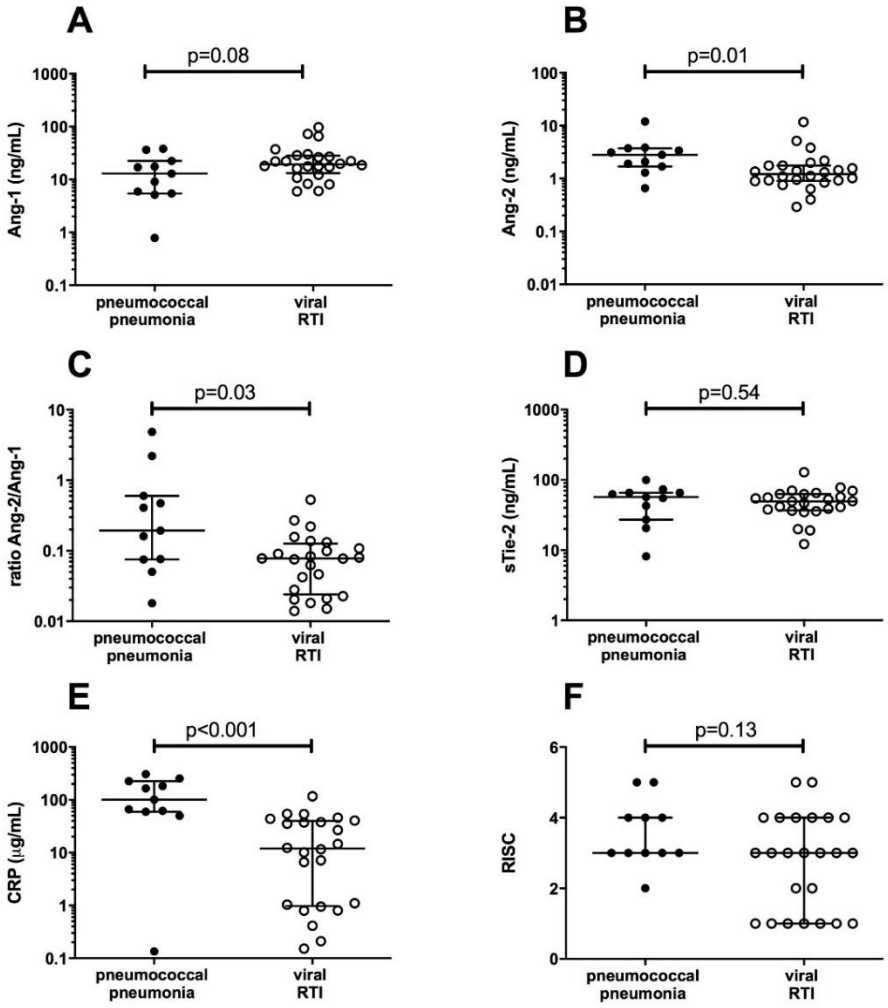

Figure 2. The concentration of Ang-1 (**A**), Ang-2 (**B**), Ang-2/Ang-1 (**C**), sTie-2 (**D**), CRP (**E**), and RISC (**F**) score were observed in cases of pneumococcal pneumonia and viral RTI. Ang-2 (p=0.013) and Ang-2/Ang-1 (p=0.033) were decreased in viral RTI pneumonia cases compared to cases of pneumococcal pneumonia. The horizontal line represents the median; upper and lower error bars represent the interquartile range.

3.3.Angiopoietins correlate with circulating lactate level

Lactic acidosis is a marker of tissue hypoxia and anaerobic cellular metabolism. Lactate levels are elevated in severe and fatal pneumonia.[31-36] We hypothesized that endothelial activation and dysregulated angiopoietins would be associated with elevated levels of lactate in our cohort. To test this hypothesis, we examined paired measurements of lactate and angiopoietins (Fig 3). We found statistically significant correlations between levels of lactate and levels of Ang-1 (ρ=-0.40; p=0.001), Ang-2 (ρ =0.39; p=0.002), and the Ang-2/Ang-1 ratio (ρ = 0.48; p<0.001). Circulating levels of CRP (ρ=-0.011; p=0.93) and RISC (ρ=0.11; p=0.41) were not correlated with lactate levels.

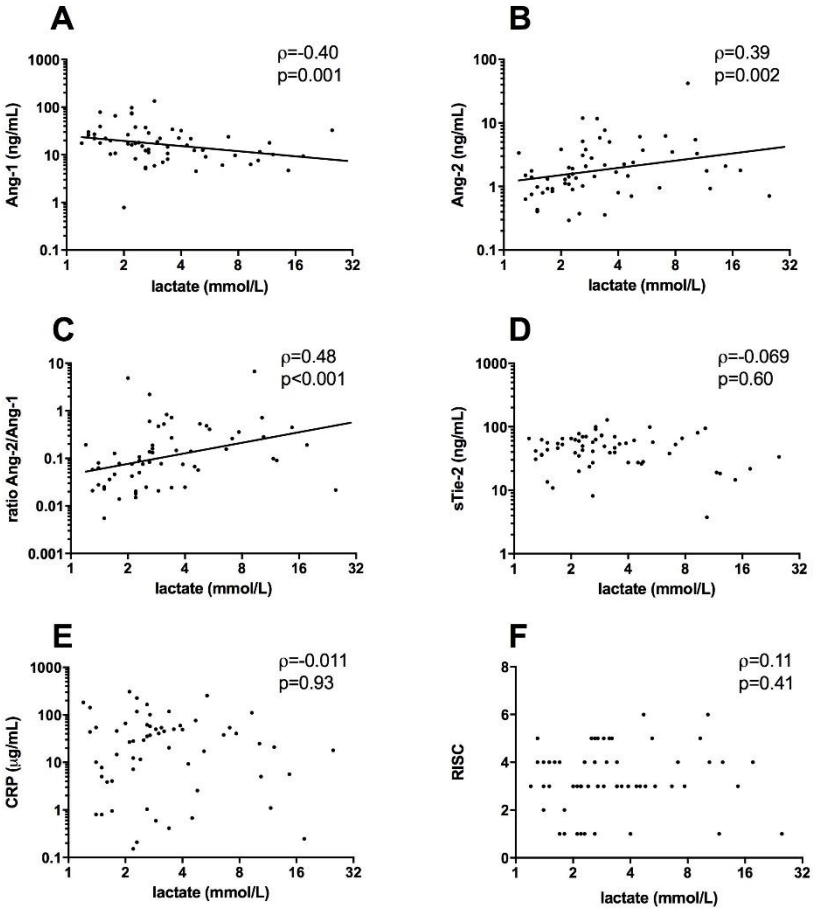

Figure 3. Correlation between Ang levels and venous lactate. **A.** Negative correlation between Ang-1 and lactate (ρ=-0.40; p=0.001). **B.** Positive correlation between Ang-2 and lactate (ρ=0.39; p=0.002). **C.** Positive correlation between the Ang-2/Ang-1 ratio and lactate (ρ=0.48; p<0.001), correlated directly with lactate levels.

3.4.Predictive value of endothelial biomarkers

Altered admission Ang levels were predictive of prolonged recovery times. Higher admission levels of Ang-2 (>1.5 ng/ml) were associated with prolonged tachypnea (HR 0.37 (95%CI 0.21-0.65); p=0.002), longer duration of oxygen therapy (HR 0.41 (95%CI 0.24-0.72); p=0.001), and hospital stay (HR 0.33 (95%CI 0.19-0.57), p <0.001) (Supplemental Table 1). Elevated ratios of Ang-2/Ang-1 (>0.084) were associated with prolonged tachypnea (HR 0.50 (95%CI 0.29-0.87); p=0.02), fever (HR 0.56 (95%CI 0.33 to 0.96); p=0.02), longer duration of oxygen therapy (HR 0.59 (95%CI 0.35-0.99); p=0.04), and hospital stay (HR 0.43 (95%CI 0.25-0.74); p=0.001; Fig 4). In contrast, higher composite clinical severity score (RISC) and elevated admission CRP levels were not significantly associated with any of these clinical endpoints (Supplementary Table 1). Details of prognostic biomarkers and multiple clinical outcomes are given in Supplemental Table 1, with the cohort dichotomized according to median admission biomarker values.

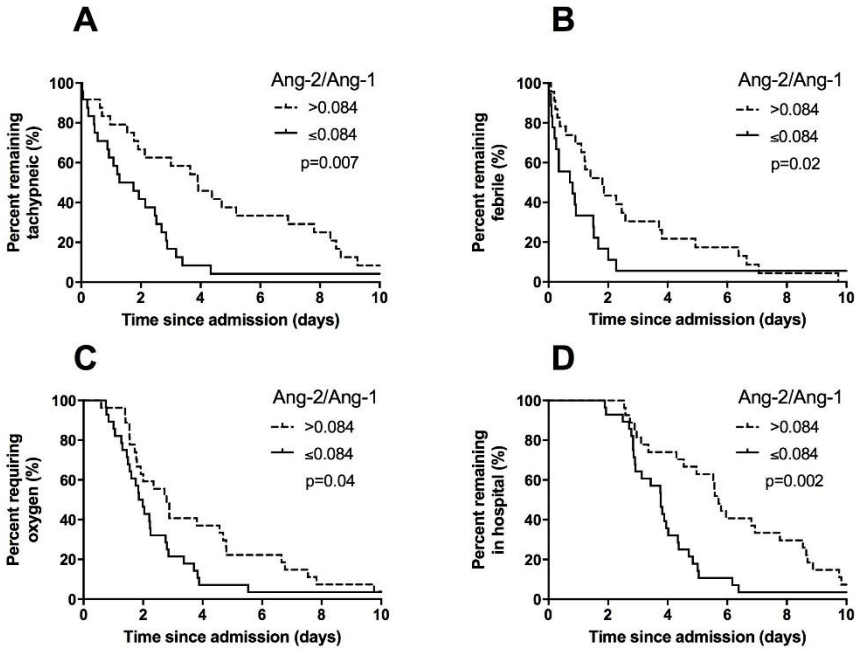

Figure 4. Time to resolution of tachypnea (**A**), and fever (**B**), duration of oxygen therapy (**C**), and the hospital length of stay (**D**) were prolonged in children with elevated Ang-2/Ang-1 ratio (>0.084) at admission (p<0.05 for all comparisons).

3.5.Angiopoietins are dysregulated in fatal hypoxemic pneumonia

We compared the concentrations of Ang-1, Ang-2, Ang-2/Ang-1 ratio and sTie-2 in cases of fatal and non-fatal hypoxemic pneumonia. Ang-1 concentrations were significantly lower in cases of fatal pneumonia (8.5 ng/ml [IQR: 6.9-9.6] vs. 18 ng/ml [IQR: 11-27]; p= 0.009; Fig 5). In contrast, the Ang-2/Ang-1 ratio was higher in cases of fatal pneumonia compared to the non-fatal group (0.36 [IQR: 0.17-0.58] vs. 0.077 [IQR: 0.025-0.19]; p= 0.05; Fig 5). There was no significant difference between the groups when comparing RISC score or levels of CRP (Fig 5).

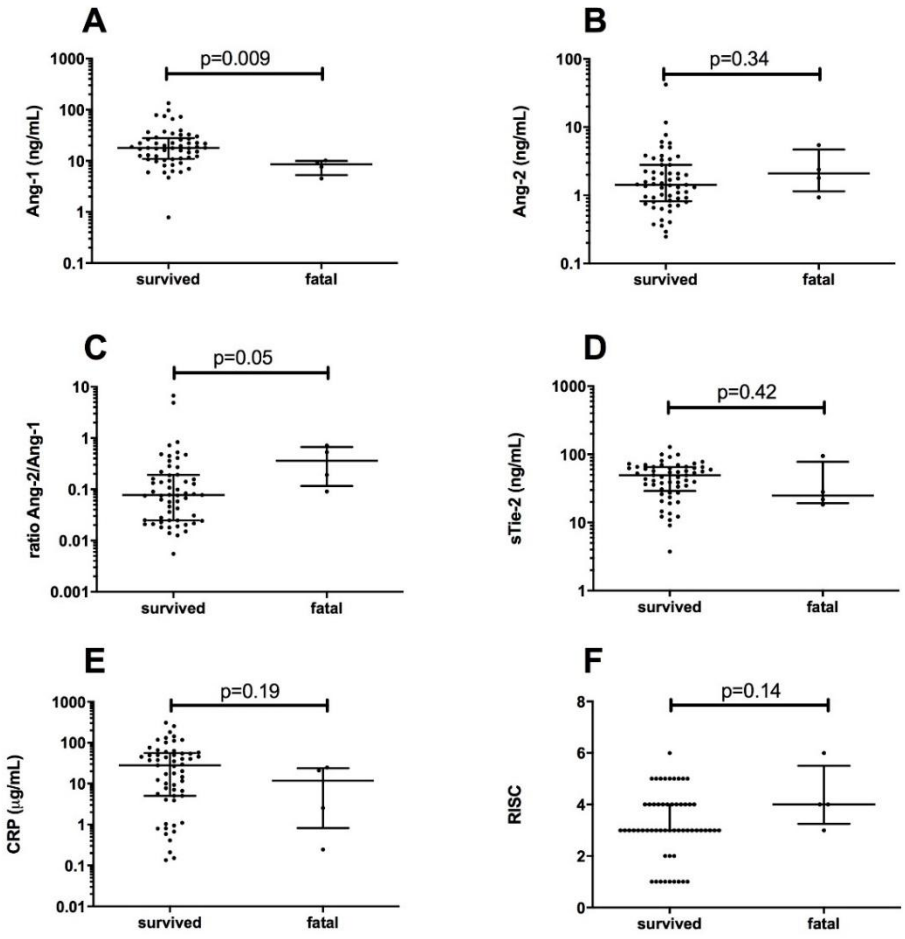

Figure 5. Comparison of angiopoietin levels in surviving and fatal cases of pediatric hypoxemic pneumonia. Concentrations of Ang-1 (**A)**were decreased (p=0.009) significantly, while the Ang-2/Ang-1 ratio (**C**) was significantly augmented (p=0.047) in fatal cases compared to surviving cases. The horizontal bar represents the median; upper and lower error bars represent the interquartile range.

4. Discussion

Here we show that altered levels of Ang-1 and Ang-2 are associated with disease severity, prolonged recovery time, and mortality in children hospitalized with hypoxemic pneumonia in Uganda. Our findings support past observations that angiopoietins, as modulators of endothelial activation, play a pathogenic role in the capillary leak and alveolar consolidation that characterize hypoxemic pneumonia.[23] Furthermore, angiopoietins measured at hospital admission may be clinically informative diagnostic and prognostic biomarkers, distinguishing between pneumococcal pneumonia and viral RTI, and associated with prolonged illness and mortality.

The clinical characteristics of our cohort were broadly consistent with prior studies of children with pneumonia in low-income settings. In our study, the proportion of children that were hypoxemic, had chest x-ray consolidation, and had a fatal outcome (Table 1) were comparable with the multi-centre Pneumonia Etiology Research for Child Health (PERCH) study (36% hypoxemic, 44% with chest x-ray consolidation, 6% mortality) and the Drakenstein Child Health Study (16% hypoxemic, 75% with chest x-ray consolidation, 1% mortality).[54-56] The median levels of Ang-1, Ang-2, and the Ang-2/Ang-1 ratio in our pediatric pneumonia cohort were lower (Table 2) than 41 ng/ml, 4.5 ng/ml, and 0.12 respectively, values previously reported in adults with pneumonia. , [23] Overall, our findings may be generalizable to children with pneumonia in other resource-limited hospitals.

Pneumonia involves inflammation of lung tissue and accumulation of fluid and immune cells in the alveolar space.[37,38] In response to infection and inflammation, the endothelium of the pulmonary microvasculature becomes activated, a state of increased permeability and expression of adhesion molecules.[6,39-42] Ang-1 and -2 regulate endothelial activation through interaction with the cell surface receptor Tie-2. In our study, high admission Ang-2/Ang-1 ratio, reflecting a state of systemic endothelial activation, was associated with greater disease severity, tissue hypoxia, prolonged recovery, and mortality.

Endothelial activation and increased peripheral circulation of endothelial biomarkers are not unique to pneumonia. Consistent with our findings in pediatric pneumonia, dysregulation of angiopoietins has been reported in other systemic infections including malaria and sepsis.[10,19,21,47-49] The Ang-2/Ang-1 ratio is elevated in severe malaria relative to uncomplicated malaria, as well as severe and fatal sepsis.[50,51] Thus, our findings are consistent with previous studies that demonstrated endothelial activation in systemic pediatric infections.

Angiopoietins discriminated between pneumococcal pneumonia and viral RTI. This may suggest a potential clinical application as diagnostic biomarkers to guide antibiotic therapy. In addition, angiopoietins provided prognostic information on recovery times and subsequent mortality. Other host biomarkers found to have diagnostic and prognostic significance in pediatric pneumonia include inflammatory proteins CRP, procalcitonin, tumor necrosis factor-related apoptosis-inducing ligand, plasma interferon-γ protein-10, and haptoglobin-related protein.[5,45,46]

CRP is valuable in distinguishing bacterial from viral pneumonia; indeed, data from the present study confirm the discriminatory power of CRP in differentiating pneumococcal pneumonia

from viral RTI (Fig 2). Although the Ang-2/Ang-1 ratio differentiates bacterial from viral pneumonia less accurately than CRP, our findings suggest that it is a better predictor of recovery times and mortality (Fig 4 and 5). Thus, angiopoietins measured at admission might assist with triage and allocation of resources to the sickest patients.

Limitations of our study include the restricted diagnostic methods used to identify the microbial etiology of pneumonia. In the absence of blood and sputum culture, lung aspirate, or bronchoscopy at our resource-limited hospital, we used PCR-based pathogen detection from stored NP swab. Nonetheless, multi-national studies in low- and middle-income countries using molecular (PCR) diagnostics have shown good agreement in pathogens detected in the upper and lower respiratory tract.[29] The lack of access to a central microbiology laboratory is typical of many low-resource hospitals and is the context in which point-of-care protein-based diagnostics could have the greatest utility. In comparing angiopoietin levels with patient outcome, the association we found was based on only four deaths and should be confirmed in larger studies. The modest sample size and number of hospitals included may limit the generalizability of this study, although these facilities are typical of severely resource-constrained hospitals within the public health sector in sub-Saharan Africa. Non-Gaussian (left-skewed) distribution of biomarker levels required that we use non-parametric, rather than parametric comparative statistics.

5. Conclusions

Here we show that biomarkers of the Ang-Tie-2 system, and particularly the Ang-2/Ang-1 ratio, are dysregulated in hypoxemic pneumonia, reflecting a state of endothelial activation. It is tempting to speculate that angiopoietins may have clinical utility as diagnostic and prognostic biomarkers. Angiopoietins provided equivalent or superior prediction of disease progression and mortality than any single clinical sign or composite clinical risk score. Angiopoietins may provide accurate risk stratification to guide allocation of scarce healthcare resources in resource-limited settings. Although we used enzyme immunoassays in this study, circulating protein biomarkers like the angiopoietins can be readily adapted to a low-cost lateral flow immunochromatographic platform. As an example, the malaria rapid diagnostic test is an accurate and inexpensive point-of-care diagnostic tool suitable for resource-limited settings.[57] Likewise, high abundance angiopoietins would be readily measurable through a finger-prick blood test. Integrating a measurement of angiopoietins within pneumonia management algorithms may be a promising strategy to improve case management and reduce global childhood mortality, particularly in low-income countries in Africa and Asia, where the majority of global deaths related to pneumonia occur.[1]

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Table 1: Time to clinical endpoint in African children hospitalized with hypoxemia, dichotomized by median biomarker values (survivors)

Ang1			
	≤18ng/mL	**>18ng/mL**	**P-value**
Time to resolution of tachypnea	3.6 (0.88-6.2)	1.2 (0.38-2.8)	0.082
Time to resolution of tachycardia	1.7 (0-4.9)	0.56 (0-2.1)	0.21
Time to resolution of fever	1.4 (0.3-3.4)	0.3 (0-1.4)	0.026
Time to wean oxygen	2.7 (1.8-4.3)	2.2 (1.5-4.7)	0.56
Time to discharge	5.6 (3.9-7.8)	3.9 (2.9-5.2)	0.05
Ang2			
	≤1.5ng/mL	**>1.5ng/mL**	**P-value**
Time to resolution of tachypnea	1.2 (0.21-2.6)	4.4 (1.2-7.8)	0.0017
Time to resolution of tachycardia	1.2 (0.042-2.2)	1.1 (0-4.9)	0.81
Time to resolution of fever	0.35 (0-1)	1.7 (0.23-3.7)	0.0089
Time to wean oxygen	1.8 (1.4-2.8)	4.3 (2-5.5)	0.0014
Time to discharge	3.8 (2.8-4.5)	5.8 (4.8-8.6)	3.50E-05
Ang2/Ang1			
	≤0.084	**>0.084**	**P-value**
Time to resolution of tachypnea	1.3 (0.24-2.8)	3.9 (0.9-7.3)	0.02
Time to resolution of tachycardia	0.88 (0-1.9)	1.7 (0-4.9)	0.33
Time to resolution of fever	0.35 (0-1.5)	1.3 (0.28-3.5)	0.021
Time to wean oxygen	2 (1.4-3.4)	2.9 (1.8-5)	0.041
Time to discharge	3.8 (2.8-4.8)	5.8 (4.3-8.6)	0.0014
CRP			
	≤28µg/mL	**>28µg/mL**	**P-value**
Time to resolution of tachypnea	1.3 (0.21-2.8)	3.9 (0.68-5.4)	0.029
Time to resolution of tachycardia	0.67 (0-1.8)	1.7 (0-4.9)	0.16
Time to resolution of fever	0.13 (0-1.1)	1.4 (0.37-2.8)	0.0015
Time to wean oxygen	2.8 (1.8-3.8)	2 (1.5-4.9)	0.59
Time to discharge	4.3 (3.4-5.7)	5.1 (2.9-7.1)	0.46
RISC			
	≤3	**>3**	**P-value**
Time to resolution of tachypnea	1.9 (0.59-3.8)	2.9 (0.15-4.7)	0.59
Time to resolution of tachycardia	1.6 (0.025-3.2)	0.4 (0-3.5)	0.45
Time to resolution of fever	0.58 (0-1.9)	1.1 (0.14-2.2)	0.45
Time to wean oxygen	2.8 (1.7-4.6)	2.2 (1.6-4)	0.64
Time to discharge	4.4 (3.3-6.2)	4.7 (2.9-6.2)	0.83

Times to endpoint (days) are expressed as median (IQR)