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High serum levels of endothelial cell adhesion molecules and their shedding enzymes do not discriminate between early onset sepsis and healthy newborns in Suriname.

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Short title: Adhesion molecule shedding in Surinamese newborns

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What is already known on this topic?

- Sepsis is associated with an Angiopoietin (Ang)-1 and Ang-2 serum level disbalance and increased shedding of soluble endothelial adhesion molecules (sCAMs).
- Recently, we established an association of the Ang-1/Ang-2 disbalance with blood culture positive early onset sepsis (EOS) in newborns.

What this study adds?

- The Ang-1/Ang-2 disbalance in blood culture positive EOS is not paralleled by increased levels of sCAMs and their sheddases.
- Levels of sCAMs and their sheddases are high after birth and do not discriminate EOS from healthy newborns.

ABSTRACT

Background: Early Onset Sepsis (EOS) is defined as onset of sepsis within 72 hours after birth. Leukocyte-endothelial interactions play a pivotal part in EOS pathophysiology. Endothelial cell adhesion molecules orchestrate these interactions and their soluble isoforms (sCAMs) are released into the vasculature by enzymes called sheddases.

Purpose: This study was undertaken to explore further the pathophysiology of EOS and to investigate the potential of sCAMand their sheddases as potential biomarkers for EOS.

Methods: Soluble CAMs sP-selectin, sE-selectin, vascular cell adhesion molecule-1 (sVCAM-1), intercellular adhesion molecule-1 (sICAM-1) and platelet and endothelial cell adhesion molecule-1 (sPECAM-1), sheddases matrix metalloproteinase-9 (MMP-9) and neutrophil elastase (NE), and sheddase antagonist tissue-inhibitor of metalloproteinases-1 (TIMP-1) were measured simultaneously in serum of 71 Surinamese newborns suspected of EOS and 20 healthy newborns, all included within 72 hours after birth.

Results: Six (8.5%) newborns had a positive blood culture. At start of antibiotic treatment and after 48-72 hours no differences were found in levels of sCAMs and sheddases between blood culture positive EOS and controls.

Conclusions: Our data indicate that endothelial CAM shedding is not increased in EOS and that levels of sCAMs and sheddases remain unchanged in early life in newborns, suggesting not a role in the pathophysiology of EOS. Therefore, these markers have limited clinical utility as biomarkers for EOS.

Keywords: newborns; early onset sepsis; adhesion molecules; shedding; Suriname.

INTRODUCTION

Early onset sepsis (EOS) in newborns within 72 hours after birth remains a clinical challenge with high morbidity and mortality [1-3]. The majority of global neonatal deaths due to EOS occur in developing countries [4]. The diagnosis of EOS is complicated, resulting in late recognition, or overtreatment of newborns with antibiotics. These dilemmas arise because the pathophysiology of EOS is poorly understood.

A hallmark of sepsis pathophysiology is endothelial cell activation followed by leukocyte recruitment into tissues [5]. The current model describes the occurrence of a shift in balance in Tie2 receptor ligands Angiopoietin (Ang)-1 and Ang-2 affecting endothelial integrity, and increased expression of endothelial cell adhesion molecules, in particular P-selectin, E-selectin, vascular cell adhesion molecule (VCAM-1), and intercellular adhesion molecule (ICAM-1) to facilitate this recruitment [6,7]. These endothelial cell adhesion molecules orchestrate leukocyte rolling on, adhesion to, and diapedesis across the endothelium [7,8]. Also, platelet and endothelial cell adhesion molecule (PECAM-1), expressed at endothelial cell junctions has a function in facilitating paracellular transmigration of leukocytes across the endothelium [9]. After intravenous administration of endotoxin in healthy adults as a sepsis model, peak levels of Ang-2 prelude the release of soluble isoforms of cell adhesion molecules (sCAMs) into the systemic circulation [10]. Endothelial cell adhesion molecules are released through ectodomain shedding by enzymes called sheddases, in particular matrix metalloproteinase-9 (MMP-9) and neutrophil elastase, released from granules in neutrophils [7,11]. Both MMP-9 and neutrophil elastase prepare the extracellular matrix for transmigration of leukocytes into inflammatory sites [12]. MMP-9 activity is balanced by sheddase antagonist tissue-inhibitor of metalloproteinases-1 (TIMP-1) [12-14].

Recently, we showed in a cohort of near term and term Surinamese newborns that a systemic circulation dysbalance in Ang-2/Ang-1 levels was associated with blood culture

positive EOS [15]. This study was undertaken to examine if this dysbalance is paralleled by increased levels of sCAMs and sheddases in this cohort of newborns with EOS to explore further the pathophysiology of EOS and to investigate their potential as biomarkers for EOS. We hypothesized that sCAMs and sheddases circulate at higher levels in blood culture positive EOS in newborns and that they are useful as biomarkers for EOS.

MATERIALS & METHODS

Study design, subjects and clinical protocol

For this study, we used a Surinamese cohort of 20 healthy newborns and 71 newborns with suspected EOS from an earlier reported study (Supplemental Table 1, previously published) [15]. All newborns were included between April 1 2015 and May 31 2016. Included were newborns with a gestational age equal to or above 34 weeks in whom antibiotics were started within the first 72 hours of life for suspected EOS. Informed consent was obtained from at least one parent for the use of residual serum and clinical information. The study protocol was made available on clinicaltrials.gov (NCT02486783) and was approved by the Surinamese Medical Ethical Board (VG-021-14A) including permission of one parent.

The management of these patients was described before [15]. In short, healthy control newborns and newborns suspected of EOS were included (t=0) within 72 hours after birth and clinically reevaluated 48-72 hours later (t=48-72h). At t=0 and t=48-72h blood was drawn for separation and storage of serum. This time point was chosen because the result of blood culture became available. Controls were newborns without signs of infection receiving blood draws for hyperbilirubinemia (n=20). Newborns with suspected EOS receiving treatment with intravenous antibiotics were divided in two groups based on result from blood culturing: blood culture negative EOS (n=65) and blood culture positive EOS (n=6).

Sample collection, preparation and analysis

At t=0 blood samples were collected during the insertion of a venous cannula and after 48-72 hours of treatment with antibiotics a second blood sample was obtained using capillary collection. After clotting at room temperature and centrifugation at 2,300x*g* for 8 minutes the serum was harvested and the residual sample was stored at -80°C until further analysis. Measurement of sP-selectin, sE-selectin, sVCAM-1, sICAM-1, and sPECAM-1 was performed on serum samples using the Human Magnetic Bead Adhesion 6-plex panel performance assay (LHC0016M, Thermo Scientific, Waltham, MA USA) according to the manufacturer's instructions. ELISA was used on aliquots of the same samples for measurement of neutrophil elastase (HK319-02, Hycult Biotech, Uden, The Netherlands), MMP-9 (Quantikine DMP900, R&D systems, Minneapolis, MN USA), and TIMP-1 (Quantikine DTM100, R&D systems), each according to the manufacturers' instructions. For each molecule, a standard curve was established via which concentrations in neonatal serum were determined. Levels below or above the linear part (for MMP-9 n=11 (7.7%) samples, for TIMP-1 n=2 (1.4%) samples, and for neutrophil elastase n=9 (6.3%) samples) of this standard curve were reported as the lowest or highest value of the standard curve, respectively. We measured intra-assay variation between plates used in the same assay by calculating coefficient of variation between levels of each molecule in samples from the same patient divided over those plates and accepted a maximum of 20%.

Statistical analysis

Categorical variables were presented as numbers and percentages with 95% CI and continuous variables, due to the nonparametric nature of the data, as median with interquartile range (IQR). The Chi-square test was used to compare categorical variables. A Mann-Whitney U test and Kruskal-Wallis test with Dunn's correction for multiple comparisons

were used for analysis of continuous variables. Because timing of inclusion after birth varied between groups (Supplemental Table 1), we investigated whether postnatal sampling day (i.e., for both t=0 and t=48-72h between day 1 and 6 after birth) correlated with sCAM and sheddase levels and calculated Spearman's *rho.* P-values <0.05 were considered statistically significant. All analyses were done using Prism version 7.0a (Graphpad Software Inc., San Diego, CA USA).

RESULTS

Demographic variables of the study cohort (n=91) are given in Supplemental Table 1. Blood culture results revealed that 6 of 71 newborns with suspected EOS (8.5%; 95% CI 3.9-17.2) had a positive blood culture with gram-negative pathogens *Klebsiella pneumoniae* (n=2), *Enterobacter cloacae* (n=2) and *Escherichia coli* (n=2). One newborn had EOS due to a spontaneous bacterial peritonitis. For n=4 others cause of EOS was unknown, but they presented with neonatal jaundice (n=1), perinatal asphyxia (n=1), meconium aspiration (n=1), and hypoglycaemia (n=1). We included 20 control newborns without signs of infection receiving blood draws for hyperbilirubinemia.

Serum levels of soluble endothelial cell adhesion molecules and their sheddases

Serum samples (n=142) were available of all 91 newborns at t=0 and of 51 at t=48-72h. Due to the limited amount of serum available, not all molecules could be measured in all samples. Measurement of levels of MMP-9 and TIMP-1 was performed in n=90 and n=51 of newborns at t=0 and t=48-72h, respectively. We were able to measure sCAMs and neutrophil elastase levels in n=80 and n=36 newborns at t=0 and 48-72h, respectively.

We found no differences between median levels of sCAMs between blood culture positive EOS, blood culture negative EOS, and control groups at either t=0 or t=48-72h

(Table 1). Median levels of sCAMs within a group did also not change between t=0 and t=48-72h. Of all sCAMs only median levels of sP-selectin in pooled (n=115) samples correlated negatively with later sampling day (*rho* -0.21; 95% CI -0.38 to -0.02; P=0.03). No differences were found in median levels of MMP-9, TIMP-1, and neutrophil elastase between blood culture positive EOS, blood culture negative EOS, and controls at either t=0 or t=48-72h. Also, no differences between median levels of the sheddases within a group between t=0 and t=48-72h were found. No correlation was found between levels of the sheddases and sampling day.

DISCUSSION

In this study we investigated whether sCAMs and their sheddases circulate at higher levels in newborns with blood culture positive EOS. We serially measured levels of sCAMs and sheddases in a cohort of near and at term newborns. In contrast to our hypothesis, none of the molecules showed any difference in serum levels between blood culture positive EOS, blood culture negative EOS, and controls, neither at start of antibiotic treatment nor after 48-72 hours. These data indicate that levels of sCAM and sheddases are not associated in the pathophysiology of EOS and have no clinical utility as early biomarkers for EOS in newborns.

Previously, we found evidence for endothelial cell activation in blood culture positive EOS in the same newborns used for this study, represented by a dysbalance in Ang-2/Ang-1 ratio [15]. Since the current data demonstrate that this dysbalance was not paralleled by increased release of sCAM or sheddases in EOS we conclude that endothelial cell adhesion molecule shedding is not or to a lesser extent involved in the pathophysiology of EOS. For interpretation of our data we reviewed and summarized available data on sCAMs and sheddases in newborns with sepsis in Supplemental Table 2 [16-34]. Comparison of our

results with other existing data is complicated because of heterogenic make up of chosen cohorts. Only one study reported a comparable cohort of near and at term newborns with suspected EOS within 72 hours after birth, in whom increased levels of sICAM-1 and neutrophil elastase levels were associated with blood culture positive EOS [19]. Other earlier studies compared levels of sCAMs in heterogenic cohorts consisting of newborns with different gestational and postnatal ages, either having EOS (based on varying definitions), or sepsis after 72 hours after birth (i.e., late onset sepsis). This variation in inclusion criteria is an important confounding factor in the interpretation of the observed levels in septic and healthy newborns. Overall, our results are in line with these studies that show that clinical utility of levels of sCAMs and sheddases in EOS is very limited.

In an earlier review by our group we pooled published data on sCAM levels in newborns [7]. Soluble CAM levels in the current study corresponded well with levels discussed in our review and those established in earlier studies in uninfected healthy newborns with similar gestational and postnatal age [7, 23,24,29-32]. However, MMP-9, TIMP-1, and neutrophil elastase levels were different and up to 4, 2, and 10-fold higher, respectively, than those reported in earlier studies [17,18,21,22,25,30], which may have been due to other methods used (see limitations). Furthermore, our earlier review and earlier data indicated that significant age-related discrepancies exist in sCAM levels between newborns, children and adults. As an example, in at term newborns sVCAM-1 concentrations in the first postnatal week were almost twice the levels in healthy adults, and equally high compared to septic adults, suggesting that sVCAM-1 levels start of high in early newborn life and then decrease with increasing age [7,31]. In our study, levels of sCAMs and sheddases during the first 6 days of life in our study remained stable for 7 out of analyzed 8 molecules, which was in contrast with earlier work in healthy newborns showing that sE-selectin decreased, and sICAM-1 and sVCAM-1 increased between day 1 and 5 after birth, while sPECAM-1 levels

did not change [29-32]. Even though some discrepancies with earlier reports exist, overall one can conclude that these and our data indicate that levels of sCAMs and sheddases measured within 72 hours after birth are high and do not discriminate between septic and healthy newborns, which limits their use as biomarkers for early identification or exclusion of EOS.

Our and pre-existing data suggest that overall high sCAM and sheddase levels in newborns are the result of other perinatal factors than EOS. Several pathophysiological processes may explain this premise. Birth may induce a 'pro-adhesive' state of the endothelium leading to increased endothelial cell adhesion molecules expression on, and shedding from, its surface. Additionally, the increase in overall leukocyte numbers and inflammatory activation of subsets associated with human birth, which was shown to be positively associated with increased perinatal stress [35-37], may cause higher intensity of leukocyte-endothelial interactions and subsequent increases endothelial cell adhesion molecules shedding. Aberrant adhesion of activated leukocytes to activated endothelium is associated with endothelial dysfunction and increased vascular permeability [38,39]. Shedding of endothelial cell adhesion molecules may then result in prevention of aberrant leukocyte adhesion on two complementary levels, namely 1) to lower endothelial cell adhesion molecules density to prevent adhesion or promote de-adhesion of already adhering leukocytes and 2) to release circulating sCAMs that act as 'decoy receptors' to capture leukocytes in the vasculature to limit leukocyte-endothelial interactions [7,10]. Whether this occurs in real life and what the contribution is to sCAM and sheddase levels in newborns remains unknown and could be studied in neonatal animal models [40-42].

Our study has some limitations. First, sample size at t=48-72h was relatively small due to limited clinical need for additional blood draws in controls and death of patients. As a result, logistic regression analysis of other factors, such as maternal perinatal factors or method of birth, potentially influencing levels of sCAMs and sheddases, was precluded.

Larger studies in countries such as Suriname, where the incidence of EOS is relatively high in comparison to Western countries, are necessary and can contribute to better insight in the vascular pathophysiology of EOS. Second, the use of serum in our study may have caused release of stored pools of MMP-9, TIMP-1, and neutrophil elastase from disrupted leukocytes during the clotting process, which could have accounted for higher levels of these molecules than reported in earlier studies.

In conclusion, our data indicate that serum levels of sCAMs and sheddases are not increased during EOS in Surinamese near and at term newborns. Other mechanisms, such as perinatal stress during birth, may drive overall high levels in all newborns which precludes discrimination between septic and healthy newborns based on levels of these molecules. For these reasons sCAMs and sheddases studied have no utility as biomarkers for EOS.

ABBREVIATIONS AND DEFINITIONS

EOS = Early onset sepsis

ICAM-1 = Intercellular adhesion molecule-1

VCAM-1= Vascular cell adhesion molecule-1

PECAM-1= Platelet and endothelial cell adhesion molecule-1

MMP-9 = Matrix metalloproteinase-9

TIMP-1 = Tissue-inhibitor of metalloproteinases-1

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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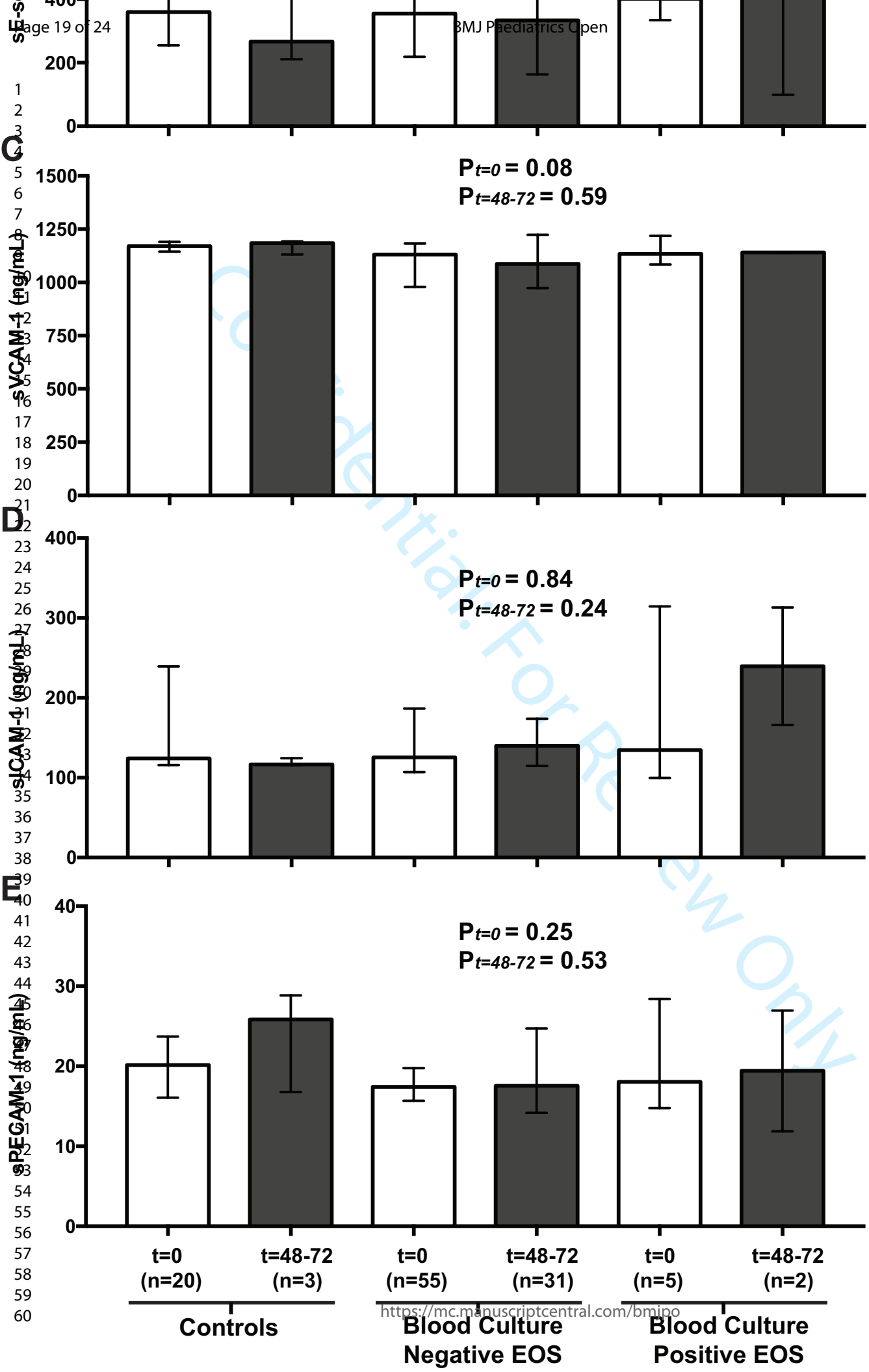
FIGURE LEGENDS

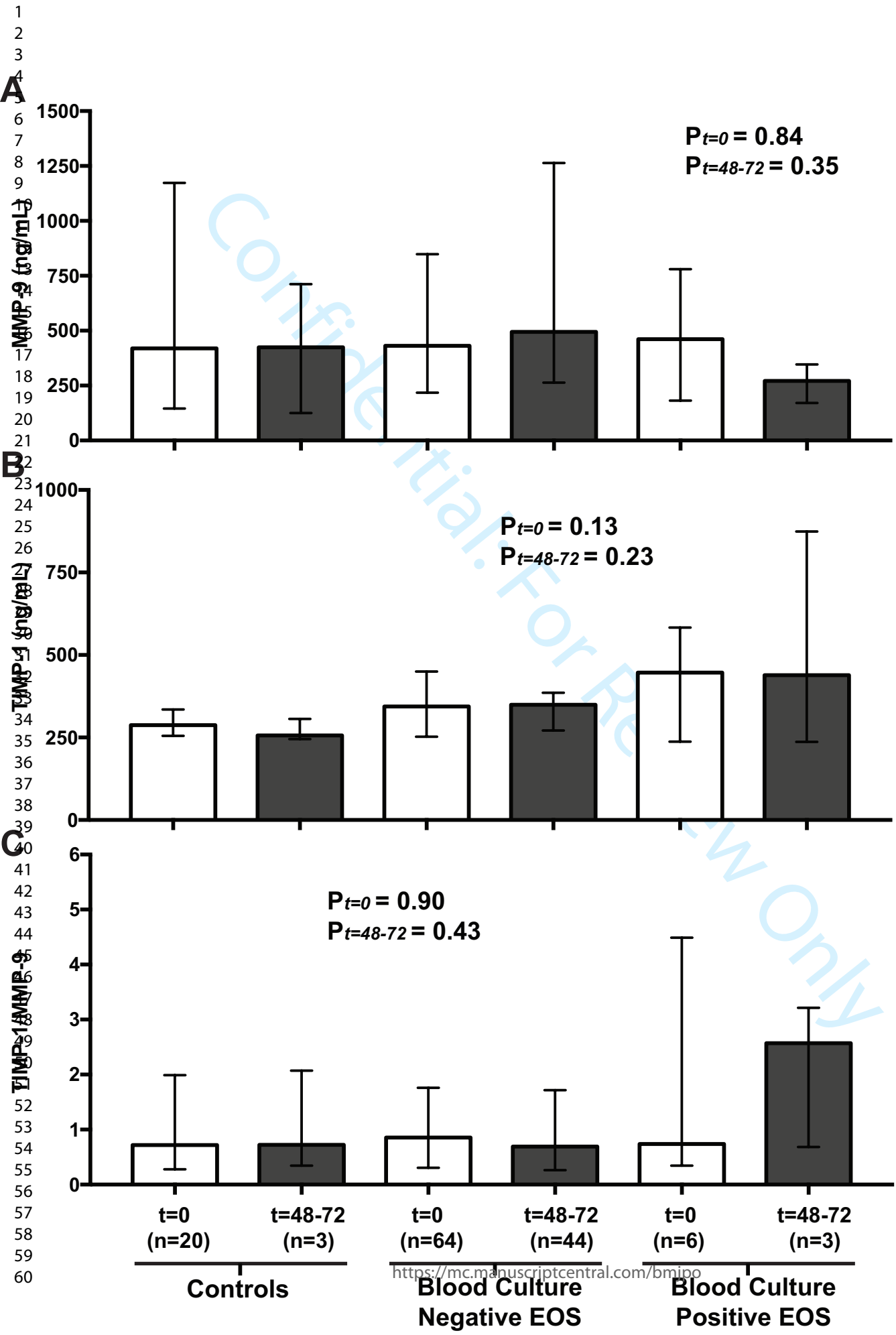
Figure 1.

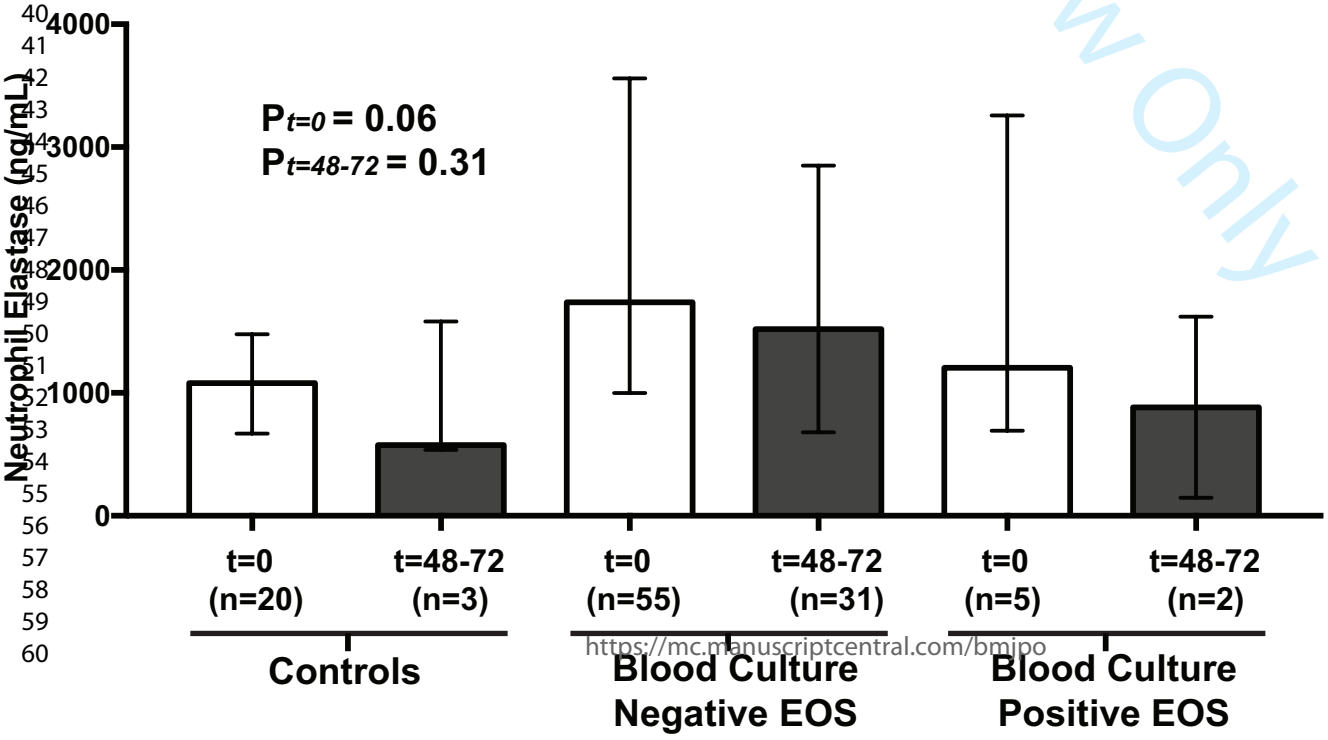
Circulating levels of endothelial adhesion molecules sP-selectin, sE-selectin, sVCAM-1, sICAM-1, and sPECAM-1 in Surinamese newborns. A: sP-selectin **B:** sE-selectin **C:** soluble vascular cell adhesion molecule-1 (sVCAM-1); **D:** soluble intercellular adhesion molecule-1 (sICAM-1); **E:** soluble platelet and endothelial cell adhesion molecule-1 (sPECAM-1). Data report levels in serum sampled at t=0 (white bars) and t=48-72h (grey bars) and are analyzed with a Kruskal-Wallis test between all groups at t=0 ($P_{t=0}$) and at t=48-72 ($P_{t=48-72}$). P<0.05 is considered statistically significant. Bars represent median values and error bars interquartile range.

Figure 2. Circulating levels of MMP-9 and TIMP-1, and TIMP-1/MMP-9 ratios in Surinamese newborns. A: Matrix metalloproteinase-9 (MMP-9); **B:** Tissue inhibitor of metalloproteinase (TIMP-1); **C:** TIMP-1/MMP-9 ratios. Data report levels in serum sampled at t=0 (white bars) and t=48-72h (grey bars) and are analyzed with a Kruskal-Wallis test between all groups at t=0 ($P_{t=0}$) and at t=48-72 ($P_{t=48-72}$). P<0.05 is considered statistically significant. Bars represent median values and error bars interquartile range.

Figure 3. Circulating levels of neutrophil elastase in Surinamese newborns. Data report levels in serum sampled at t=0 (white bars) and t=48-72h (grey bars) and are analyzed with a Kruskal-Wallis test between all groups at t=0 ($P_{t=0}$) and at t=48-72 ($P_{t=48-72}$). P<0.05 is considered statistically significant. Bars represent median values and error bars interquartile range.







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

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

This table was previously published in reference 15. CPAP = continuous positive airway pressure; N/A = not applicable.

[a] Presence of pregnancy-induced hypertension, preeclampsia or diabetes mellitus;

[b] Defined as intrapartum fever or administration of antibiotics;

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

Supplemental Table 2: Studies reporting levels of endothelial cell adhesion molecules and shedding enzymes in newborns

Study (ref.), year	CAM or shedding enzyme	Cohort Characteristics	Gestational age (weeks)	Postnatal age	Main Results	AnalysisMethod	Healthy (ng/mL)[1]	Septic (ng/mL)[1]
Fatah et al. (16), 2017	sE-selectin	EOS and LOS	NS	NS	sE-selectin **elevated** in BCPS	ELISA	148.9 ± 7.9	177.1 ± 3.5
Weitkamp et al. (17), 2016	MMP-9	EOS and LOS	25-36	< and ≥ 3 days	MMP-9 levels **lower** in BCPS	Multiplex bead assay	NS	NS
Wynn et al. (18), 2015	MMP-9	Chorioamnionitis	25-36	NS	MMP-9 levels **lower** in chorioamnionitis	Multiplex bead assay	NS	NS
Sugitharini et al. (19), 2013	sICAM-1, NE	EOS	34-42	0-72 hours	sICAM-1 and NE **elevated** in EOS	ELISA Antibody array	sICAM-1/ NE: NS	sICAM-1: NS NE:499.2±22.0
Edgar et al. (20), 2010	sICAM-1, sE-selectin	EOS and LOS	24-41	NS	sICAM-1 and sE-selectin **elevated**	ELISA	sICAM-1: 165 (130-290) sE-selectin: 71 (51-118)	sICAM-1: 405 (252-666) sE-selectin 158 (94-207)
Fukanaga et al. (21), 2009	MMP, TIMP-1	Uninfected newborns	<30	Cord blood	No difference	ELISA	MMP-9: 22 (16-48) TIMP-1: 122 (86-249)	NS
Sunagawa et al. (22), 2009	MMP-9, TIMP-1	Uninfected newborns	35-41	1-2 days	NA	ELISA	NS	NA
Figueras et al. (23), 2007	sICAM-1, sVCAM-1, sP-selectin,	EOS and LOS	32-40	1-32 days	sICAM-1 and sVCAM-1 **increased** over time.	ELISA	sICAM-1: 156 (150-194) sVCAM-1: 856 (742-960) sP-selectin: 272 (152-288)	sICAM-1: 394 (342-600) sVCAM-1: 1153 (726-1307) sP-selectin: 244 (170-324)
Sitaru et al. (24), 2005	sP-selectin	Chorioamnionitis	25-40	Cord blood	sP-selectin **elevated** in chorioamnionitis	ELISA	104 ± 71	222 ± 128
Schulz et al. (25), 2004	MMP-9, TIMP-1	Uninfected newborns	25-40	1-28 days	MMP-9 highest in preterm TIMP-1 highest in at term	ELISA	NS	NA
Edgar et al.	sICAM-1	EOS and LOS	24-42	NS	sICAM-1 **elevated** in	ELISA	205 (146-343)	406 (345-1180)

(26), 2002					BCPS			
Apostolou et al. (27), 2002	sICAM-1	EOS and LOS	25-42	NS	sICAM-1 **elevated** in BCPS	ELISA	358.4 ± 28.9	710.7 ± 56.6
Dollner et al. (28), 2001	sICAM-1, sE-selectin	EOS and LOS	30-42	1-7 days	sICAM-1 and sE-selectin **elevated** in BCPS	ELISA	sICAM-1: 244.0 (92.5-500) sE-selectin: 91.4 (<2.0-217.8)	sICAM-1: 357.4 (141.6-500) sE-selectin: 151.7 (37.0-362.2)
Malamitsi et al. (29), 2000	sVCAM-1, sPECAM-1	Uninfected newborns	37-40	1-5 days	No change between day 1 and 5	ELISA	sVCAM-1: 1340 ± 58.3 sPECAM-1: 17.5 ± 0.7	NA
Giannaki et al. (30), 2000	sE-selectin	Uninfected newborns	At term	1-5 days	sE-selectin **decreases** between day 1 and 5	ELISA	139 ± 48	NA
Giannaki et al. (31), 1999	sICAM-1, sVCAM-1	Uninfected newborns	At term	1-5 days	sICAM-1 and sVCAM-1 **increase** between day 1 and 5	ELISA	sICAM: 179 ±56.1 sVCAM-1: 1125.0 ± 281.0	NA
Phocas et al. (32), 1998	sICAM-1	Uninfected newborns	35-42	1-30 days	sICAM-1 **increases** between day 1, 5 and 30	ELISA	137.3 ± 62.0	NA
Berner et al. (33), 1998	sICAM-1	EOS	26-42	0-96 hours	sICAM-1 **lower** in EOS. sICAM-1 **increases** over time	ELISA	421 (291-459)	446 (171-534)
Austgulen et al. (34), 1997	sICAM-1, sVCAM-1, sE-selectin	EOS and LOS, pneumonia	24-42	0-162 hours	sE-selectin and sICAM-1 **elevated** in infected neonates	ELISA	sE-selectin: 84.2 (21.6-231.3) sICAM-1: 2131.3 (1449.5-3500.0) sVCAM-1: 237.0 (122.0-500.0)	NS

CAM = endothelial cell adhesion molecule; sVCAM-1 = soluble Vascular Cell Adhesion Molecule-1; sICAM-1 = soluble Intercellular Adhesion Molecule-1; NE = neutrophil elastase NA = Not available; NS = Not specified; EOS = Early Onset Sepsis; LOS = Late Onset Sepsis; BCPS = Blood Culture Positive Sepsis.

[a] Levels are in mean ± SD, mean ± SEM, median (interquartile range), or median (range).

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Serum concentrations of endothelial cell adhesion molecules and their shedding enzymes and early onset sepsis in newborns in Suriname

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Serum concentrations of endothelial cell adhesion molecules and their shedding enzymes and early onset sepsis in newborns in Suriname

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ABSTRACT

Background: Early Onset Sepsis (EOS) is defined as onset of sepsis within 72 hours after birth. Leukocyte-endothelial interactions play a pivotal part in EOS pathophysiology. Endothelial cell adhesion molecules (CAMs) orchestrate these interactions and their soluble isoforms (sCAMs) are released into the vasculature by enzymes called sheddases.

Purpose: This study was undertaken to explore further the pathophysiology of EOS and to investigate the potential of sCAM and their sheddases as potential biomarkers for EOS.

Methods: Stored serum aliquots were used from 71 Surinamese newborns suspected of EOS and 20 healthy newborns from an earlier study. Serum had been collected within 72 hours after birth and six (8.6%) newborns had a positive blood culture with gram-negative pathogens. Concentrations of sCAMs sP-selectin, sE-selectin, vascular cell adhesion molecule-1 (sVCAM-1), intercellular adhesion molecule-1 (sICAM-1) and platelet and endothelial cell adhesion molecule-1 (sPECAM-1), sheddases matrix metalloproteinase-9 (MMP-9) and neutrophil elastase (NE), and sheddase antagonist tissue-inhibitor of metalloproteinases-1 (TIMP-1) were measured simultaneously with Luminex and ELISA.

Results: MMP-9 and TIMP-1 levels were measured in serum of n=91 newborns and sCAMs and NE levels in serum of n=80 newborns, respectively. We found no differences in median concentrations of sCAMs, MMP-9 and TIMP-1, or NE between blood culture positive EOS, blood culture negative EOS, and control groups at start of antibiotic treatment.

Conclusions: Our data indicate that serum concentrations of sCAMs and their sheddases have no clinical utility as biomarkers for EOS.

Keywords: newborns; early onset sepsis; adhesion molecules; shedding; Suriname.

KEY MESSAGES

What is already known on this topic?

- Recently, we established an association of the Ang-1/Ang-2 disbalance with blood culture positive early onset sepsis (EOS) in newborns.
- The relationship between this Ang-1/Ang-2 disbalance and serum levels of sCAMs and their sheddases is unclear.

What this study adds?

- The Ang-1/Ang-2 disbalance in blood culture positive EOS is not paralleled by increased levels of sCAMs and their sheddases.
- Levels of sCAMs and their sheddases are high after birth and do not discriminate EOS from healthy newborns.

INTRODUCTION

Early onset sepsis (EOS) in newborns within 72 hours after birth remains a clinical challenge with high morbidity and mortality [1-3]. The majority of global neonatal deaths due to EOS occur in developing countries [4]. The diagnosis of EOS is complicated, resulting in late recognition, or overtreatment of newborns with antibiotics. These dilemmas arise because the pathophysiology of EOS is poorly understood.

A hallmark of sepsis pathophysiology is endothelial cell activation followed by leukocyte recruitment into tissues [5]. The current model describes the occurrence of a shift in balance in Tie2 receptor ligands Angiopoietin (Ang)-1 and Ang-2 affecting endothelial integrity, and increased expression of endothelial cell adhesion molecules, in particular P-selectin, E-selectin, vascular cell adhesion molecule (VCAM-1), and intercellular adhesion molecule (ICAM-1) to facilitate this recruitment [6,7]. These endothelial cell adhesion molecules orchestrate leukocyte rolling on, adhesion to, and diapedesis across the endothelium [7,8]. Also, platelet and endothelial cell adhesion molecule (PECAM-1), expressed at endothelial cell junctions has a function in facilitating paracellular transmigration of leukocytes across the endothelium [9]. After intravenous administration of endotoxin in healthy adults as a sepsis model, peak concentrations of Ang-2 prelude the release of soluble isoforms of cell adhesion molecules (sCAMs) into the systemic circulation [10]. Endothelial cell adhesion molecules are released through ectodomain shedding by enzymes called sheddases, in particular matrix metalloproteinase-9 (MMP-9) and neutrophil elastase (NE), released from granules in neutrophils [7,11]. Both MMP-9 and NE prepare the extracellular matrix for transmigration of leukocytes into inflammatory sites [12]. MMP-9 activity is balanced by sheddase antagonist tissue-inhibitor of metalloproteinases-1 (TIMP-1) [12-14].

Recently, we showed in a cohort of near term and term Surinamese newborns that a systemic dysbalance in Ang-2/Ang-1 concentrations was associated with blood culture

positive EOS [15]. This study was undertaken to examine if this dysbalance is paralleled by increased serum concentrations of sCAMs and sheddases in this cohort of newborns with EOS to explore further the pathophysiology of EOS. We hypothesized that sCAM and sheddases concentrations measured at start of antibiotic treatment for suspected EOS are higher in newborns with blood culture positive EOS than in healthy controls.

MATERIALS & METHODS

Study design, subjects and clinical protocol

For this study, we used a Surinamese cohort of 20 healthy newborns and 71 newborns with suspected EOS from an earlier reported study [15]. All newborns were included after admission between April 1 2015 and May 31 2016 to the neonatal care facility of Academic Pediatric Center Suriname at the Academic Hospital Paramaribo in Suriname. Included were newborns with a gestational age equal to or above 34 weeks in whom antibiotics were started within the first 72 hours of life for suspected EOS. Suspicion of EOS was based on the attending physicians decision to start antibiotic treatment. Informed consent was obtained from at least one parent for the use of residual serum and clinical information. The study protocol was made available on clinicaltrials.gov (NCT02486783) and was approved by the Surinamese Medical Ethical Board (VG-021-14A) including permission of one parent.

The management of these patients was described before [15]. In short, healthy control newborns and newborns suspected of EOS were included at start of antibiotic treatment within 72 hours after birth. At start of antibiotic treatment blood was collected for separation and storage of serum. Controls were newborns without signs of infection receiving blood draws for hyperbilirubinemia (n=20). Newborns with suspected EOS receiving treatment with intravenous antibiotics were divided in two groups based on result from blood culturing: blood culture negative EOS (n=65) and blood culture positive EOS (n=6).

Sample collection, preparation and analysis

In the previous study, serum had been collected from whole blood collected after insertion of a venous cannula in newborns suspected of EOS and after capillary collection in controls [15]. Frozen serum samples had been transported on dry ice to the Netherlands and aliquoted and frozen again upon arrival. A stored aliquot was used for the measurement of sP-selectin, sE-selectin, sVCAM-1, sICAM-1, and sPECAM-1 using the Human Magnetic Bead Adhesion 6-plex panel performance assay (LHC0016M, Thermo Scientific, Waltham, MA USA) according to the manufacturer's instructions. ELISA was used on the same aliquots for measurement of neutrophil elastase (HK319-02, Hycult Biotech, Uden, The Netherlands), MMP-9 (Quantikine DMP900, R&D systems, Minneapolis, MN USA), and TIMP-1 (Quantikine DTM100, R&D systems), each according to the manufacturers' instructions. For each molecule, a standard curve was established via which concentrations in neonatal serum were determined. Levels below or above the linear part of this standard curve were reported as the lowest or highest value of the standard curve, respectively. We measured intra-assay variation between plates used in the same assay by calculating coefficient of variation between levels of each molecule in samples from the same patient divided over those plates and accepted a maximum of 20%.

Statistical analysis

A Kruskal-Wallis test with Dunn's correction for multiple comparisons were used for analysis between the blood parameters and the three groups (blood culture positive EOS, blood culture negative EOS, and control groups). P-values <0.05 were considered statistically significant. All analyses were done using Prism version 7.0a (Graphpad Software Inc., San Diego, CA USA).

RESULTS

Demographic variables of the whole study cohort (n=91) can be found in Table 1 of reference 15. Of baseline characteristics birth weight, age at presentation (between 0 and 72 hours after birth), and Apgar score at 5 minutes were distributed unevenly amongst the three groups ($P<0.05$). Blood culture results revealed that 6 of 70 newborns with suspected EOS (8.6%; 95% CI 1.9-15.3%) had a positive blood culture with gram-negative pathogens *Klebsiella pneumoniae* (n=2), *Enterobacter cloacae* (n=2) and *Escherichia coli* (n=2). One newborn had EOS due to a spontaneous bacterial peritonitis. For n=4 others cause of EOS was unknown, but they presented with neonatal jaundice (n=1), perinatal asphyxia (n=1), meconium aspiration (n=1), and hypoglycaemia (n=1).

Serum concentrations of soluble endothelial cell adhesion molecules and their sheddases

Due to the limited amount of serum available, not all molecules could be measured in all samples. We were able to measure MMP-9 and TIMP-1 levels in serum of n=90 newborns and sCAMs and NE levels in serum of n=80 newborns, respectively. We found no differences in median concentrations of sCAMs (Figure 1), MMP-9 and TIMP-1 (Figure 2), or NE (Figure 3) between blood culture positive EOS, blood culture negative EOS, and control groups at start of antibiotic treatment within 72 hours after birth.

DISCUSSION

In this study we investigated whether sCAMs and their sheddases circulate at higher concentrations in near and at term newborns with blood culture positive EOS at start of antibiotic treatment. In contrast to our hypothesis, none of the molecules showed any difference in serum concentrations between blood culture positive EOS, blood culture

negative EOS, and controls within 72 hours after birth. Our data indicate that serum concentrations of sCAMs and their sheddases have no clinical utility as biomarkers for EOS nor to guide the start of antibiotic treatment.

Previously, we found evidence for endothelial cell activation in blood culture positive EOS in the same newborns used for this study, represented by a dysbalance in Ang-2/Ang-1 ratio [15]. Since the current data demonstrate that this dysbalance was not paralleled by increased release of sCAM or sheddases in EOS we conclude that endothelial cell adhesion molecule shedding is not or to a lesser extent involved in the pathophysiology of EOS. For interpretation of our data we reviewed and summarized available data on sCAMs and sheddases in newborns with sepsis in Supplemental Table 1 [16-34]. Comparison of our results with other existing data is complicated because of heterogenic make up of chosen cohorts. Only one study reported a comparable cohort of near and at term newborns with suspected EOS within 72 hours after birth, in whom increased concentrations of sICAM-1 and neutrophil elastase were associated with blood culture positive EOS [19]. Other earlier studies compared concentrations of sCAMs in heterogenic cohorts consisting of newborns with different gestational and postnatal ages, either having EOS (based on varying definitions), or sepsis after 72 hours after birth (i.e., late onset sepsis). This variation in inclusion criteria is an important confounding factor in the interpretation of the observed concentrations in septic and healthy newborns. Overall, our results are in line with these studies that show that clinical utility of sCAMs and sheddases in EOS is very limited.

In an earlier review by our group we pooled published data on sCAM concentrations in newborns [7]. Soluble CAM concentrations in the current study corresponded well with concentrations discussed in our review and those established in earlier studies in uninfected healthy newborns with similar gestational and postnatal age [7, 23,24,29-32]. However, MMP-9, TIMP-1, and neutrophil elastase concentrations were different and up to 4, 2, and

10-fold higher, respectively, than those reported in earlier studies [17,18,21,22,25,30], which may have been due to other methods used (see limitations). Furthermore, our earlier review and earlier data indicated that significant age-related discrepancies exist in sCAM concentrations between newborns, children and adults. As an example, in at term newborns sVCAM-1 concentrations in the first postnatal week were almost twice the concentrations in healthy adults, and equally high compared to septic adults, suggesting that sVCAM-1 concentrations start of high in early newborn life and then decrease with increasing age [7,31]. In our study, concentrations of sCAMs and sheddases during the first 3 days of life in our study remained stable, which was in contrast with earlier work in healthy newborns showing that sE-selectin decreased, and sICAM-1 and sVCAM-1 increased between day 1 and 5 after birth, while sPECAM-1 levels did not change [29-32]. Even though some discrepancies with earlier reports exist, overall one can conclude that these and our data indicate that concentrations of sCAMs and sheddases measured within 72 hours after birth are high and do not discriminate between septic and healthy newborns, which limits their use as biomarkers for early identification or exclusion of EOS.

Our and pre-existing data suggest that overall high sCAM and sheddase concentrations in newborns are the result of other perinatal factors than EOS. Several pathophysiological processes may explain this premise. Birth may induce a 'pro-adhesive' state of the endothelium leading to increased endothelial cell adhesion molecules expression on, and shedding from, its surface. Additionally, the increase in overall leukocyte numbers and inflammatory activation of subsets associated with human birth, which was shown to be positively associated with increased perinatal stress [35-37], may cause higher intensity of leukocyte-endothelial interactions and subsequent increases endothelial cell adhesion molecules shedding. Aberrant adhesion of activated leukocytes to activated endothelium is associated with endothelial dysfunction and increased vascular permeability [38,39].

Shedding of endothelial cell adhesion molecules may then result in prevention of aberrant leukocyte adhesion on two complementary levels, namely 1) to lower endothelial cell adhesion molecules density to prevent adhesion or promote de-adhesion of already adhering leukocytes and 2) to release circulating sCAMs that act as 'decoy receptors' to capture leukocytes in the vasculature to limit leukocyte-endothelial interactions [7,10]. Whether this occurs in real life and what the contribution is to sCAM and sheddase concentrations in newborns remains unknown and could be studied in neonatal animal models [40-42].

Our study has some limitations. First, sample size was relatively small. As a result, logistic regression analysis of other factors, such as maternal perinatal factors or method of birth, potentially influencing levels of sCAMs and sheddases, was precluded. Larger studies in countries such as Suriname, where the incidence of EOS is relatively high in comparison to Western countries [43], are necessary and can contribute to better insight in the vascular pathophysiology of EOS. Second, the use of serum in our study may have caused release of stored pools of MMP-9, TIMP-1, and neutrophil elastase from disrupted leukocytes during the clotting process, which could have accounted for higher levels of these molecules than reported in earlier studies. Last, repeated freeze-thaw cycles may have affected quality of serum samples with regards to reproducibility of NE concentrations.

In conclusion, our data indicate that serum concentrations of sCAMs and sheddases are not higher in Surinamese newborns with EOS versus controls at start of antibiotic treatment. Although concentrations may still increase significantly more in newborns with EOS, other mechanisms, such as perinatal stress during birth, may drive overall high concentrations in all newborns which precludes discrimination between septic and healthy newborns.

ABBREVIATIONS AND DEFINITIONS

EOS = Early onset sepsis

ICAM-1 = Intercellular adhesion molecule-1

VCAM-1= Vascular cell adhesion molecule-1

PECAM-1= Platelet and endothelial cell adhesion molecule-1

MMP-9 = Matrix metalloproteinase-9

TIMP-1 = Tissue-inhibitor of metalloproteinases-1

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest

AUTHOR CONTRIBUTIONS

RZ, MvM, GM, and FBP conceived and designed the study. RZ and AJ collected clinical data and collected the samples. RZ, RMJ, and MvM prepared the samples and performed the sample analysis. RZ and MvM analyzed the final database. RZ, MvM, GM, and FBP drafted the manuscript. All authors co-authored and approved the final manuscript.

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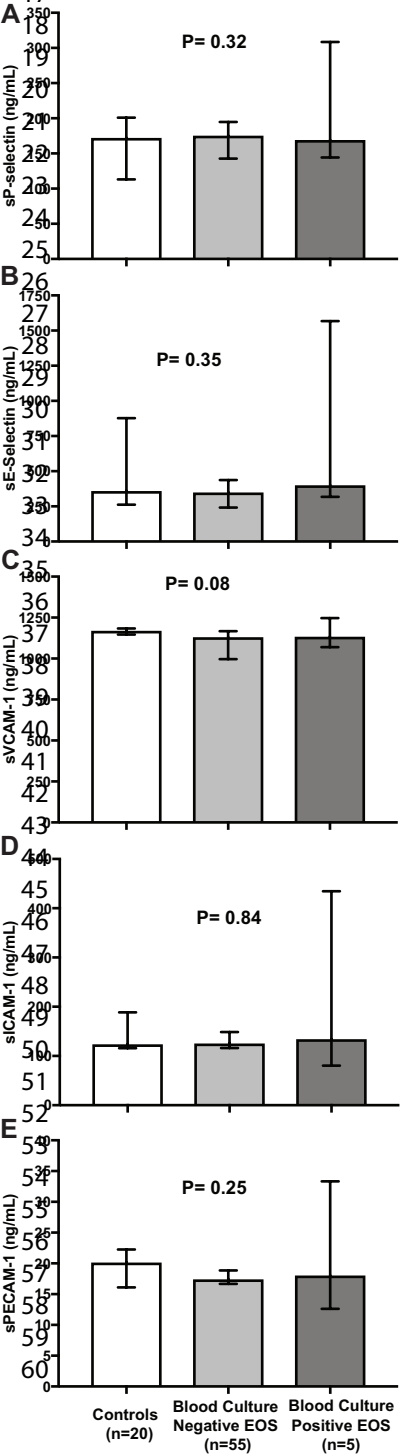
FIGURE LEGENDS

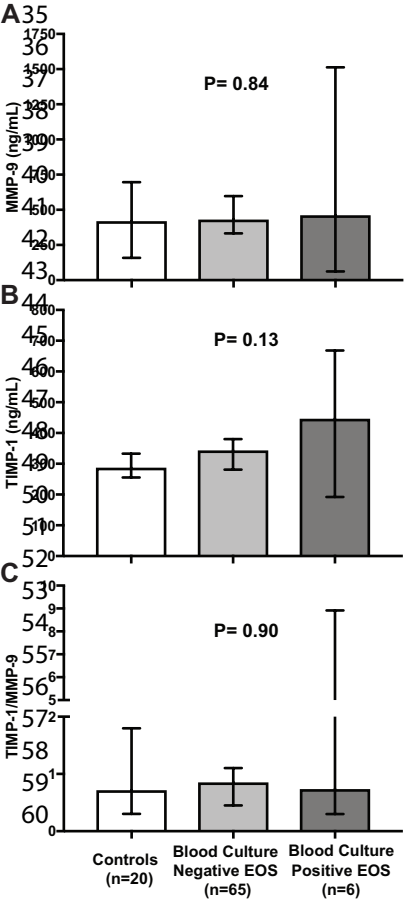
Figure 1.

Circulating levels of endothelial adhesion molecules sP-selectin, sE-selectin, sVCAM-1, sICAM-1, and sPECAM-1 in Surinamese newborns. A: sP-selectin **B:** sE-selectin **C:** soluble vascular cell adhesion molecule-1 (sVCAM-1); **D:** soluble intercellular adhesion molecule-1 (sICAM-1); **E:** soluble platelet and endothelial cell adhesion molecule-1 (sPECAM-1). Bars represent median values and error bars 95% confidence intervals. P-values <0.05 were considered statistically significant.

Figure 2. Circulating levels of MMP-9 and TIMP-1, and TIMP-1/MMP-9 ratios in Surinamese newborns. A: Matrix metalloproteinase-9 (MMP-9); **B:** Tissue inhibitor of metalloproteinase (TIMP-1); **C:** TIMP-1/MMP-9 ratios. Bars represent median values and error bars 95% confidence intervals. P-values <0.05 were considered statistically significant.

Figure 3. Circulating levels of neutrophil elastase in Surinamese newborns. Bars represent median values and error bars 95% confidence intervals. P-values <0.05 were considered statistically significant.





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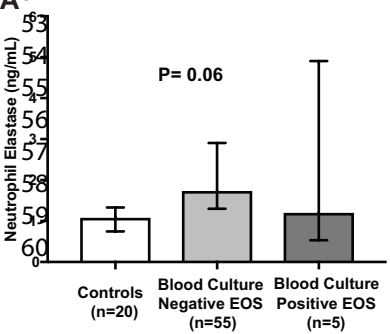

Supplemental Table 2: Studies reporting levels of endothelial cell adhesion molecules and shedding enzymes in newborns

Study (ref.), year	CAM or shedding enzyme	Cohort Characteristics	Gestational age (weeks)	Postnatal age	Main Results	AnalysisMethod	Healthy (ng/mL)[1]	Septic (ng/mL)[1]
Fatah et al. (16), 2017	sE-selectin	EOS and LOS	NS	NS	sE-selectin **elevated** in BCPS	ELISA	148.9 ± 7.9	177.1 ± 3.5
Weitkamp et al. (17), 2016	MMP-9	EOS and LOS	25-36	< and ≥ 3 days	MMP-9 levels **lower** in BCPS	Multiplex bead assay	NS	NS
Wynn et al. (18), 2015	MMP-9	Chorioamnionitis	25-36	NS	MMP-9 levels **lower** in chorioamnionitis	Multiplex bead assay	NS	NS
Sugitharini et al. (19), 2013	sICAM-1, NE	EOS	34-42	0-72 hours	sICAM-1 and NE **elevated** in EOS	ELISA Antibody array	sICAM-1/ NE: NS	sICAM-1: NS NE:499.2±22.0
Edgar et al. (20), 2010	sICAM-1, sE-selectin	EOS and LOS	24-41	NS	sICAM-1 and sE-selectin **elevated**	ELISA	sICAM-1: 165 (130-290) sE-selectin: 71 (51-118)	sICAM-1: 405 (252-666) sE-selectin 158 (94-207)
Fukanaga et al. (21), 2009	MMP, TIMP-1	Uninfected newborns	<30	Cord blood	No difference	ELISA	MMP-9: 22 (16-48) TIMP-1: 122 (86-249)	NS
Sunagawa et al. (22), 2009	MMP-9, TIMP-1	Uninfected newborns	35-41	1-2 days	NA	ELISA	NS	NA
Figueras et al. (23), 2007	sICAM-1, sVCAM-1, sP-selectin,	EOS and LOS	32-40	1-32 days	sICAM-1 and sVCAM-1 **increased** over time.	ELISA	sICAM-1: 156 (150-194) sVCAM-1: 856 (742-960) sP-selectin: 272 (152-288)	sICAM-1: 394 (342-600) sVCAM-1: 1153 (726-1307) sP-selectin: 244 (170-324)
Sitaru et al. (24), 2005	sP-selectin	Chorioamnionitis	25-40	Cord blood	sP-selectin **elevated** in chorioamnionitis	ELISA	104 ± 71	222 ± 128
Schulz et al. (25), 2004	MMP-9, TIMP-1	Uninfected newborns	25-40	1-28 days	MMP-9 highest in preterm TIMP-1 highest in at term	ELISA	NS	NA
Edgar et al.	sICAM-1	EOS and LOS	24-42	NS	sICAM-1 **elevated** in	ELISA	205 (146-343)	406 (345-1180)

(26), 2002					BCPS			
Apostolou et al. (27), 2002	sICAM-1	EOS and LOS	25-42	NS	sICAM-1 **elevated** in BCPS	ELISA	358.4 ± 28.9	710.7 ± 56.6
Dollner et al. (28), 2001	sICAM-1, sE-selectin	EOS and LOS	30-42	1-7 days	sICAM-1 and sE-selectin **elevated** in BCPS	ELISA	sICAM-1: 244.0 (92.5-500) sE-selectin: 91.4 (<2.0-217.8)	sICAM-1: 357.4 (141.6-500) sE-selectin: 151.7 (37.0-362.2)
Malamitsi et al. (29), 2000	sVCAM-1, sPECAM-1	Uninfected newborns	37-40	1-5 days	No change between day 1 and 5	ELISA	sVCAM-1: 1340 ± 58.3 sPECAM-1: 17.5 ± 0.7	NA
Giannaki et al. (30), 2000	sE-selectin	Uninfected newborns	At term	1-5 days	sE-selectin **decreases** between day 1 and 5	ELISA	139 ± 48	NA
Giannaki et al. (31), 1999	sICAM-1, sVCAM-1	Uninfected newborns	At term	1-5 days	sICAM-1 and sVCAM-1 **increase** between day 1 and 5	ELISA	sICAM: 179 ±56.1 sVCAM-1: 1125.0 ± 281.0	NA
Phocas et al. (32), 1998	sICAM-1	Uninfected newborns	35-42	1-30 days	sICAM-1 **increases** between day 1, 5 and 30	ELISA	137.3 ± 62.0	NA
Berner et al. (33), 1998	sICAM-1	EOS	26-42	0-96 hours	sICAM-1 **lower** in EOS. sICAM-1 **increases** over time	ELISA	421 (291-459)	446 (171-534)
Austgulen et al. (34), 1997	sICAM-1, sVCAM-1, sE-selectin	EOS and LOS, pneumonia	24-42	0-162 hours	sE-selectin and sICAM-1 **elevated** in infected neonates	ELISA	sE-selectin: 84.2 (21.6-231.3) sICAM-1: 2131.3 (1449.5-3500.0) sVCAM-1: 237.0 (122.0-500.0)	NS

CAM = endothelial cell adhesion molecule; sVCAM-1 = soluble Vascular Cell Adhesion Molecule-1; sICAM-1 = soluble Intercellular Adhesion Molecule-1; NE = neutrophil elastase NA = Not available; NS = Not specified; EOS = Early Onset Sepsis; LOS = Late Onset Sepsis; BCPS = Blood Culture Positive Sepsis.

[a] Levels are in mean ± SD, mean ± SEM, median (interquartile range), or median (range).