Early onset sepsis in Suriname

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Low Serum Angiopoietin-1, high Angiopoietin-2, and high Ang-2/Ang-1 Protein Ratio are Associated with Early Onset Sepsis in Surinamese Newborns

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ABSTRACT

Purpose

Vascular inflammation and leakage in sepsis is mediated by Angiopoietin-1 (Ang-1) and -2 (Ang-2) and their phosphorylation of the endothelial Tie-2 receptor. Levels of Ang-2 change in adults and children during sepsis, which is associated with severity of sepsis. This study investigates levels of Ang-1 and Ang-2 in newborns to gain insight in the vascular pathophysiology of early onset sepsis (EOS) within 72 hours after birth.

Methods

A prospective cohort study was performed amongst 71 Surinamese newborns treated with antibiotics for suspected EOS and 20 control newborns. Newborns with suspected EOS were divided in two groups: blood culture negative and positive EOS. Ang-1 and Ang-2 levels were measured in serum obtained at start of antibiotic treatment and at reevaluation after 48-72 hours.

Results

In this cohort 8.5% of newborns had a positive blood culture. At start of antibiotic treatment Ang-1 serum levels were lower ($P<0.01$), and Ang-2 and Ang-2/Ang-1 serum protein ratios higher ($P<0.01$ and $P<0.01$, respectively) in newborns with blood culture positive EOS than in controls. These levels were not dependent on timing of first blood draw after birth. After 48-72 hours levels of Ang-1 further decreased in blood culture positive EOS, while in the other groups no change was observed.

Conclusions

Our findings support the hypothesis that a dysbalance in the Angiopoietins plays a role in the vascular pathophysiology of EOS.

INTRODUCTION

Sepsis is a syndrome with physiologic, pathological and biochemical changes induced by an infection, and occurs in all age groups [1]. Early onset sepsis (EOS) in newborns, defined as onset of sepsis within 72 hours after birth, remains a clinical diagnostic and therapeutic challenge due to its non-specific clinical presentation. This is associated with late discovery and undertreatment of septic newborns or overtreatment with antibiotics of uninfected ones [2-4]. These diagnostic and therapeutic problems arise because the pathophysiology of EOS is not completely understood [3-5].

One of the pathological changes in septic patients is microvascular dysfunction leading to increased vascular inflammation and leakage [6]. Vascular endothelial cells control these changes through the Angiopoietin/Receptor Tyrosine Kinase (Tie)-2 endothelial receptor system, which is severely disturbed in sepsis [6,7-9]. The system consists of the ligands Angiopoietin-1 (Ang-1) and -2 (Ang-2) [9]. In health, Ang-1-Tie2 binding promotes intracellular Tie-2 phosphorylation, which prevents the occurrence of vascular inflammation and vascular leakage [10]. During sepsis, Ang-2 dose dependently competes with Ang-1, which inhibits Tie-2 phosphorylation and induces destabilizing vascular inflammation and leakage [11,12]. In sepsis in children and adults, higher Ang-2 levels and Ang-2/Ang-1 ratios in blood are associated with presence, severity and outcome of sepsis, while changes in Ang-1 levels are less uniformly present [13-17]. To date, there is insufficient knowledge if disturbances in the Angiopoietin/Tie2 system also reflect the activation state of the endothelium during EOS in newborns [18]. Furthermore, no data exists on the Angiopoietins during EOS from non-Western countries, such as Suriname.

Therefore, we studied the levels and behavior of Ang-1 and Ang-2 at start of antibiotic treatment and at reevaluation between 48 to 72 hours in Surinamese newborns with suspected EOS. We hypothesized that lower Ang-1 and higher Ang-2 and Ang-2/Ang-1 protein ratio were associated with blood culture positive EOS.

MATERIALS AND METHODS

Study Design and Subjects

A prospective observational cohort study was performed at the neonatal care facility of the Academic Pediatric Center Suriname at the Academic Hospital Paramaribo. Patients were included in a 14-month period between April 1st 2015 and May 31st 2016. Newborns with a gestational age (GA) equal to or above 34 weeks in whom antibiotics were started within the first 72 hours of life for suspected EOS were included. Excluded were neonates of whom no serum was obtained or not enough information was available after the study period to confirm outcomes. Written informed consent was obtained from at least one parent for the use of residual serum and clinical information. The study protocol was approved by the Surinamese Medical-Ethical Board (VG-021-14A) and was made available on clinicaltrials.gov (Trial registration: NCT02486783 registered 27/6/2015).

Clinical Protocol

For all newborns, the standard local protocol for the management of suspected EOS was followed. This included the start of antibiotics after blood collection for culture and serial laboratory testing of infectious parameters (t=0). Intravenous ampicillin (50-75 mg/kg/day) and gentamycin (5 mg/kg/day) were started based on the presence of maternal risk factors for infection (i.e., positive group B *streptococcus* culture, (premature) prolonged rupture of membranes, intrapartum fever or intrapartum antibiotics) and/or clinical signs of infection of the newborn. Controls were newborns without signs of infection receiving blood draws for hyperbilirubinemia. In these controls, no antibiotics were started. Newborns in whom antibiotics were started were divided in two groups based on blood culture result: 1) blood culture negative EOS and 2) blood culture positive EOS.

Data Collection

For all newborns maternal information (i.e., history, pregnancy complications (i.e., presence of diabetes mellitus, pregnancy-induced hypertension (PIH) or preeclampsia (PE)) and maternal risk factors for infection) were recorded, along with gestational age (if unknown according to Ballard), Apgar scores, birth weight, gender, ethnicity, results from laboratory testing (white blood cell counts and CRP levels), duration of antibiotic treatment, blood culture results, hospital course, and mortality.

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. After 48-72 hours of treatment with antibiotics a second blood sample was obtained using capillary collection. This time point was labeled t=48-72. CRP and hematological parameters were determined routinely at the clinical laboratory of the Academic Hospital Paramaribo. Blood was allowed to clot at room temperature and serum was separated by centrifugation at 2,300x*g* for 8 minutes, the serum was harvested 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. Measurement of levels of Ang-1 and Ang-2 was performed using the Human Luminex Screening Assay LXSAH (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. We determined inter-assay coefficients of variation (CV) and accepted a maximum of 20%. Median inter-assay CV ranged from 7.3% to 10.5% for Ang-1 and 4.6% to 10.3% for Ang-2, respectively.

Statistical Analysis

Categorical variables were presented as numbers and percentages with 95% CI and compared with chi-square. Continuous variables were presented as median and interquartile range (IQR) Due to the nonparametric nature of the data a Mann-Whitney or Kruskal-Wallis test with Dunn's correction for multiple comparisons was used for analysis of continuous variables. Spearman's

rho was used to assess bivariable associations between CRP levels and Ang-1 and Ang-2 levels, respectively. P-values <0.05 were considered statistically significant. All analyses were performed using Prism version 7.0a (Graphpad Software Inc., San Diego, CA, USA).

RESULTS

Demographics

Of 101 eligible newborns 8 newborns were excluded for incomplete clinical information and 2 for insufficient serum. For the 91 included newborns demographics are given in Table 1. Birth weight, age at presentation, Apgar score and clinical course at t=48-72h were distributed unevenly amongst the three groups. Six (8.5%; 95% CI 3.9-17.2) newborns receiving antibiotic treatment had a positive blood culture (all gram-negative bacteria, *Klebsiella pneumoniae* (n=2), *Enterobacter cloacae* (n=2) and *Escherichia coli* (n=2). Newborns with EOS received respiratory and circulatory support more often than controls (P<0.001). A total of five newborns with EOS died. White blood cell, neutrophil and trombocyte counts and CRP levels were not different between groups (Table 2).

Levels of Angiopoietins

At t=0, median levels of Ang-1 were significantly lower in blood culture positive EOS (28.3 (28.0) ng/mL) versus controls (77.4 (65.2) ng/mL), P<0.01 (Table 2) (Figure 1A). Median Ang-2 levels were higher in blood culture EOS (21.1 (13.3) ng/mL) versus controls (10.2 (1.9) ng/mL), P<0.001, respectively) (Figure 1B). The Ang-2/Ang-1 protein ratio was higher in blood culture positive EOS (median (IQR) 0.77 (0.77) versus controls (median (IQR) 0.13 (0.13) (P<0.01) (Figure 1C). There was no difference in median levels of Ang-1, Ang-2 and Ang-2/Ang-1 protein ratio between blood culture negative EOS and controls.

At t=48-72h, median Ang-1 levels had decreased 21-fold in blood culture positive EOS from levels at t=0 (P=0.10), while median Ang-2 levels remained high (P=0.99) (Table 2) (Figure 1A-B). Median levels of Ang-1, Ang-2 and Ang-2/Ang-1 protein ratio were not different when comparing blood culture positive or blood culture negative EOS with controls.

Levels of Ang-1 and Ang-2 were tested for dependency on timing of first blood draw (t=0) after birth. For controls and EOS (blood culture negative plus blood culture positive EOS) median levels at t=0 were not different between newborns if t=0 was before 24 hours or between 24-72 hours after birth (Figure 2A-B).

Because CRP levels at t=48-72h increased from levels at t=0 in blood culture negative and positive EOS (Table 2), correlation of Ang-1 and Ang-2 with CRP was assessed amongst 44 newborns with blood culture negative (n=42) and positive (n=2) EOS in whom all data had been recorded (Figure 3A-B). Lower median Ang-1 (*rho* -0.46; 95% CI -0.67 to -0.19), but not higher Ang-2, correlated with higher CRP at t=48-72h.

Table 1. Descriptive statistics of the study group (n=91)

			Early Onset Sepsis		
		Controls (n=20)	**Blood Culture Negative (n=65)**	**Blood Culture Positive (n=6)**	**P-value**
Pregnancy, *n (%)*	Complications[1]	3 (15)	16 (25)	1 (17)	0.63
	Chorioamnionitis[2]	0	18 (28)	0	
Mode of delivery, *n (%)*	Vaginal	12 (60)	46 (75)	4 (67)	0.54
	Caesarean	8 (40)	19 (25)	2 (33)	
Sex, *n (%)*	Male	9 (45)	29 (45)	5 (83)	0.19
	Female	11 (55)	36 (55)	1 (17)	
Ethnicity, *n (%)*	Maroon and Creole	12 (60)	44 (68)	4 (67)	0.61
	Hindo-Surinamese	3 (15)	14 (21)	1 (17)	
	Other[3]	5 (25)	7 (11)	1 (17)	
Gestational age, *n (%) (weeks)*	34-37	1 (5)	22 (34)	0	0.06
	37-40	14 (70)	30 (46)	4 (67)	
	≥40	5 (25)	13 (20)	2 (33)	
Apgar score, *n (%)*	<5	0	5 (8)	2 (33)	0.03
Birth weight, Median (IQR) (*grams*)		3130 (700)	2840 (835)	3500 (906)	0.02
Age at presentation, *n (%) (hours)*	<24	4 (20)	43 (66)	2 (33)	<0.01
	24-48	7 (35)	13 (20)	1 (17)	
	48-72	9 (45)	9 (14)	3 (50)	
Clinical course (at 48-72h), *n (%)*	CPAP	0	9 (14)	0	<0.001
	Mechanical Ventilation	0	7 (11)	2 (33)	
	Cardiotonics	0	5 (8)	1 (17)	
	Mortality	0	3 (5)	2 (33)	

CPAP = continuous positive airway pressure; N/A = not applicable.

[1] Presence of pregnancy-induced hypertension, preeclampsia or diabetes mellitus.

[2] Defined as intrapartum fever or administration of antibiotics.

[3] Includes: Javanese, Chinese, Caucasian and Amerindian.

DISCUSSION

In this study, we investigated the serum levels of Ang-1 and Ang-2 to better understand the vascular pathophysiology in near-term and term Surinamese newborns treated for EOS. Lower levels of Ang-1, higher Ang-2, and a higher Ang-2/Ang-1 protein ratio in serum of newborns was associated with blood culture positive EOS at start of antibiotic treatment. Levels of Ang-1 further decreased over time in newborns with blood culture positive EOS and correlated negatively with higher levels of CRP. These results indicate a role for the Angiopoietins in vascular inflammation during EOS in Suriname. An estimated 5-10% of total EOS data is from non-Western countries such as Suriname, while there is strong indication that over 90% of global deaths due to EOS occurs in these settings [2,19,20]. Thus, our data add critical basic and clinical knowledge on the true global impact of EOS.

Table 2. Infection biomarkers in baseline controls and newborns with suspected and blood culture positive early onset sepsis

				Early Onset Sepsis		
	Time Point	**n (%)**	**Controls**	**Blood Culture Negative**	**Blood Culture Positive**	**P-value**
White blood cells (x10^9/L)[1]	t=0	88 (97)	15.3 (8.2)	17.5 (9.7)	21.9 (82.4)	0.68
Neutrophils (x10^9/L)[1]	t=0	72 (79)	7.1 (8.5)	9.2 (7.9)	10.2 (34.1)	0.57
Platelets (x10^9/L)[1]	t=0	83 (91)	235 (82)	239 (60)	74 (164.5)	0.07
C-reactive protein (mg/dL)	t=0	75 (82)	<0.5 (0)	<0.5 (0.7)	0.7 (4.8)	0.34
	t=48-72h	44 (48)	N/A	0.7 (1.8)	1.4 (16.3)	0.81[1]
	Delta	44 (48)	N/A	0.1 (1.3)	1.7 (3.4)	0.46[1]
Angiopoietin-1 (ng/mL)	t=0	91 (100)	77.4 (65.2)	82.2 (45.7)	28.3 (28.0)	<0.01
	t=48-72h	49 (54)	68.9 (44.5)	73.6 (67.3)	1.3 (16.1)	0.02[1]
Angiopoietin-2 (ng/mL)	t=0	91 (100)	10.2 (1.9)	11.2 (6.9)	21.1 (13.3)	<0.01
	t=48-72h	49 (54)	9.9 (1.5)	11.8 (4.7)	19.0 (18.9)	0.07[1]

N/A = not applicable.
Data presented as median (IQR) and analyzed with a Kruskal-Wallis test between all groups or [1]with a Mann-Whitney test between blood culture negative and positive EOS groups.

Our results of Ang-1 levels are in line with other studies that reported reduced Ang-1 levels in children associated with septic shock and death [21,22]. The mechanism for low Ang-1 remains poorly understood. While Ang-1 levels are low, the levels of its soluble ligand sTie-2 are higher in the blood of septic patients. Soluble Tie 2 may acts as a decoy receptor binding Ang-1 with high affinity, thereby decreasing its circulating levels. On the other hand, increasing Ang-1 levels, thereby increasing endothelial Tie-2 receptor phosphorylation may help to inhibit vascular inflammation and leakage. In a clinically relevant murine model, intravenous recombinant Ang-1 treatment was sufficient to improve sepsis-associated organ dysfunctions and survival time, most likely by preserving endothelial barrier function [23].

Higher levels of Ang-2 may be reflective of vascular inflammation and vascular leakage. Intravenous lipopolysaccharide injection in human volunteers, adult human sepsis, and secondary infection in critically ill patients causes higher levels of circulating Ang-2 and higher Ang-2/Ang-1 ratios [24-28]. As intracellular Tie-2 phosphorylation cannot be assessed in patients, an increased Ang-2/Ang-1 ratio might be predictive for reduced endothelial Tie-2 receptor phosphorylation with subsequent vascular inflammation and leakage.

EOS can occur following colonization of the newborn with bacterial pathogens following intra-uterine infection or in the birth canal during labor [4]. Two studies found higher maternal and amniotic fluid levels of Ang-2 in cases of intra-uterine infection in at term and preterm birth [29,30]. To our knowledge, placental Ang-2 crossing has not been described. The presence of intra-uterine infection may result in EOS and cause subsequent suppression of neonatal levels of Ang-1 and release of neonatal Ang-2 from endothelial cells. Our finding that levels of Ang-1

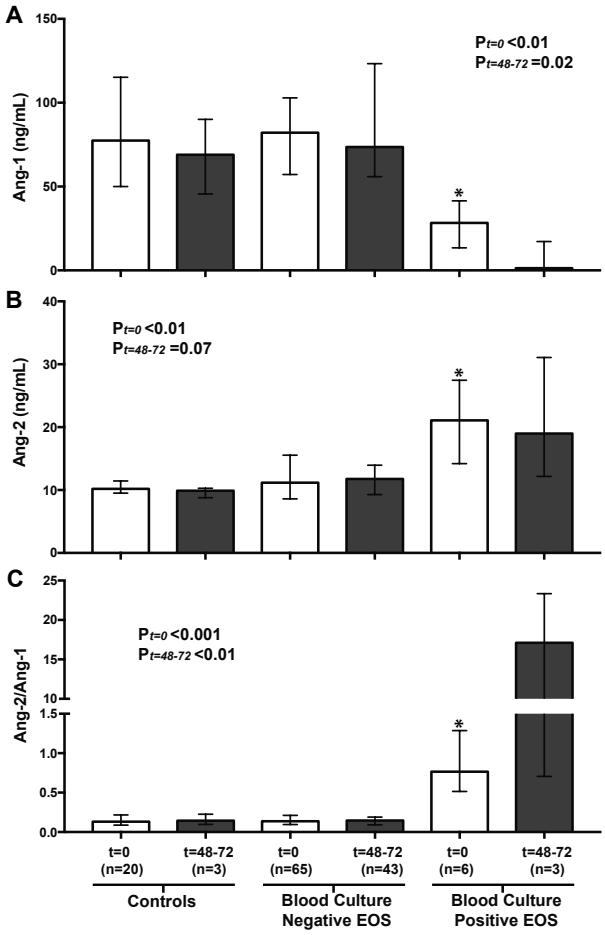

Figure 1. Serum levels of Angiopoietin-1 and Angiopoietin-2 of controls and newborns with blood culture negative and positive early onset sepsis (EOS). A: Angiopoietin-1 (Ang-1); **B:** Angiopoietin-2 (Ang-2); **C:** Ang-2/Ang-1protein ratio; Data represent levels in serum sampled at t=0 (white bars) and t=48-72h (grey bars) and are analyzed with a Kruskal-Wallis test with Dunn's correction for multiple comparisons between all groups at t=0 ($P_{t=0}$) and at t=48-72 ($P_{t=48-72}$). * P<0.05 when groups are separately compared to controls. Bars represent median values and error bars interquartile range.

and Ang-2 are similar between infected newborns included directly after birth and after 24 hours supports this hypothesis.

A remarkable finding in our study was that levels of Ang-1 in newborns were up to a 10-fold higher, specifically in healthy newborns and those with blood culture negative EOS, than in children or adults in earlier studies [13-17,21,22]. Placental levels of Ang-1 and Ang-2 are high during pregnancy and then quickly drop after birth [31,32]. Only one earlier study compared both antepartum and post caesarean maternal samples with neonatal umbilical cord blood samples [32]. Ang-1 concentrations were significantly higher in umbilical samples, suggesting separate

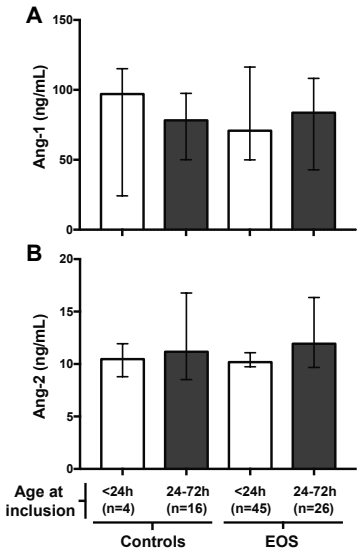

Figure 2. Serum levels of Angiopoietin-1 and Angiopoietin-2 at inclusion in newborns included before and after the first 24 hours of life. A: Angiopoietin-1 (Ang-1); **B:** Angiopoietin-2 (Ang-2); Data represent pooled levels at t=0 from newborns considered uninfected (controls) and from newborns considered infected (blood culture negative and positive EOS), included before 24h (light grey bars) versus 24-72h (checked light grey bars) after birth. Data was analyzed with a Mann-Whitney test. Bars represent median values and error bars interquartile range.

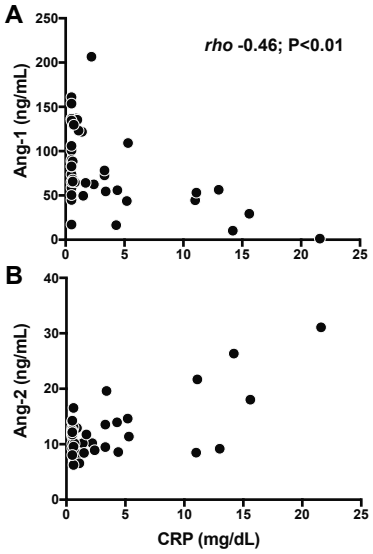

Figure 3. Correlations of serum levels of Angiopoietin-1 and Angiopoietin-2 with serum levels of C-reactive protein in newborns with blood culture negative or positive early onset sepsis (EOS). Correlations of CRP with **A:** Angiopoietin-1 (Ang-1); **B:** Angiopoietin-2 (Ang-2); Data represent levels of Ang-1, Ang-2 and CRP in serum sampled at t=48-72h from newborns (in whom data on levels was universally available) with blood culture negative (n=42) and positive (n=2) EOS. Spearman's *rho* was used to assess correlations. Correlation (*rho*) is given when significant.

Angiopoietin regulation in the newborn. Animal models of pregnancy may help elucidate the exact dynamics of Angiopoietins in newborns [33]. These animal models may also be instrumental in detecting endothelial Tie-2 receptor phosphorylation in different microvascular beds.

From a clinical perspective, our findings indicate that serial measurement of Angiopoietins may predict or exclude bacteremia in newborns before blood culture results are known. High serial Ang-1 and low serial Ang2/Ang-1 ratio may be extra arguments to discontinue antibiotics, alongside serial measurement of CRP. A known limitation to CRP is its slow synthesis limiting its utility in early prediction of EOS. To overcome this issue, inflammatory mediators that precede CRP synthesis, such as Interleukin (IL)-1ß, IL-6, IL-8 and Tumor-necrosis Factor (TNF)-a have been of interest in EOS research [34-36]. These mediators have short half-lives, which limits their clinical use and establishment of appropriate cut-off values. In our study, levels of Ang-1 remained high in healthy and low in the sickest newborns at reevaluation 48-72 hours after start of antibiotics, indicating persistent association with severity of disease over time and clinical utility. Additionally, TNF-a has been shown to correlate with Ang-2 levels in adult patients with sepsis [24,37]. For these reasons it would be interesting to evaluate temporal relations of the Angiopoietins with a panel of inflammatory mediators, such as TNF-α, Il-6 and IL-1ß in EOS.

Our study has several limitations. First, our sample size was relatively small to assess relevance of the Angiopoietins as clinical biomarkers and results may have been confounded by birth weight and asphyxia, which were distributed unevenly amongst the groups. Second, as levels of the Angiopoietins were determined with a Luminex Screening Assay we were unable to compare levels with results from other studies measured with ELISA [21,28], and small sample volumes acquired in neonates precluded assessment of other inflammatory mediators. Future studies will focus on eliminating these limitations to enable us to validate the current observations in newborns in Surinamese newborns.

In summary, our data show changes in the ligands of the Angiopoietin/Tie2 endothelial receptor system Ang-1 and Ang-2 in EOS and support the hypothesis that increased vascular inflammation and increased vascular leakage leads to microvascular dysfunction in the pathophysiology of EOS. The potential impact of intra-uterine-infection deserves attention in future investigations to further elucidate dynamics of Angiopoietins in newborns with and without EOS.

Abbreviations

EOS = early onset sepsis
Ang-1 = Angiopoietin-1
Ang-2 = Angiopoietin-2
Tie-2 = TEK tyrosine kinase endothelial-2 receptor

Conflict of Interest

The authors declare that they have no conflict of interest.

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