PEER REVIEW HISTORY

BMJ Paediatrics Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

This paper was submitted to another journal from Archives of Disease in Childhood but declined for publication following peer review. The authors addressed the reviewers' comments and submitted the revised paper to BMJ Paediatrics Open. The paper was subsequently accepted for publication at BMJ Paediatrics Open.

ARTICLE DETAILS

TITLE (PROVISIONAL)	Serum concentrations of endothelial cell adhesion molecules and their shedding enzymes and early onset sepsis in newborns in Suriname
AUTHORS	Zonneveld, Rens; Jongman, Rianne; Juliana, Amadu; Molema, Grietje; Meurs, Matijs; Plötz, Frans

VERSION 1 - REVIEW

REVIEWER	Reviewer name: Van Rossum, Annemarie M.C. Reviewer Affiliation: Erasmus MC University Medical Center-Sophia Children's Hospital, Department of Pediatrics, Division of Infectious Diseases and Immunology Rotterdam Competing interest statement: No conflicts of interests
REVIEW RETURNED	30-Mar-2018

GENERAL COMMENTS	This is a comprehensive original article in which the results of an observational study on a panel of soluble CAMs and their shedding enzymes in serum of 91 newborns was measured. The aim of the study is not very consistent and clearly articulated, but early onset neonatal sepsis is an important topic in which additional knowledge on pathophysiology and biomarkers to discriminate diseased from healthy neonates is very much needed. The investigators report negative findings that are important to report. Comments: Page 3, line 31: the objective of this study could be more specific. Why did the investigators want to know whether the increase in Ang-2 levels was paralleled by an increase in sCAMs and sheddase levels. Was the hypothesis that these levels could discriminate better than Ang-2 levels? Are these levels easier to measure? Or was it interest in underlying mechanisms as is suggested in the first sentence of the discussion? - page 3, line 48. Is informed consent obtained from only one parent allowed in Surinam? Or was there a waiver? - Page 4, line 5 blood sample taken at t=0 and t=48-72 uur. Why has this time-point been chosen? Was this based on kinetics or because of clinical relevance for example? Please add. - Page 4, line 27-33: the number of available serum samples should be moved to the results section. - Methods section: add when and how bloodcultures have been done.

	- Page 5 Results section: baseline clinical characteristics are lacking. I would like to see baseline characteristics for blood-culture positive, blood culture negative and controls. - Page 5 How was the suspicion for EOS in the blood culture negative EOS patients defined? This should be added to the methods section. - Page 5: results section. please add which pathogens were found in the patients with positive blood culture. - Page 6, line5-12: this could be more specific: how many studies have been done, how many reported positive, how many negative results and add references. - Page 6, line 42 "Larger studies in countries as Surinam, where we expect incidence of EOS to be relatively high.." Expect or is high? What is the incidence? - Page 6, line 48 "...higher levels of these molecules than reported earlier": have these studies also used serum or have they used plasma? - page 9 Table 1 this table is not very helpful. I would suggest to use a graph in stead

REVIEWER	Reviewer name: Mazzucchelli, Iolanda Reviewer Affiliation: University of Pavia, Department of Internal Medicine and Therapeutics Competing interest statement: I declare that I have no conflicts of interest
REVIEW RETURNED	09-Apr-2018

GENERAL COMMENTS	The authors reported data of soluble cell adhesion molecules (sCAMs) and their shedding enzymes in Suriname newborns, healthy or affected by EOS and they tried to evaluate if plasma levels of these factors were correlated with septic condition. In the third point of “What is already know” the authors have written “The relationship between ……..and their sheddases is unclear”: this suggests it isn’t known. They have to cut this point. The type of study design has to be reported. The studied population consists of 6 newborns affected by EOS with blood culture positive, 65 affected by EOS with blood culture negative (how did the authors make the diagnosis of EOS?) and 20 healthy controls: 6 newborns affected by culture-proven EOS looks a very small population to reach the aim of the study. Are the authors sure that their data are sufficient to evaluate the role of sCAMs in the pathophysiology of sepsis? CAMs is an acronym of Cell Adhesion Molecules not of Endothelial Cell Adhesion Molecules nor endothelial adhesion molecules. The authors have to report that sCAMs were quantified by Luminex. The authors have to clarify better how many evaluations they made for each group, for each protein and for each time. The kit used to measure sCAMs tests at the same time also PAI-1 protein. The authors didn’t report any information about this protein, why? The authors reported that “they investigated whether sCAMs and their sheddases are involved in the pathophysiology of EOS.” Reading the paper I understand that the authors have quantified sCAMs and sheddases in healthy newborns (n=20)

	in newborns with proven EOS (n=6) and in suspected EOS (n=65, that represents 71% of the studied population). I think that to give information on the pathophysiology of sepsis and the possible relationship with sCAMs the large cohort of 65 newborns should be better assessed for their diagnosis/clinical condition.

VERSION 1 – AUTHOR RESPONSE

Reviewer #1

This is a comprehensive original article in which the results of an observational study on a panel of soluble CAMs and their shedding enzymes in serum of 91 newborns was measured. The aim of the study is not very consistent and clearly articulated, but early onset neonatal sepsis is an important topic in which additional knowledge on pathophysiology and biomarkers to discriminate diseased from healthy neonates is very much needed. The investigators report negative findings that are important to report.

Comments:

> Page 3, line 31: the objective of this study could be more specific. Why did the investigators want to know whether the increase in Ang-2 levels was paralleled by an increase in sCAMs and sheddase levels. Was the hypothesis that these levels could discriminate better than Ang-2 levels? Are these levels easier to measure? Or was it interest in underlying mechanisms as is suggested in the first sentence of the discussion?

REPLY: To underscore the objectives of our study we rewrote the introduction, in particular to emphasize the involvement of Angiopoietins and Cellular Adhesion molecules during sepsis. However, most studies are performed in adults and little is known about the pathophysiology of early onset sepsis in newborns. This is also confirmed by the reviewer in the general comments. Our present study was therefore undertaken to further explore the pathophysiology of EOS. We rewrote the purpose of our study: “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.”

> - page 3, line 48. Is informed consent obtained from only one parent allowed in Surinam? Or was there a waiver?

REPLY: The Surinamese Medical-Ethical Board approved a single parent informed consent.

> - Page 4, line 5 blood sample taken at t=0 and t=48-72 uur. Why has this time-point been chosen? Was this based on kinetics or because of clinical relevance for example? Please add.

REPLY: We choose this time point because according to clinical protocol the results of blood culture became available and a decision was made to stop or to continue antibiotics. We added in the manuscript: “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.”

> - Page 4, line 27-33: the number of available serum samples should be moved to the results section.

REPLY: As suggested by the reviewer we have moved this to the result section: “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.”

> - Methods section: add when and how blood cultures have been done.

REPLY: We have added this in the methods section: “At t=0, before start of antibiotic treatment, 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.”

> - Page 5 Results section: baseline clinical characteristics are lacking. I would like to see baseline characteristics for blood-culture positive, blood culture negative and controls.

REPLY: Since this study used a Surinamese cohort of 20 healthy newborns and 71 newborns with suspected EOS from an earlier reported study we prefer to refer for baseline characteristics to our previously paper as well as to supplemental data provided. We added Supplementary Table 1 to the manuscript.

> - Page 5 How was the suspicion for EOS in the blood culture negative EOS patients defined? This should be added to the methods section.

REPLY: We have added this in the methods section by adding “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).”

> - Page 5: results section. please add which pathogens were found in the patients with positive blood culture.

REPLY: We have added this in the results section, namely “… blood culture with gram-negative pathogens Klebsiella pneumoniae (n=2), Enterobacter cloacae (n=2) and Escherichia coli (n=2).”

> - Page 6, line5-12: this could be more specific: how many studies have been done, how many reported positive, how many negative results and add references.

REPLY: provide a comprehensive overview of these studies we have added Supplemental Table 2 to the manuscript in which we summarize a total of 19 papers on this subject. To our knowledge, these are all studies written in English on EOS and the involvement of CAMs and sheddases.

> - Page 6, line 42 "Larger studies in countries as Surinam, where we expect incidence of EOS to be relatively high.." Expect or is high? What is the incidence?

REPLY: The only available detailed information on incidence of EOS in Suriname can be found in a retrospective study from members of our group (Zonneveld et al., “Improved referral and survival of newborns after scaling up of intensive care in Suriname.” BMC Pediatr 2017;17(1):189). In this study we report an incidence between 30 and 40% of total sepsis at the hospital (Neonatal care facility of the Academic Pediatric Center Suriname) where this study was undertaken. Half of this number was EOS and about 30% of admitted newborns empirically received antibiotics. These numbers indicate a huge burden of EOS, but are not yet conclusive for the whole Surinamese situation, since they do not represent EOS occurring at other hospitals and birth clinics in the country. We are currently prospectively evaluating true incidence of EOS, and we expect incidence to be high based on our initial data.

> - Page 6, line 48 "...higher levels of these molecules than reported earlier": have these studies also used serum or have they used plasma?

REPLY: The majority (four out of six) of the studies referenced here used plasma, instead of serum.

> - Page 9 Table 1 this table is not very helpful. I would suggest to use a graph instead

REPLY: As suggested by the reviewer we included three graphs to show our data.

Reviewer #2

The authors reported data of soluble cell adhesion molecules (sCAMs) and their shedding enzymes in Suriname newborns, healthy or affected by EOS and they tried to evaluate if plasma levels of these factors were correlated with septic condition.

> In the third point of “What is already know” the authors have written “The relationship between ……..and their sheddases is unclear”: this suggests it isn’t known. They have to cut this point.

REPLY: We have changed this into the text below.

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.

> The type of study design has to be reported.

REPLY: We refer in the methods section that for this study, we used a Surinamese cohort of 20 healthy newborns and 71 newborns with suspected EOS from an earlier reported study, which was an observational study.

> The studied population consists of 6 newborns affected by EOS with blood culture positive, 65 affected by EOS with blood culture negative (how did the authors make the diagnosis of EOS?) and 20 healthy controls: 6 newborns affected by culture-proven EOS looks a very small population to reach the aim of the study. Are the authors sure that their data are sufficient to evaluate the role of sCAMs in the pathophysiology of sepsis?

REPLY: We agree with the reviewer that the number of blood culture positive patients is small. However, this number is significantly higher than reported in Western or developed countries. Incidence rates than are approximately 0,1%. In this respect, our reported number is high, in particular compared to total sample number. The diagnosis of suspected EOS remains difficult. We choose to define newborns in whom antibiotics were started within 72 hours after birth because of maternal risk factors and/or clinical condition of the newborn, but in whom blood culture results were negative after 48-72 hours after sampling.

In contrast to our previous report where we did find significant changes in levels of the Angiopoietins, we were now unable to find any differences between all three groups. Interestingly, absolute numbers in all groups were higher than reported in adult septic patients suggesting other factors involved in these newborns. We think, despite a negative finding, it is well worth to publish these results, in agreement with the first reviewer.

> CAMs is an acronym of Cell Adhesion Molecules not of Endothelial Cell Adhesion Molecules nor endothelial adhesion molecules.

REPLY: The reviewer is correct and we have changed this at its first mentioning and throughout the manuscript.

> The authors have to report that sCAMs were quantified by Luminex.

REPLY: In the methods section we added how the assays were performed: “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.”

> The authors have to clarify better how many evaluations they made for each group, for each protein and for each time.

REPLY: Please also the comments made by the first reviewer. We have added this in the results section: ”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.”

> The kit used to measure sCAMs tests at the same time also PAI-1 protein. The authors didn’t report any information about this protein, why?

REPLY: Although part of the Human Magnetic Bead Adhesion 6-plex, PAI-1 is essentially a marker of fibrinolysis and intravascular coagulation and it has been evaluated as a marker for human sepsis. However, for this study, we were interested in the endothelial cellular adhesion molecules and their sheddases as markers of leukocyte-endothelial interaction and endothelial integrity during EOS. To our knowledge PAI-1 is not similarly involved in these pathophysiological processes. Please also see our introduction were we explain the ratio and purpose of our study for focusing on endothelial cellular adhesion molecules and sheddases.

> The authors reported that “they investigated whether sCAMs and their sheddases are involved in the pathophysiology of EOS.” Reading the paper I understand that the authors have quantified sCAMs and sheddases in healthy newborns (n=20) in newborns with proven EOS (n=6) and in suspected EOS (n=65, that represents 71% of the studied population). I think that to give information on the pathophysiology of sepsis and the possible relationship with sCAMs the large cohort of 65 newborns should be better assessed for their diagnosis/clinical condition.

REPLY: Please also see our previous reply on sample size. The diagnosis of EOS remains difficult. We chose to define newborns in whom antibiotics were started within 72 hours after birth because of maternal risk factors and/or clinical condition of the newborn, but in whom blood culture results were negative after 48-72 hours after sampling.

VERSION 2 – REVIEW

REVIEWER	Reviewer name: AMC van Rossum Institution and Country: Erasmus MC University Medical Center Rotterdam, the Netherlands Competing interests:no
REVIEW RETURNED	11-Jun-2018

GENERAL COMMENTS	Overall the authors did a good job in rewriting and addressing the main issues with this manuscript. Specific comments: TEXT: - page 3, line 63 in the methods section of the abstract the blood cultures drawn (essential in this study) are lacking - page 7, line 171: serum samples were available of all 91 newborns at t=0, but only of 51 at t= 48-72h. The authors should analyze and report whether the group with a sample taken at t = 48-72 hours is similar (with respect to baseline characteristics) to the group without a sample taken at this timepoint to make a selection bias less likely. - page 8, line 196-197. This sentence suggests that serum for the current study has been frozen before analysis as the serum of neonates of a already published study has been used. If that is correct, how sure are the authors that freeze-thaw cycle did not affect the results of this study. Please add to the discussion either as a limitation or as what is known. - Page 11, line 261-262 "Other mechanisms, ..., may drive overall high levels in all newborns, which precludes discrimination between septic and healthy newborns etc.". This might preclude discrimination, but does not necessarily precludes discrimination. Basline levels could be higher in neonates, but still increase significantly more in septic neonates as compared to healthy neonates as is the case in other biomarkers used in neonates. Please correct this in the conclusion of the main body text. - Suppl table 1. What is the p value for, the difference between what, controls versus EOS, or bloodculture negative vs blood culture positive EOS? - Suppl table 1: would suggest to reference to the paper in reference 15 and only summarize the baseline characteristics if there are copyright and/or space issues. AUTHORS REPLY TO COMMENTS: - Reply to "page 5 how was the suspicion for EOS in the blood culture negative EOS patients defined?" does not answer what the suspicion was based on. This was probably pragmatic, when according to the attending physician antibiotics were started. This approach of how neonates were suspected of EOS should be added. - Page 6, line 5-12 "this could be more specific: how many studies have been done, how many reported positive, how many negative with references". The added Table 2 is impressive, but also extensive. If there is space for this, I think it has added value, but otherwise this could still be summarized as mentioned in the text of the discussion.

	- Reply to Page 6, line 42 incidence in Surinam. Please add the reference to the manuscript.

REVIEWER	Reviewer name: Eirini Koutoumanou Institution and Country: UCL, UK Competing interests: none
REVIEW RETURNED	29-Jun-2018

GENERAL COMMENTS	This is a rather heavy report on the clinical and technical front and my opinion is that it might be best suited to an alternative journal. My biggest concern is the fact that the authors have not presented nor analysed the data in a paired manner. Data were collected serially at time 0 and 48-72 hours later. This serial nature has been lost in the presentation (line graphs should have been presented instead of bar graphs of the means) and paired tests should have been used to compare the differences of various outcomes. These two issues will have to be rectified first before any further comments can be made on the content of this paper. Some additional smaller comments can be found below: - It would have been useful to know which hospital/area the data came from without having to refer to the study that contained more details about the original data. - IQR is not an alternative to confidence intervals (CI) for non-normal data. Instead CIs for medians can be calculated and they should be presented alongside any medians. - Graphs A and B of Figure 1 have not printed well and cannot be seen/seen clearly

VERSION 2 – AUTHOR RESPONSE

Reviewer: 1

Overall the authors did a good job in rewriting and addressing the main issues with this manuscript.

Reply: We would like to take the opportunity to thank Dr. Van Rossum for reviewing our manuscript. Her comments were very helpful for improving the revised manuscript. We have addressed her specific comments below.

Specific comments:

Page 3, line 63 in the methods section of the abstract the blood cultures drawn (essential in this study) are lacking

Reply: We agree that this information is essential for the abstract. We have changed the methodology section of the abstract accordingly.

Page 7, line 171: serum samples were available of all 91 newborns at t=0, but only of 51 at t= 48-72h. The authors should analyze and report whether the group with a sample taken at t = 48-72 hours is similar (with respect to baseline characteristics) to the group without a sample taken at this time point to make a selection bias less likely.

Reply: We thank the reviewer for this suggestion. For the revision of this paper the second reviewer suggested analysis of the serial results in a paired manner. However, the sample size at t=48-72h was too small, which led us to exclude this time point from the analysis. As a result, the baseline characteristics at t=48-72h needed no further analysis.

Page 8, line 196-197. This sentence suggests that serum for the current study has been frozen before analysis as the serum of neonates of a already published study has been used. If that is correct, how sure are the authors that freeze-thaw cycle did not affect the results of this study? Please add to the discussion either as a limitation or as what is known.

Reply: Samples were frozen once after collection and thawed upon arrival after transport to the Netherlands before aliquoting. Aliquots were used for final analysis. Thus, freeze-thaw cycles were limited to two. Earlier reports have indicated up to five repeated freeze-thaw cycles did not significantly affect serum and plasma concentrations of soluble adhesion molecules (Nash et al., Clin Exp Immunol 1995, 101:13-7 and Hosnijeh et al., Biomarkers 2010, 15(2):140-148) for different assays (i.e., bead assays and ELISA). Six repeated freeze-thaw cycles did not affect TIMP-1 (Kisand et al., Clin Chem Lab Med 2011, 49(2):229-35), and eight repeated cycles did not affect MMP-9 (Lourbakos et al., Sci Rep 2017, 7(1):17888) serum concentrations. For neutrophil elastase no data exists. We have added a statement regarding this to the limitations part in the discussion section on page 11, line 241-242.

- Page 11, line 261-262 "Other mechanisms, ..., may drive overall high levels in all newborns, which precludes discrimination between septic and healthy newborns etc.". This might preclude discrimination, but does not necessarily precludes discrimination. Basline levels could be higher in neonates, but still increase significantly more in septic neonates as compared to healthy neonates as is the case in other biomarkers used in neonates. Please correct this in the conclusion of the main body text.

Reply: We agree with the reviewer and changed the final sentence of the manuscript.

- Supplemental table 1. What is the p value for, the difference between what, controls versus EOS, or blood culture negative vs blood culture positive EOS?

Reply: For reasons described below, we have removed supplemental table 1. The original p value referred to the comparison between all three groups. We added a statement on distribution of variables between the study groups to the Results section on page 8, line 153-156.

Reviewer: 2

This is a rather heavy report on the clinical and technical front and my opinion is that it might be best suited to an alternative journal.

My biggest concern is the fact that the authors have not presented nor analysed the data in a paired manner. Data were collected serially at time 0 and 48-72 hours later. This serial nature has been lost in the presentation (line graphs should have been presented instead of bar graphs of the means) and paired tests should have been used to compare the differences of various outcomes. These two issues will have to be rectified first before any further comments can be made on the content of this paper.

Reply: We would like to thank Dr. Eirini Koutoumanou for reviewing our manuscript. After careful review of the data, we presented this matter to our institutional clinical epidemiologist (acknowledged in the new version of the paper). We found that the number of samples at t=48-72 hours is too small for a proper serial analysis with meaningful results. We have decided to remove these samples from our analysis.

Results remain the same for the first time point and, in our opinion, the conclusions are still valid for the first 72 hours after birth. We amended our conclusions in the abstract and final conclusions of the paper accordingly.

Some additional smaller comments can be found below:

It would have been useful to know which hospital/area the data came from without having to refer to the study that contained more details about the original data.

Reply: We agree with the reviewer and added a statement to the Study design, subjects and clinical protocol paragraph in the Materials & Methods section on page 4. We also changed the Statistical Analysis paragraph accordingly on page 7.

IQR is not an alternative to confidence intervals (CI) for non-normal data. Instead CIs for medians can be calculated and they should be presented alongside any medians.

Reply: We have calculated 95% confidence intervals for all medians and presented them in the figures. We have changed all Figure Legends accordingly on page 19.

Graphs A and B of Figure 1 have not printed well and cannot be seen/seen clearly

Reply: We apologize for this and we hope all new figures have printed properly.