**Airway angiopoietin-2 in ventilated very preterm infants:
association with prenatal factors and neonatal outcome**

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1 Airway angiopoietin-2 in ventilated very preterm infants: association with prenatal factors and
2 neonatal outcome

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23 Abbreviated title: Angiopoietin-2 in airways of ventilated preterm infants

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Abstract

Objective: Pulmonary angiogenesis is a prerequisite for lung development. Angiopoietin-2 (Ang2) destabilizes endothelial cells through its endothelial receptor TIE-2, enabling vascular sprouting. Ang1 stabilizes new blood vessels. Soluble TIE-2 (sTIE-2) modulates these effects. We hypothesized that histological funisitis is associated with alterations of Ang2 in airways and of the systemic angiopoietin-TIE-2 homeostasis in very low birth weight infants (VLBW), contributing to pulmonary morbidity and mortality.

Methods: We measured Ang2 in tracheobronchial aspirate fluid (TAF) of 42 VLBW < 30 weeks of gestation from day 1 through 15 and Ang1, Ang2 and sTIE-2 in umbilical cord serum of 28 infants by ELISA. Histological examination distinguished 3 groups: funisitis, chorioamnionitis and controls.

Results: Funisitis was associated with lower Ang2 values in TAF but not with changes of Ang1, Ang2 and sTIE-2 in umbilical cord serum. Infants who developed BPD or died had a persistently decreased ratio of previously measured Ang1 to Ang2 in TAF on days 1-5 and increased cord serum concentrations of sTIE-2. Moderate BPD/death was associated with an increase of Ang2 in TAF on day 10 and decreased Ang1/Ang2 ratio from day 3-15. Small for gestational age (SGA) infants had increased Ang2 in TAF on day 1-7 and a lower Ang1/Ang2 ratio on days 5-7.

Conclusions: The predominance of Ang2 in airway fluid of infants with BPD/death and SGA infants suggests a link between disrupted placental and fetal pulmonary angiogenesis. Histological funisitis with reduced Ang2 in TAF was of minor relevance for outcome in our cohort.

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4 1 **Introduction**

5
6 2 Angiogenesis plays a pivotal role in organ development and maturation¹. In extremely
7
8 3 premature infants disturbed pulmonary angiogenesis accompanies alveolar simplification,
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10 4 eventually leading to bronchopulmonary dysplasia (BPD)²⁻³.
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12 5 *Angiopoietin-2* (Ang2) belongs to a family of angiogenic growth factors which bind
13
14 6 specifically to the *tyrosine kinase receptor with immunoglobulin and epidermal growth factor*
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16 7 *homology domain 2* (TIE-2) on endothelial cells. Ang2 destabilizes the endothelium by
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18 8 decreasing the phosphorylation status of TIE-2, leading to a more responsive endothelium, a
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20 9 prerequisite for sprouting of new vessels⁴. The endothelial destabilization also leads to
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22 10 vascular leakage which is a feature of inflammation⁵. Binding of the Ang2 antagonist
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24 11 *angiopoietin-1* (Ang1) to TIE-2 induces tightening of endothelial intercellular junctions,
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26 12 leading to stabilization and maturation of newly formed vessels⁶. Both angiopoietins act
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28 13 synergistically in the process of angiogenesis⁴. A soluble form of TIE-2 (sTIE-2), released
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30 14 from the endothelium after proteolytical cleavage of the receptor's extracellular domain,
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32 15 interferes with the angiopoietin-TIE-2 signaling by binding the free ligands⁷.
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34 16 A differential spatial and temporal regulation of TIE-2 by Ang1 and Ang2 in the lung could
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36 17 be shown a prerequisite for a stable pulmonary vasculature and normal lung development and
37
38 18 maturation in a murine model⁸. Increased concentrations of Ang2 in tracheobronchial aspirate
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40 19 fluid (TAF) have been associated with the development of BPD or death in ventilated
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42 20 premature infants⁹.
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44 21 The role of angiopoietins and TIE-2 in systemic inflammatory processes and pulmonary
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46 22 injury has been extensively addressed. In a pediatric population, high plasma Ang2 levels and
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48 23 low Ang1 levels were associated with septic shock¹⁰. Serum concentrations of sTIE-2 have
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50 24 been shown elevated in adult septic patients at risk for adult respiratory distress syndrome
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52 25 compared to non-septic patients¹¹. However, data about blood levels of the angiopoietins and
53
54 26 their receptor in very premature infants exposed to prenatal inflammation do not exist.

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1 Histological funisitis is regarded as morphological counterpart of a fetal systemic
2 inflammatory response¹² and has been shown associated with down-regulation of angiogenic
3 factors in placental tissue¹³. This fetal inflammatory response has been identified as risk factor
4 for BPD¹⁴ and mortality¹⁵ after premature birth.

5 The primary aim of our exploratory pilot study was to assess the association between
6 histological funisitis and postnatal airway concentrations of Ang2 in ventilated very
7 premature infants. We also investigated concentrations of the factors Ang1, Ang2 and sTIE-2
8 in umbilical cord blood. We speculated that alterations of pulmonary Ang2 and a disturbed
9 homeostasis of the angiopoietin-TIE-2 system after funisitis might contribute to pulmonary
10 morbidity and neonatal mortality of this high risk group of patients.

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4 1 **Material and Methods**

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6 2 *Patients*

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8 3 42 infants with a birth weight <1500 g and a gestational age < 30 weeks, who were admitted
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10 4 to the intensive care unit of the Department of Neonatology, Poznań University of Medical
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12 5 Sciences, Poland, between November 2004 and August 2005, were enrolled in this
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14 6 prospective study after informed consent of the parents. Patients were inborns, had to be
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16 7 mechanically ventilated for respiratory failure within the first 48 hours of life and had no
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18 8 major congenital malformations. Respiratory support was adjusted to reach a SpO₂ between
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20 9 85% and 94%. Infants with supplemental oxygen for at least 28 days were assigned to have
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22 10 BPD¹⁶. Depending on the need for oxygen or respiratory support at a postmenstrual age of 36
23
24 11 weeks, BPD was graded as mild, moderate or severe¹⁶. The study was approved by the Ethical
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26 12 Committees of the Poznań University of Medical Sciences and the Faculty of Medicine,
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28 13 University of Wuerzburg, Germany.
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37 15 *Histological examinations*

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39 16 For each infant at least 2 sections of umbilical cord, 2 sections of placental parenchyma and 1
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41 17 section of membrane rolls were evaluated for the presence and severity of neutrophilic
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43 18 infiltrates by two pathologists, unaware of clinical data, according to a protocol¹⁷. Patients
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45 19 were assigned to three groups according to histological diagnosis. The first group was
46
47 20 characterized by chorioamnionitis and funisitis and will be referred to as funisitis group
48
49 21 (n=17). In the second group chorioamnionitis without funisitis was diagnosed
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51 22 (chorioamnionitis group, n=9). The control group consisted of 16 patients without histological
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53 23 signs of inflammation in placenta, membranes and umbilical cord.
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4 1 *Sampling, preparation and storage*

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6 2 Tracheobronchial aspiration was performed in a standardized manner up to 3 times per day on
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8 3 days 1, 3, 5, 7, 10 and 15 of life. After instillation of 0.5 ml NaCl 0.9% into the endotracheal
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10 4 tube a suction catheter was inserted slightly beyond the distal tip of the tube and airway
11
12 5 secretions were suctioned and collected in sterile mucus traps (Unomedical, Birkerød,
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14 6 Denmark). Each specimen was diluted with NaCl 0.9% to a volume of 0.5 ml. Specimens of
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16 7 one day were pooled. They were spun at 3,000 revolutions per minute (rpm) for 5 minutes.
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18 8 Supernatants were centrifuged again twice at 10,000 rpm for 5 minutes. Specimens were
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20 9 cooled during processing, including centrifugation. The resulting clear supernatants
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22 10 were divided into aliquots and stored at -80°C until further analysis.
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27 11 Umbilical cord blood could be collected from the umbilical cord vein of 28 infants. Blood
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29 12 was centrifuged at 3,000 rpm for 10 minutes and the serum stored at -80°C until analysis.
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34 14 *Measurement of factors and secretory component for IgA*

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36 15 Concentrations of Ang1, Ang2 and sTIE-2 in umbilical cord serum and levels of Ang2 in
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38 16 TAF were measured with commercially available enzyme-linked immunosorbent assays
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40 17 (ELISA) (R&D Systems, Minneapolis, USA). To correct for different dilutions,
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42 18 concentrations in TAF were related to the secretory component for IgA (SC). SC levels in
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44 19 TAF of preterm infants are largely independent of capillary leakage, gestational and postnatal
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46 20 age as well as respiratory disease in the first weeks of life which makes it a valid dilutional
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48 21 marker of epithelial lining fluid¹⁸⁻¹⁹. SC was measured by ELISA as previously described²¹.
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55 23 *Statistical analysis*

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57 24 Continuous variables were analyzed with one-way analysis of variance (ANOVA), if they
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59 25 showed a normal distribution within groups and homogeneity of variance. Data with a skewed
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1 distribution were primarily assessed with nonparametric tests (Kruskal-Wallis test, Mann-
2 Whitney-U-test). We used post hoc Bonferroni method to correct for multiple comparisons.
3 Sequential values were compared with Friedman test and categorical or nominal values
4 assessed with χ^2 -test. Correlations were analyzed using Spearman's rank correlation
5 coefficients. All tests were two-sided, a value of $p<0.05$ was considered significant. Statistical
6 evaluation was performed with SPSS 18.0 for Windows (SPSS Inc., Chicago, USA).

1 Results

2 Patients

3 Clinical characteristics and outcome of patients in the 3 groups control, chorioamnionitis and
4 funisitis are summarized in table 1. Infants of the chorioamnionitis group had lower birth
5 weights than those in the control and funisitis group ($p=0.01$). Gestational age and proportions
6 of small for gestational age infants (SGA, i.e. birth weight < 10. percentile for gestational age
7 and gender) did not differ significantly between groups. Most infants born after maternal
8 clinical signs of amnion infection and premature rupture of membranes belonged to the
9 funisitis group. The initial blood leukocyte count was higher and the interval between rupture
10 of membranes and birth was longer in the funisitis group compared to chorioamnionitis and
11 control ($p<0.01$, respectively).

12 Thirty-six infants had received prenatal steroids. Within the first 15 days of life a single dose
13 of hydrocortisone was administered to 4 infants (days 1, 3, 6 and 9 respectively) and a single
14 dose of dexamethasone was given to 2 infants (day 8 and 10, respectively). Twenty-six infants
15 were treated with a natural surfactant preparation up to 3 times. Administration of prenatal
16 and postnatal corticosteroids or surfactant did not differ between groups.

17 There was no difference in respiratory outcome measures, such as duration of mechanical
18 ventilation, incidence of BPD or duration of oxygen therapy, between groups. TAF was
19 cultured for *Ureaplasma urealyticum* in 40 infants. Positive cultures (11) did not differ
20 between groups and were not associated with respiratory outcome either.

21 Mortality was highest in the chorioamnionitis group. Overall, 10 infants of the study cohort
22 died until day of life 25. They had a significantly lower gestational age (mean±standard
23 deviation: 25.7 ± 1.7 weeks vs. 27.9 ± 1.5 weeks, $p<0.01$) and birth weight (857 ± 256 g vs.
24 1067 ± 199 g, $p<0.05$) than survivors and were all mechanically ventilated from the day of
25 birth until death.

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Nineteen infants developed BPD, 13 had mild, 6 moderate and no infant severe BPD. Infants, who developed BPD or died, had a lower gestational age and birth weight and were more likely to be males ($p<0.05$, respectively) than those who survived without BPD. They were mechanically ventilated for a longer period ($p<0.01$) and needed higher peak oxygen supplementation as well as higher peak inspiratory pressure on mechanical ventilation ($p<0.05$, respectively, table 2). All 6 SGA infants either developed BPD (5) or died (1).

Ang2/SC in TAF

Ang2/SC in TAF of the 42 infants decreased over time. Day 1, median: 110.4 pg/ μ g (interquartile range: 44.1-218.9); day 3, 71.8 pg/ μ g (44.3-106.6); day 5, 39.8 pg/ μ g (21.3-114.0); day 7, 30.1 pg/ μ g (16.9-62.4); day 10, 32.1 pg/ μ g (16.9-49.0) and day 15, 23.8 pg/ μ g (7.7-35.6); $p < 0.001$.

Figure 1 illustrates values for Ang2/SC in the 3 groups control, chorioamnionitis and funisitis. The funisitis group had significantly lower values in TAF than the control group on days 1-3 ($p<0.01$) and 10 ($p<0.05$) and lower values than the chorioamnionitis group on days 1-7 ($p<0.01$, respectively), whereas Ang2/SC did not differ significantly between the chorioamnionitis and control group on any day.

Infants who developed a moderate form of BPD or died showed significantly higher Ang2/SC on day 10 (49.5 pg/ μ g [38.2-72.5]) compared to the remainder (24.2 pg/ μ g [15.8-36.5], $p < 0.05$).

Ang2/SC did not correlate with gestational age and did not differ between genders. On days 1-7 SGA infants had significantly higher Ang2/SC levels than those infants whose birth weight was appropriate for gestational age (AGA) (figure 2). Surfactant therapy, pre- and postnatal corticosteroids and colonization with *Ureaplasma urealyticum* were not associated with alterations of Ang2/SC.

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4 1 *Ratio Ang1/Ang2*

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6 2 We used TAF concentrations of the Ang2 counterpart Ang1, which we had previously
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8 3 measured in the same cohort, to calculate the ratio between the two factors³². The Ang1/Ang2
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10 4 ratio did not differ significantly between the three groups control, chorioamnionitis and
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12 5 funisitis (data not shown).
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14
15 6 On days 1-5, the ratio was significantly lower in infants who developed BPD or died ($p < 0.05$,
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17 7 respectively). Patients, who developed moderate BPD or died, had significantly lower values
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19 8 on days 3-15 compared to the remaining infants ($p < 0.05$, respectively) (figure 3). The ratio
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21 9 was significantly decreased in SGA infants compared to AGA infants on days 5 and 7 ($p <$
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23 10 0.05).
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27 11 *Umbilical cord serum concentrations*

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29 12 Umbilical cord blood could be obtained from 28 infants of our cohort. They did not differ
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31 13 significantly in any demographic or outcome parameter from the remaining 14 infants.
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33 14 Umbilical cord serum concentrations of Ang1, Ang2 and sTie-2 were not different between
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35 15 the 3 groups control, chorioamnionitis and funisitis. They did not differ between SGA and
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37 16 AGA infants either. Patients, who developed BPD or died, had significantly higher sTIE-2
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39 17 concentrations ($p < 0.05$) than those that survived without BPD (figure 4).
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Discussion

In our exploratory study histological funisitis was associated with decreased concentrations of Ang2 in TAF of ventilated very premature infants compared to infants exposed to chorioamnionitis or to no inflammation in utero. Pulmonary angiogenesis is a prerequisite for alveolarisation^{2,21} and Ang2 is responsible for endothelial cell sprouting and promotes vascular remodeling²². In vitro, proangiogenic signaling of Ang2 by activation of TIE-2 has been shown concentration dependent²³. One might therefore speculate that decreased proangiogenic signaling by Ang2 in the lungs of very premature infants with funisitis - reflecting a fetal systemic inflammatory response¹² - may predispose them for disturbed pulmonary angiogenesis along with impaired alveolar development, eventually contributing to the risk of BPD¹⁴⁻¹⁵.

However, we found higher Ang2 concentrations in TAF of ventilated VLBW infants on day 10 to be associated with the outcome moderate BPD or death along with a reduced ratio of Ang1 to Ang2 in TAF on many of the first postnatal days in infants who developed mild to moderate BPD or died. These findings suggest that a preponderance of Ang2 in airway fluid of ventilated very premature infants plays a role for pulmonary outcome and neonatal mortality. Increased Ang2 concentrations in TAF in the first week of life of ventilated VLBW infants have recently been shown associated with the combined outcome moderate BPD or death⁹. Our findings confirm and amend these data.

In recent years, the association between histological funisitis and respiratory morbidity in very premature infants has been questioned²⁴⁻²⁶. In our cohort funisitis was not associated with the outcome BPD or death. This may be due to a lack of power to detect such an association with our samples size. Moreover, funisitis does not represent a homogeneous disease²⁷. Its risk is modified by factors, like duration of membrane rupture, which by themselves interfere with neonatal outcome. Need for sustained mechanical ventilation and higher oxygen supplementation, postnatal risk factors which have been described to modulate the association

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1 between prenatal inflammation and BPD^{26,28-29}, were both associated with the combined
2 outcome BPD or death in our cohort. Hyperoxia has been shown to induce pulmonary Ang2
3 expression on transcriptional and translational level in an animal model of acute lung injury
4 and to decrease Ang1 mRNA expression simultaneously³⁰. In this setting the abundance of
5 Ang2 seems to play an important role for pulmonary oxidative injury, inflammation and cell
6 death³⁰. These experimental data are also in accordance with our findings and underscore the
7 relevance of postnatal factors as potential risk modulators for BPD²⁸.
8 Treatment strategies targeting to reduce pulmonary Ang2 expression may have potential
9 benefits for respiratory outcome³⁰. Ang2 concentrations in TAF of VLBW infants have been
10 decreased after administration of dexamethasone⁹. However, our data suggest that the overall
11 balance between different types of factors - i.e. between Ang1, which stabilizes endothelium
12 and leads to vascular maturation⁶, and Ang2, which destabilizes vascular endothelium⁴⁻⁵ - may
13 be as important for respiratory outcome of very premature infants as changes in one single
14 factor. This is in accordance with data from a model of lipopolysaccharide-induced acute lung
15 injury³¹. Likewise, we have recently demonstrated an imbalance between Ang1 and the
16 angiostatic factor endostatin in TAF of extremely premature infants to be associated with the
17 combined outcome BPD or death³².
18 The functions of angiopoietins are pleiotropic and context-dependent¹. Ang2 synergizes with
19 vascular endothelial growth factor (VEGF) to stimulate angiogenesis but causes endothelial
20 cell death and vessel regression in the absence of sufficient angiogenic signals¹. VEGF has
21 been shown decreased in TAF of ventilated very premature infants developing BPD and in
22 pulmonary tissue of infants who had died from the disease³³⁻³⁵. In light of these data our
23 findings would support the concept of impaired vascular signaling in the pathogenesis of
24 BPD.
25 Interestingly, Ang2 levels in TAF of SGA infants were increase compared to AGA infant
26 during the first week of life in our cohort, along with an imbalance between Ang1 and Ang2

on day 5 and 7. All SGA infants either developed BPD or died. These findings are in line with 2 recent studies, which identified preeclampsia and intrauterine growth retardation (IUGR), but not histological chorioamnionitis with funisitis, as risk factors for BPD in extremely premature infants^{24,36}.

Regulation of vascular development in the fetomaternal unit is disturbed in preeclampsia, leading to disturbed placentation and eventually to IUGR. Similarly, disrupted pulmonary vascularisation plays a crucial role for BPD^{2,3,21}. Our data complement existing evidence that an imbalance between Ang1 and Ang2 in different fetomaternal compartments is a common denominator of impaired placental as well as fetal pulmonary vascular development. In maternal plasma at midgestation, IUGR has been found associated with increased Ang2 concentrations³⁷ and preeclampsia with a decrease of the Ang1/Ang2 ratio³⁸. A decrease of Ang2 concentrations along with an increase of the Ang1/Ang2 ratio in the amniotic cavity seems to be critical to prevent regression, destabilization and leakage of placental vessels during normal gestation³⁹⁻⁴⁰. Intraamniotic Ang2 might be at least partially of fetal pulmonary origin^{30,39}. Higher Ang2 level in TAF during the initial phase of life in SGA infants might therefore reflect both disturbed placental and fetal pulmonary angiogenesis⁴¹.

Remarkably, intraamniotic inflammation/infection - assessed by either the presence of soluble markers of the innate immune system, increased intraamniotic IL-6 or positive amniotic fluid cultures - have been shown associated with higher intraamniotic Ang2 levels in women presenting with preterm labor or preterm premature rupture of membranes in 2 studies³⁹⁻⁴⁰.

Both studies failed to demonstrate an association between histological funisitis and higher intraamniotic Ang2. In placentas of very premature infants angiogenic factors have been demonstrated to be decreased after histological chorioamnionitis and funisitis¹³. However, histological evidence of inflammation was not associated with morphological alterations of the placental vasculature in this study. These data underscore a limited relevance of histological chorioamnionitis with funisitis for angiogenic homeostasis in maternal and fetal

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1 tissues. Likewise, histological funisitis was not associated with changes of umbilical cord
2 serum concentrations of Ang1, Ang2 and sTIE-2 concentrations in our cohort.
3 However, sTIE-2 levels in umbilical cord serum were higher in infants who developed BPD
4 or died. In critically ill adult patients concentrations of sTIE-2 in blood have been associated
5 with increased pulmonary vascular permeability¹¹, which plays a role in the early phase of
6 BPD^{16,20,29}. sTIE-2 is released into the blood after proteolytical cleavage of the extracellular
7 domain of the endothelial receptor, either constitutively or after proangiogenic stimulation^{7,42}.
8 sTIE-2 is believed to act as a decoy for angiopoietins, affecting the signaling pathways
9 induced by binding of the ligands to the membrane-bound receptor⁴². As Ang1 has been
10 shown to bind to sTIE-2 with a much higher affinity than Ang2 in vitro⁴³, high sTIE-2
11 concentrations may shift the Ang1/Ang2 ratio in favour of Ang2, eventually contributing to
12 systemic and pulmonary endothelial activation and destabilization¹¹.
13 Lower gestational and birth weight as well as sustained mechanical ventilation of infants who
14 died in our cohort represent important risk factors for all grades of BPD. We therefore
15 combined the outcome BPD and death for analysis. The higher mortality in the
16 chorioamnionitis group compared to both other groups can be attributed to the lower birth
17 weight in this group as important predictor of mortality in VLBW infants⁴⁴.
18 Our clinical explorative study has limitations. We did not assess the activity but only
19 concentrations of factors involved in angiogenesis. Moreover, our sample size made inclusion
20 of potential confounders in a multivariate analysis not applicable as the power to detect
21 associations between independent factors and concentrations of the angiogenic factors would
22 have been less than 80% in most cases. Therefore, our study can only be regarded preliminary
23 and suitable to generate new hypotheses, which have to be proved by experimental studies
24 and clinical studies with appropriate patient numbers to allow multivariate comparisons,
25 before definite conclusions can be drawn.

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1 To conclude, our data suggest that an increase of Ang2 and an imbalance with its counterpart
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4 1 Ang1 in airway fluid potentially contributes to a sustained disturbance of pulmonary vascular
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8 3 and alveolar development in ventilated very preterm infants who develop BPD or die. The
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10 4 presence of such an imbalance in SGA infants implies an association between disrupted
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12 5 placental and fetal pulmonary angiogenesis. We demonstrated that histological funisitis
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14 6 associated with reduced Ang2 concentrations in airway fluid is of minor relevance for
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16 7 outcome. Further ante- and postnatal factors are likely to modulate the risk of disturbed
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18 8 pulmonary vascularisation and alveolarisation in very premature infants.
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17 No author has competing interests to declare.

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Figure legends

Figure 1: Boxplots of Ang2/SC (A) in TAF of the three groups control (n=16), chorioamnionitis (n=9) and funisitis (n=17). The bold bars indicate the median, the boxes the interquartile range and the error bars the 5th and 95th percentile. Circles indicate outliers (1.5-3 x interquartile range from upper or lower quartile), rhombs extreme values (> 3 x interquartile range from upper or lower quartile). Ang2/SC in the funisitis group was significantly lower than in the control group on days 1, 3 and 10 and lower than in the chorioamnionitis group on days 1-10.

Figure 2: Boxplots of Ang2/SC in TAF for small for gestational age infants (SGA) compared to appropriate for gestational age infants (AGA). SGA infants had significantly higher Ang2/SC on days 1-7 than AGA infants ($p < 0.05$, respectively)

Figure 3: Boxplots of the ratio Ang1/Ang2 in TAF for the outcome moderate BPD or death. The ratio was significantly decreased on days 3-10 for infants who developed BPD or died.

Figure 4: Mean concentrations with standard deviations of Ang1, Ang2 and sTIE-2 in umbilical cord serum of infants who survived without BPD and infants who developed BPD or died. sTIE-2 concentrations were significantly higher for infants who developed BPD or died.

Table 1: Clinical characteristics and short term outcome

	Control n=16	Chorioamnionitis n=9	Funisitis n=17
Gestational age (wk)*	28.1 ± 1.2	26.5 ± 1.8	27.1 ± 2.1
Birth weight (g)*	1,048 ± 186	827 ± 186 [#]	1,088 ± 241
Male	5 (31%)	6 (67%)	7 (41%)
SGA	4 (25%)	2 (22%)	0 (0%)
Cause of preterm birth [†]			
Multiple gestation	7 (44%)	1 (11%)	1 (6%)
AIS/PROM	1 (6%)	4 (44%)	14 (82%)
PIH/Preeclampsia	3 (19%)	2 (22%)	1 (6%)
others	5 (31%)	2 (22%)	1 (6%)
Initial leukocyte count (*1,000/μl)	8.15 (5.85-12.13)	8.0 (6.45-9.75)	18.5 (9.4-29.9) [§]
Interval ROM – birth (hours)**	0 (0-6)	0 (0-0)	71 (20-432) ^{&}
Prenatal steroids	13 (81%)	8 (89%)	15 (88%)
Postnatal steroids	5 (31%)	3 (33%)	7 (41%)
Surfactant therapy	11 (69%)	7 (78%)	8 (47%)
<i>U. urealyticum</i> in TAF	2 (13%)	2 (22%)	7 (41%)
Death ^{\$}	1 (6%)	5 (56%)	4 (24%)
BPD in survivors			
No	7 (44%)	1 (11%)	5 (29%)
Mild	5 (31%)	2 (22%)	6 (35%)
moderate	3 (19%)	1 (11%)	2 (12%)

SGA	small for gestational age
AIS	amnion infection syndrome
(P)ROM	(premature) rupture of membranes;
PIH	pregnancy induced hypertension

* Values are means $\pm$ SD

[†] p<0.01 (χ^2 -Test)[§]p<0.05 vs. control and chorioamnionitis

^{\$}p<0.05 (χ^2 -Test)

** Values are medians (lower-upper quartile)

#p=0.01 vs. control and funisitis

[&] p<0.01 vs. control and chorioamnionitis

Table 2: Differences in clinical characteristics and respiratory support between infants who developed BPD or died and the remainder.

	BPD/death n = 29 (mean $\pm$ SD)	Survivors without BPD, n = 13 (mean $\pm$ SD)	p-value
Gestational age (wk)	26.9 $\pm$ 1.8	28.3 $\pm$ 1.4	<0.05
Birth weight (g)	954 $\pm$ 237	1155 $\pm$ 132	<0.05
Duration of mechanical ventilation (days)	27.0 $\pm$ 24.1	13.3 $\pm$ 5.1	<0.01
Maximum peak oxygen supplementation for > 0.5 hours (%)	85 $\pm$ 21	65 $\pm$ 26	<0.05
Maximum peak inspiratory pressure (kPa)	211 $\pm$ 41	181 $\pm$ 27	<0.05

254x190mm (96 x 96 DPI)

Figure 2

254x190mm (96 x 96 DPI)

296x209mm (96 x 96 DPI)

Figure 4

190x254mm (96 x 96 DPI)

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1 **Airway** angiopoietin-2 in ventilated very preterm infants: **association with prenatal factors and**
2 **neonatal outcome**

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16 Key words: bronchopulmonary dysplasia, lung injury, angiogenesis,
17 vascular endothelial growth factor, small for gestational age infant

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23 Abbreviated title: Angiopoietin-2 **in airways of ventilated** preterm infants

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Abstract

Objective: Pulmonary angiogenesis is a prerequisite for lung development. Angiopoietin-2 (Ang2) destabilizes endothelial cells through its endothelial receptor TIE-2, enabling vascular sprouting. Ang1 stabilizes new blood vessels. Soluble TIE-2 (sTIE-2) modulates these effects.

We hypothesized that histological funisitis is associated with alterations of Ang2 in airways and of the systemic angiopoietin-TIE-2 homeostasis in very low birth weight infants (VLBW), contributing to pulmonary morbidity and mortality.

Methods: We measured Ang2 in tracheobronchial aspirate fluid (TAF) of 42 VLBW < 30 weeks of gestation from day 1 through 15 and Ang1, Ang2 and sTIE-2 in umbilical cord serum of 28 infants by ELISA. Histological examination distinguished 3 groups: funisitis, chorioamnionitis and controls.

Results: Funisitis was associated with lower Ang2 values in TAF but not with changes of Ang1, Ang2 and sTIE-2 in umbilical cord serum. Infants who developed BPD or died had a persistently decreased ratio of previously measured Ang1 to Ang2 in TAF on days 1-5 and increased cord serum concentrations of sTIE-2. Moderate BPD/death was associated with an increase of Ang2 in TAF on day 10 and decreased Ang1/Ang2 ratio from day 3-15. Small for gestational age (SGA) infants had increased Ang2 in TAF on day 1-7 and a lower Ang1/Ang2 ratio on days 5-7.

Conclusions: The predominance of Ang2 in airway fluid of infants with BPD/death and SGA infants suggests a link between disrupted placental and fetal pulmonary angiogenesis. Histological funisitis with reduced Ang2 in TAF was of minor relevance for outcome in our cohort.

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4 **1 Introduction**

5
6 2 Angiogenesis plays a pivotal role in organ development and maturation¹. In extremely
7
8 3 premature infants disturbed pulmonary angiogenesis accompanies alveolar simplification,
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10 4 eventually leading to bronchopulmonary dysplasia (BPD)²⁻³.
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12 5 *Angiopoietin-2* (Ang2) belongs to a family of angiogenic growth factors which bind
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14 6 specifically to the *tyrosine kinase receptor with immunoglobulin and epidermal growth factor*
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16 7 *homology domain 2* (TIE-2) on endothelial cells. Ang2 destabilizes the endothelium by
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18 8 decreasing the phosphorylation status of TIE-2, leading to a more responsive endothelium, a
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20 9 prerequisite for sprouting of new vessels⁴. The endothelial destabilization also leads to
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22 10 vascular leakage which is a feature of inflammation⁵. Binding of the Ang2 antagonist
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24 11 *angiopoietin-1* (Ang1) to TIE-2 induces tightening of endothelial intercellular junctions,
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26 12 leading to stabilization and maturation of newly formed vessels⁶. Both angiopoietins act
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28 13 synergistically in the process of angiogenesis⁴. A soluble form of TIE-2 (sTIE-2), released
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30 14 from the endothelium after proteolytical cleavage of the receptor's extracellular domain,
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32 15 interferes with the angiopoietin-TIE-2 signaling by binding the free ligands⁷.
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34 16 A differential spatial and temporal regulation of TIE-2 by Ang1 and Ang2 in the lung could
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36 17 be shown a prerequisite for a stable pulmonary vasculature and normal lung development and
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38 18 maturation in a murine model⁸. Increased concentrations of Ang2 in tracheobronchial aspirate
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40 19 fluid (TAF) have been associated with the development of BPD or death in ventilated
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42 20 premature infants⁹.
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44 21 The role of angiopoietins and TIE-2 in systemic inflammatory processes and pulmonary
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46 22 injury has been extensively addressed. In a pediatric population, high plasma Ang2 levels and
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48 23 low Ang1 levels were associated with septic shock¹⁰. Serum concentrations of sTIE-2 have
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50 24 been shown elevated in adult septic patients at risk for adult respiratory distress syndrome
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52 25 compared to non-septic patients¹¹. However, data about blood levels of the angiopoietins and
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54 26 their receptor in very premature infants exposed to prenatal inflammation do not exist.

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4 1 Histological funisitis is regarded as morphological counterpart of a fetal systemic
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6 2 inflammatory response¹² and has been shown associated with down-regulation of angiogenic
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8 3 factors in placental tissue¹³. This fetal inflammatory response has been identified as risk factor
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10 4 for BPD¹⁴ and mortality¹⁵ after premature birth.
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13 5 The primary aim of our exploratory pilot study was to assess the association between
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15 6 histological funisitis and postnatal airway concentrations of Ang2 in ventilated very
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17 7 premature infants. We also investigated concentrations of the factors Ang1, Ang2 and sTIE-2
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19 8 in umbilical cord blood. We speculated that alterations of pulmonary Ang2 and a disturbed
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21 9 homeostasis of the angiopoietin-TIE-2 system after funisitis might contribute to pulmonary
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23 10 morbidity and neonatal mortality of this high risk group of patients.
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4 1 **Material and Methods**

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6 2 *Patients*

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8 3 42 infants with a birth weight <1500 g and a gestational age < 30 weeks, who were admitted
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10 4 to the intensive care unit of the Department of Neonatology, Poznań University of Medical
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12 5 Sciences, Poland, **between November 2004 and August 2005**, were enrolled in this
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14 6 prospective study after informed consent of the parents. Patients were inborns, had to be
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16 7 mechanically ventilated for respiratory failure within the first 48 hours of life and had no
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18 8 major congenital malformations. Respiratory support was adjusted to reach a SpO₂ between
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20 9 85% and 94%. Infants with supplemental oxygen for at least 28 days were assigned to have
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22 10 BPD¹⁶. Depending on the need for oxygen or respiratory support at a postmenstrual age of 36
23
24 11 weeks, BPD was graded as mild, moderate or severe¹⁶. The study was approved by the Ethical
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26 12 Committees of the Poznań University of Medical Sciences and the Faculty of Medicine,
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28 13 University of Wuerzburg, Germany.
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37 15 *Histological examinations*

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39 16 For each infant at least 2 sections of umbilical cord, 2 sections of placental parenchyma and 1
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41 17 section of membrane rolls were evaluated for the presence and severity of neutrophilic
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43 18 infiltrates by two pathologists, unaware of clinical data, according to a protocol¹⁷. Patients
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45 19 were assigned to three groups according to histological diagnosis. The first group was
46
47 20 characterized by chorioamnionitis and funisitis and will be referred to as funisitis group
48
49 21 (n=17). In the second group chorioamnionitis without funisitis was diagnosed
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51 22 (chorioamnionitis group, n=9). The control group consisted of 16 patients without histological
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53 23 signs of inflammation in placenta, membranes and umbilical cord.
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4 1 *Sampling, preparation and storage*

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6 2 Tracheobronchial aspiration was performed in a standardized manner up to 3 times per day on
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8 3 days 1, 3, 5, 7, 10 and 15 of life. After instillation of 0.5 ml NaCl 0.9% into the endotracheal
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10 4 tube a suction catheter was inserted slightly beyond the distal tip of the tube and airway
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12 5 secretions were suctioned and collected in sterile mucus traps (Unomedical, Birkerød,
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14 6 Denmark). Each specimen was diluted with NaCl 0.9% to a volume of 0.5 ml. Specimens of
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16 7 one day were pooled. They were spun at 3,000 revolutions per minute (rpm) for 5 minutes.
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18 8 Supernatants were centrifuged again twice at 10,000 rpm for 5 minutes. Specimens were
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20 9 cooled during processing, including centrifugation. The resulting clear supernatants
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22 10 were divided into aliquots and stored at -80°C until further analysis.
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27 11 Umbilical cord blood could be collected from the umbilical cord vein of 28 infants. Blood
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29 12 was centrifuged at 3,000 rpm for 10 minutes and the serum stored at -80°C until analysis.
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34 14 *Measurement of factors and secretory component for IgA*

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36 15 Concentrations of Ang1, Ang2 and sTIE-2 in umbilical cord serum and levels of Ang2 in
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38 16 TAF were measured with commercially available enzyme-linked immunosorbent assays
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40 17 (ELISA) (R&D Systems, Minneapolis, USA). To correct for different dilutions,
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42 18 concentrations in TAF were related to the secretory component for IgA (SC). SC levels in
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44 19 TAF of preterm infants are largely independent of capillary leakage, gestational and postnatal
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46 20 age as well as respiratory disease in the first weeks of life which makes it a valid dilutional
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48 21 marker of epithelial lining fluid¹⁸⁻¹⁹. SC was measured by ELISA as previously described²¹.
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55 23 *Statistical analysis*

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57 24 Continuous variables were analyzed with one-way analysis of variance (ANOVA), if they
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59 25 showed a normal distribution within groups and homogeneity of variance. Data with a skewed
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1 distribution were primarily assessed with nonparametric tests (Kruskal-Wallis test, Mann-
2 Whitney-U-test). We used post hoc Bonferroni method to correct for multiple comparisons.
3 Sequential values were compared with Friedman test and categorical or nominal values
4 assessed with χ^2 -test. Correlations were analyzed using Spearman's rank correlation
5 coefficients. All tests were two-sided, a value of $p<0.05$ was considered significant. Statistical
6 evaluation was performed with SPSS 18.0 for Windows (SPSS Inc., Chicago, USA).

1 Results

2 Patients

3 Clinical characteristics and outcome of patients in the 3 groups control, chorioamnionitis and
4 funisitis are summarized in table 1. Infants of the chorioamnionitis group had lower birth
5 weights than those in the control and funisitis group ($p=0.01$). Gestational age and proportions
6 of small for gestational age infants (SGA, i.e. birth weight < 10. percentile for gestational age
7 and gender) did not differ significantly between groups. Most infants born after maternal
8 clinical signs of amnion infection and premature rupture of membranes belonged to the
9 funisitis group. The initial blood leukocyte count was higher and the interval between rupture
10 of membranes and birth was longer in the funisitis group compared to chorioamnionitis and
11 control ($p<0.01$, respectively).

12 Thirty-six infants had received prenatal steroids. Within the first 15 days of life a single dose
13 of hydrocortisone was administered to 4 infants (days 1, 3, 6 and 9 respectively) and a single
14 dose of dexamethasone was given to 2 infants (day 8 and 10, respectively). Twenty-six infants
15 were treated with a natural surfactant preparation up to 3 times. Administration of prenatal
16 and postnatal corticosteroids or surfactant did not differ between groups.

17 There was no difference in respiratory outcome measures, such as duration of mechanical
18 ventilation, incidence of BPD or duration of oxygen therapy, between groups. TAF was
19 cultured for *Ureaplasma urealyticum* in 40 infants. Positive cultures (11) did not differ
20 between groups and were not associated with respiratory outcome either.

21 Mortality was highest in the chorioamnionitis group. Overall, 10 infants of the study cohort
22 died until day of life 25. They had a significantly lower gestational age (mean±standard
23 deviation: 25.7 ± 1.7 weeks vs. 27.9 ± 1.5 weeks, $p<0.01$) and birth weight (857 ± 256 g vs.
24 1067 ± 199 g, $p<0.05$) than survivors and were all mechanically ventilated from the day of
25 birth until death.

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Nineteen infants developed BPD, 13 had mild, 6 moderate and no infant severe BPD. Infants, who developed BPD or died, had a lower gestational age and birth weight and **were more likely to be males** ($p < 0.05$, respectively) than those who survived without BPD. They were mechanically ventilated for a longer period ($p < 0.01$) and needed higher peak oxygen supplementation as well as higher peak inspiratory pressure on mechanical ventilation ($p < 0.05$, respectively, table 2). **All 6 SGA infants either developed BPD (5) or died (1).**

Ang2/SC in TAF

Ang2/SC in TAF of the 42 infants decreased over time. Day 1, median: 110.4 pg/ μ g (interquartile range: 44.1-218.9); day 3, 71.8 pg/ μ g (44.3-106.6); day 5, 39.8 pg/ μ g (21.3-114.0); day 7, 30.1 pg/ μ g (16.9-62.4); day 10, 32.1 pg/ μ g (16.9-49.0) and day 15, 23.8 pg/ μ g (7.7-35.6); $p < 0.001$. Figure 1 illustrates values for Ang2/SC in the 3 groups control, chorioamnionitis and funisitis. The funisitis group had significantly lower values in TAF than the control group on days 1-3 ($p < 0.01$) and 10 ($p < 0.05$) and lower values than the chorioamnionitis group on days 1-7 ($p < 0.01$, respectively), whereas Ang2/SC did not differ significantly between the chorioamnionitis and control group on any day.

Infants who developed a moderate form of BPD or died showed significantly higher Ang2/SC on day 10 (**49.5 pg/ μ g [38.2-72.5]**) compared to the remainder (**24.2 pg/ μ g [15.8-36.5]**, $p < 0.05$).

Ang2/SC did not correlate with gestational age and did not differ between genders. On days 1-7 SGA infants had significantly higher Ang2/SC levels than those infants whose birth weight was appropriate for gestational age (AGA) (figure 2). Surfactant therapy, pre- and postnatal corticosteroids and colonization with *Ureaplasma urealyticum* were not associated with alterations of Ang2/SC.

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4 1 *Ratio Ang1/Ang2*

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6 2 We used TAF concentrations of the Ang2 counterpart Ang1, which we had previously
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8 3 measured in the same cohort, to calculate the ratio between the two factors³². The Ang1/Ang2
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10 4 ratio did not differ significantly between the three groups control, chorioamnionitis and
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12 5 funisitis (data not shown).
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15 6 On days 1-5, the ratio was significantly lower in infants who developed BPD or died ($p < 0.05$,
16
17 7 respectively). Patients, who developed moderate BPD or died, had significantly lower values
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19 8 on days 3-15 compared to the remaining infants ($p < 0.05$, respectively) (figure 3). The ratio
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21 9 was significantly decreased in SGA infants compared to AGA infants on days 5 and 7 ($p <$
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23 10 0.05).
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27 11 *Umbilical cord serum concentrations*

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29 12 Umbilical cord blood could be obtained from 28 infants of our cohort. They did not differ
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31 13 significantly in any demographic or outcome parameter from the remaining 14 infants.
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33 14 Umbilical cord serum concentrations of Ang1, Ang2 and sTie-2 were not different between
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35 15 the 3 groups control, chorioamnionitis and funisitis. They did not differ between SGA and
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37 16 AGA infants either. Patients, who developed BPD or died, had significantly higher sTIE-2
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39 17 concentrations ($p < 0.05$) than those that survived without BPD (figure 4).
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4 **1 Discussion**

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6 2 In our exploratory study histological funisitis was associated with decreased concentrations of
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8 3 Ang2 in TAF of ventilated very premature infants compared to infants exposed to
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10 4 chorioamnionitis or to no inflammation in utero. Pulmonary angiogenesis is a prerequisite for
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12 5 alveolarisation^{2,21} and Ang2 is responsible for endothelial cell sprouting and promotes
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14 6 vascular remodeling²². In vitro, proangiogenic signaling of Ang2 by activation of TIE-2 has
15
16 7 been shown concentration dependent²³. One might therefore speculate that decreased
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18 8 proangiogenic signaling by Ang2 in the lungs of very premature infants with funisitis -
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20 9 reflecting a fetal systemic inflammatory response¹² - may predispose them for disturbed
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22 10 pulmonary angiogenesis along with impaired alveolar development, eventually contributing to
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24 11 the risk of BPD¹⁴⁻¹⁵.
25
26 12 However, we found higher Ang2 concentrations in TAF of ventilated VLBW infants on day
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28 13 10 to be associated with the outcome moderate BPD or death along with a reduced ratio of
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30 14 Ang1 to Ang2 in TAF on many of the first postnatal days in infants who developed mild to
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32 15 moderate BPD or died. These findings suggest that a preponderance of Ang2 in airway fluid
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34 16 of ventilated very premature infants plays a role for pulmonary outcome and neonatal
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36 17 mortality. Increased Ang2 concentrations in TAF in the first week of life of ventilated VLBW
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38 18 infants have recently been shown associated with the combined outcome moderate BPD or
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40 19 death⁹. Our findings confirm and amend these data.
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42 20 In recent years, the association between histological funisitis and respiratory morbidity in very
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44 21 premature infants has been questioned²⁴⁻²⁶. In our cohort funisitis was not associated with the
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46 22 outcome BPD or death. This may be due to a lack of power to detect such an association with
47
48 23 our samples size. Moreover, funisitis does not represent a homogeneous disease²⁷. Its risk is
49
50 24 modified by factors, like duration of membrane rupture, which by themselves interfere with
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52 25 neonatal outcome. Need for sustained mechanical ventilation and higher oxygen
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54 26 supplementation, postnatal risk factors which have been described to modulate the association

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1 between prenatal inflammation and BPD^{26,28-29}, were both associated with the combined
2 outcome BPD or death in our cohort. Hyperoxia has been shown to induce pulmonary Ang2
3 expression on transcriptional and translational level in an animal model of acute lung injury
4 and to decrease Ang1 mRNA expression simultaneously³⁰. In this setting the abundance of
5 Ang2 seems to play an important role for pulmonary oxidative injury, inflammation and cell
6 death³⁰. These experimental data are also in accordance with our findings and underscore the
7 relevance of postnatal factors as potential risk modulators for BPD²⁸.
8 Treatment strategies targeting to reduce pulmonary Ang2 expression may have potential
9 benefits for respiratory outcome³⁰. Ang2 concentrations in TAF of VLBW infants have been
10 decreased after administration of dexamethasone⁹. However, our data suggest that the overall
11 balance between different types of factors - i.e. between Ang1, which stabilizes endothelium
12 and leads to vascular maturation⁶, and Ang2, which destabilizes vascular endothelium⁴⁻⁵ - may
13 be as important for respiratory outcome of very premature infants as changes in one single
14 factor. This is in accordance with data from a model of lipopolysaccharide-induced acute lung
15 injury³¹. Likewise, we have recently demonstrated an imbalance between Ang1 and the
16 angiostatic factor endostatin in TAF of extremely premature infants to be associated with the
17 combined outcome BPD or death³².
18 The functions of angiopoietins are pleiotropic and context-dependent¹. Ang2 synergizes with
19 vascular endothelial growth factor (VEGF) to stimulate angiogenesis but causes endothelial
20 cell death and vessel regression in the absence of sufficient angiogenic signals¹. VEGF has
21 been shown decreased in TAF of ventilated very premature infants developing BPD and in
22 pulmonary tissue of infants who had died from the disease³³⁻³⁵. In light of these data our
23 findings would support the concept of impaired vascular signaling in the pathogenesis of
24 BPD.
25 Interestingly, Ang2 levels in TAF of SGA infants were increased compared to AGA infant
26 during the first week of life in our cohort, along with an imbalance between Ang1 and Ang2

on day 5 and 7. All SGA infants either developed BPD or died. These findings are in line with 2 recent studies, which identified preeclampsia and intrauterine growth retardation (IUGR), but not histological chorioamnionitis with funisitis, as risk factors for BPD in extremely premature infants^{24,36}. Regulation of vascular development in the fetomaternal unit is disturbed in preeclampsia, leading to disturbed placentation and eventually to IUGR. Similarly, disrupted pulmonary vascularisation plays a crucial role for BPD^{2,3,21}. Our data complement existing evidence that an imbalance between Ang1 and Ang2 in different fetomaternal compartments is a common denominator of impaired placental as well as fetal pulmonary vascular development. In maternal plasma at midgestation, IUGR has been found associated with increased Ang2 concentrations³⁷ and preeclampsia with a decrease of the Ang1/Ang2 ratio³⁸. A decrease of Ang2 concentrations along with an increase of the Ang1/Ang2 ratio in the amniotic cavity seems to be critical to prevent regression, destabilization and leakage of placental vessels during normal gestation³⁹⁻⁴⁰. Intraamniotic Ang2 might be at least partially of fetal pulmonary origin^{30,39}. Higher Ang2 level in TAF during the initial phase of life in SGA infants might therefore reflect both disturbed placental and fetal pulmonary angiogenesis⁴¹. Remarkably, intraamniotic inflammation/infection - assessed by either the presence of soluble markers of the innate immune system, increased intraamniotic IL-6 or positive amniotic fluid cultures - have been shown associated with higher intraamniotic Ang2 levels in women presenting with preterm labor or preterm premature rupture of membranes in 2 studies³⁹⁻⁴⁰. Both studies failed to demonstrate an association between histological funisitis and higher intraamniotic Ang2. In placentas of very premature infants angiogenic factors have been demonstrated to be decreased after histological chorioamnionitis and funisitis¹³. However, histological evidence of inflammation was not associated with morphological alterations of the placental vasculature in this study. These data underscore a limited relevance of histological chorioamnionitis with funisitis for angiogenic homeostasis in maternal and fetal

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1 tissues. Likewise, histological funisitis was not associated with changes of umbilical cord
2 serum concentrations of Ang1, Ang2 and sTIE-2 concentrations in our cohort.
3 However, sTIE-2 levels in umbilical cord serum were higher in infants who developed BPD
4 or died. In critically ill adult patients concentrations of sTIE-2 in blood have been associated
5 with increased pulmonary vascular permeability¹¹, which plays a role in the early phase of
6 BPD^{16,20,29}. sTIE-2 is released into the blood after proteolytical cleavage of the extracellular
7 domain of the endothelial receptor, either constitutively or after proangiogenic stimulation^{7,42}.
8 sTIE-2 is believed to act as a decoy for angiopoietins, affecting the signaling pathways
9 induced by binding of the ligands to the membrane-bound receptor⁴². As Ang1 has been
10 shown to bind to sTIE-2 with a much higher affinity than Ang2 in vitro⁴³, high sTIE-2
11 concentrations may shift the Ang1/Ang2 ratio in favour of Ang2, eventually contributing to
12 systemic and pulmonary endothelial activation and destabilization¹¹.
13 Lower gestational and birth weight as well as sustained mechanical ventilation of infants who
14 died in our cohort represent important risk factors for all grades of BPD. We therefore
15 combined the outcome BPD and death for analysis. The higher mortality in the
16 chorioamnionitis group compared to both other groups can be attributed to the lower birth
17 weight in this group as important predictor of mortality in VLBW infants⁴⁴.
18 Our clinical explorative study has limitations. We did not assess the activity but only
19 concentrations of factors involved in angiogenesis. Moreover, our sample size made inclusion
20 of potential confounders in a multivariate analysis not applicable as the power to detect
21 associations between independent factors and concentrations of the angiogenic factors would
22 have been less than 80% in most cases. Therefore, our study can only be regarded preliminary
23 and suitable to generate new hypotheses, which have to be proved by experimental studies
24 and clinical studies with appropriate patient numbers to allow multivariate comparisons,
25 before definite conclusions can be drawn.

To conclude, our data suggest that an increase of Ang2 and an imbalance with its counterpart Ang1 in airway fluid potentially contributes to a sustained disturbance of pulmonary vascular and alveolar development in ventilated very preterm infants who develop BPD or die. The presence of such an imbalance in SGA infants implies an association between disrupted placental and fetal pulmonary angiogenesis. We demonstrated that histological funisitis associated with reduced Ang2 concentrations in airway fluid is of minor relevance for outcome. Further ante- and postnatal factors are likely to modulate the risk of disturbed pulmonary vascularisation and alveolarisation in very premature infants.

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No author has competing interests to declare.

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Figure legends

Figure 1: Boxplots of Ang2/SC (A) in TAF of the three groups control (n=16), chorioamnionitis (n=9) and funisitis (n=17). The bold bars indicate the median, the boxes the interquartile range and the error bars the 5th and 95th percentile. Circles indicate outliers (1.5-3 x interquartile range from upper or lower quartile), rhombs extreme values (> 3 x interquartile range from upper or lower quartile). Ang2/SC in the funisitis group was significantly lower than in the control group on days 1, 3 and 10 and lower than in the chorioamnionitis group on days 1-10.

Figure 2: Boxplots of Ang2/SC in TAF for small for gestational age infants (SGA) compared to appropriate for gestational age infants (AGA). SGA infants had significantly higher Ang2/SC on days 1-7 than AGA infants ($p < 0.05$, respectively)

Figure 3: Boxplots of the ratio Ang1/Ang2 in TAF for the outcome moderate BPD or death. The ratio was significantly decreased on days 3-10 for infants who developed BPD or died.

Figure 4: Mean concentrations with standard deviations of Ang1, Ang2 and sTIE-2 in umbilical cord serum of infants who survived without BPD and infants who developed BPD or died. sTIE-2 concentrations were significantly higher for infants who developed BPD or died.

Table 1: Clinical characteristics and short term outcome

	Control n=16	Chorioamnionitis n=9	Funisitis n=17
Gestational age (wk)*	28.1 ± 1.2	26.5 ± 1.8	27.1 ± 2.1
Birth weight (g)*	1,048 ± 186	827 ± 186 [#]	1,088 ± 241
Male	5 (31%)	6 (67%)	7 (41%)
SGA	4 (25%)	2 (22%)	0 (0%)
Cause of preterm birth [†]			
Multiple gestation	7 (44%)	1 (11%)	1 (6%)
AIS/PROM	1 (6%)	4 (44%)	14 (82%)
PIH/Preeclampsia	3 (19%)	2 (22%)	1 (6%)
others	5 (31%)	2 (22%)	1 (6%)
Initial leukocyte count (*1,000/μl)	8.15 (5.85-12.13)	8.0 (6.45-9.75)	18.5 (9.4-29.9) [§]
Interval ROM – birth (hours)**	0 (0-6)	0 (0-0)	71 (20-432) ^{&}
Prenatal steroids	13 (81%)	8 (89%)	15 (88%)
Postnatal steroids	5 (31%)	3 (33%)	7 (41%)
Surfactant therapy	11 (69%)	7 (78%)	8 (47%)
<i>U. urealyticum</i> in TAF	2 (13%)	2 (22%)	7 (41%)
Death [§]	1 (6%)	5 (56%)	4 (24%)
BPD in survivors			
No	7 (44%)	1 (11%)	5 (29%)
Mild	5 (31%)	2 (22%)	6 (35%)
moderate	3 (19%)	1 (11%)	2 (12%)

SGA small for gestational age

AIS amnion infection syndrome

(P)ROM (premature) rupture of membranes;

PIH pregnancy induced hypertension

* Values are means ± SD

† p<0.01 (χ²-Test)

§ p<0.05 vs. control and chorioamnionitis

§ p<0.05 (χ²-Test)

** Values are medians (lower-upper quartile)

p=0.01 vs. control and funisitis

& p<0.01 vs. control and chorioamnionitis

Reviewers' Comments to Author:

Reviewer: 2

Comments to the Author

Minor: page 5, include dates of enrollment of these infants.

The dates of enrollment were introduced in the revised version of the manuscript (page 5, line 5)

page 6: rounds per minute (rpm) is usually referred to as revolutions per minute.

The wording was changed according to the reviewers suggestion (page 6, line 7)

Reviewer: 1

Comments to the Author

Thomas and colleagues have presented a new version of the manuscript. I continue to have a few questions/issues that need to be addressed.

Major

This manuscript was difficult to read and understand the path to the conclusions that the authors reached. The authors seem to hang on to the need to try to link funisitis and Ang-Tie2 system. The authors have presented data that there is an association with increased Ang2/SC and BPD and with decreased Ang1/Ang2 ratio and BPD. It seems that funisitis does not correlate with either of these. SGA seems to be a potential confounder of these values and BPD. However, the authors go on to say none of these (Ang1/Ang2 or Ang2/SC) correlate with the development of BPD when the analysis was corrected for confounders of gestational age, SGA and gender. Also, the relationship between BPD and sTie2 became not significant when adjusted for gestational age. Yet the authors in their response to Reviewer 3 state "However, a limitation of this approach is also the power < 80% in most cases to detect a significant interaction between the confounders or an influence of the single confounder on the dependent parameter", so is it that the association with BPD is lost with the multivariate analysis or not? In other words, do the authors believe that they can preform a believable multivariate analysis with the small patient sample. If they do not believe that they have the power to perform such an analysis, then state that as a limitation of the study! This is a PILOT (preliminary) study correct? It is okay to have a PILOT study that suggests an association between an measurable factor and a clinical outcome. That is what drives the rationale to commit resources to the investigation/validation of the hypothesis generated by the PILOT study. In other words, state that these are PILOT data and the application of confounders for multivariate analysis led to a power of <80% and thus could not be applied. This would lead to a

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rationale for a call for a larger study to look at the relationship between tracheal Ang2/SC and Ang1/Ang2 ratio as a predictor of BPD. The authors may want to consider that they rewrite the manuscript as looking for an association between funisitis and Ang-Tie2 system and did not find that, but they found that Ang2/SC and Ang1/Ang2 ratio may be a predictor of BPD but a larger study with appropriate patient numbers to allow multivariate comparison for confounders must be done. They can use ref 9 (Aghai et al) to support their preliminary data about the role of Ang-Tie2 signaling is important to be considered in the risk for development of BPD.

We greatly appreciate the suggestions of the reviewer which helped us to improve the manuscript substantially.

With the approach of a multivariate analysis we followed what we understood as advice of reviewer 3 in the first review process. However, we agree with reviewer 1 that multivariate analysis with inclusion of potential confounders is of very limited value unless the power to detect associations and interactions is > 80%. This is, as quoted from our former answer to reviewer 3, the case for most of the multivariate analyses we performed. We therefore decided to omit multivariate analysis from the second revision of our manuscript. We state that as major limitation in the discussion section (page 14, lines 18-25)

Following the reviewer's suggestion we state, that histological funisitis with the associated decrease of Ang2 in TAF, is most probably of minor relevance for outcome of the infants. In this regard we wrote our discussion more precisely. Indeed our preliminary data support the findings of Aghai and colleagues. We brought this issue into a sharper focus in the revised manuscript.

We have changed the discussion section substantially for the second revision to be more stringent and precise.

1- please consider showing the Ang2/SC data referred to on p9 line 18-20.

We introduced the Ang2/SC values on day 10 for infants who developed moderate BPD or died compared to those who survived without BPD in the revised version of our manuscript (page 9, lines 18-20)

2- Figure 4 does not seem needed and can be stated in the text.

According to the reviewer's suggestion we omitted figure 4 from our revised manuscript. However, we found that presentation of individual data for Ang1, Ang2 and sTIE2 for the three groups control, chorioamnionitis and funisitis in the text would be confusing for the reader. We therefore decided not to present them

in the text. The new figure 4 (formerly figure 5) clearly depicts the range of values we measured for all 3 parameters.

3- Each figure should specify in the figure that the factor being represented is from Tracheal Aspirates or from umbilical cord blood.

The figures and legends were changed according to the reviewer's suggestion

4- the authors allude to a relationship between Ang2 and VEGF on p13 and that increased Ang2 in the presence of decreased VEGF "causes endothelial cell death and vessel regression in the absence of sufficient angiogenic signals". Did they authors measure VEGF in their trach aspirate samples? If they did and there is a correlation between increased Ang2 and decreased VEGF, that would strongly support the assertion that there is an effect of impaired vascular signals on the risk of developing BPD.

We thank the reviewer for this very important remark. We did not measure VEGF in our samples. However, 2 groups have independently demonstrated VEGF protein levels in airway secretions to be lower in extremely premature infants who developed BPD compared to those who did not develop the disease (Lassus, AJRCCM 1999 and Been, Pediatr Res 2010). Another group demonstrated VEGF mRNA to be decreased in lungs of infants who died from BPD compared to controls who died from non-pulmonary causes (Bhatt, AJRCCM 2001). Therefore we still feel comfortable with our statement on page 13, lines 1-5 and we agree with the reviewer that low VEGF along with (relatively) increased Ang2 supports the concept of impaired vascular signaling in the pathogenesis of BPD which we state on page 12, lines 22-24.

5- The authors try to make a link between intraamniotic Ang2 production and an increase in Ang2/SC at day 5 of life in the tracheal aspirates. This is quite a reach, I would expect that if there was a relationship between amniotic production and airway levels, that these should be elevated on the day of birth and day 1. Perhaps the increased Ang2/SC is related to the treatments to keep a premature infant alive, e.g. Oxygen, ventilation, etc.

We are grateful for the reviewer's comment. We do not want to hypothesize that intraamniotic production of Ang2 might be a cause of elevated airway concentrations for this parameter on day 5 of postnatal life. The reviewer is absolutely right with his statement that rather postnatal factors like mechanical ventilation or oxygen therapy might contribute to increased release of Ang2 into the epithelial lining fluid of the infants.

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In normal gestations, Buhimschi demonstrated a direct correlation of GA with intraamniotic Ang1 and an inverse correlation with Ang2 along with an increase of the intraamniotic Ang1/Ang2 ratio with increasing GA. This is thought to be mandatory for the integrity of the placental vasculature. Intraamniotic Ang1 and Ang2 is likely produced, at least to a certain extent, by the fetal lung (Buhimschi J Clin Endocrinol Metab 2010). Data on intraamniotic Ang1/Ang2 in SGA infants or on pulmonary expression of the factors in utero do not exist to our knowledge. However it is an intriguing speculation that higher Ang2 as found on day 1-7 in our cohort (figure 2) and lower pulmonary Ang1/Ang2 ratios might reflect a disturbed gestational regulation of the angiogenic factors with effects for both impaired placental vascularization as in SGA infants and impaired pulmonary vascular development as in BPD.(page 13, lines 5-16)