Influence of foetal inflammation on the development of meconium aspiration syndrome in term neonates with meconium-stained amniotic fluid

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Background: Meconium-stained amniotic fluid is observed in approximately 10-15% of all deliveries, however, only 5% of neonates with meconium-stained amniotic fluid develop meconium aspiration syndrome (MAS). Although foetal distress and subsequent sympathetic stimulation have been considered as the primary upstream events of MAS, this clinical complication sometimes occurs due to other pathologies, such as intraamniotic inflammation. The aim of this study was to investigate whether the incidence of MAS is associated with the presence of funisitis and chorioamnionitis in term neonates with meconium-stained amniotic fluid.

Methods: Between April 2013 and March 2015, 95 term neonates with meconium-stained amniotic fluid, who were hospitalized at a neonatal intensive care unit, were enrolled in the study. The placenta and umbilical cord were histopathologically examined. Clinical variables and histopathological findings associated with the incidence of MAS were studied.

Results: A total of 36 neonates developed MAS. Univariate logistic regression analysis revealed that a heavier birth weight, male sex, 1-min Apgar score ≤ 7, funisitis (but not chorioamnionitis), and elevated acute-phase inflammatory reaction score were associated with increased incidence of MAS (all p < 0.05). The multivariate model comprised funisitis (OR=5.03, 95%CI, 1.63-15.5), 1-min Apgar score ≤ 7 (OR=2.74,95%CI, 1.06-7.09), and male sex (OR=3.4, 95%CI, 1.24-9.34).

Conclusion: In neonates with meconium-stained amniotic fluid, funisitis, as well as low 1-min Apgar score and male sex, was identified as an independent variable for MAS development. Intraamniotic inflammation might be involved in the pathological mechanisms of MAS.

1 **Influence of Foetal Inflammation on the Development of Meconium**
2 **Aspiration Syndrome in Term Neonates with Meconium–Stained**
3 **Amniotic Fluid**
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45   Abstract

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47   Background: Meconium-stained amniotic fluid is observed in approximately 10-15% of all

48   deliveries, however, only 5% of neonates with meconium-stained amniotic fluid develop

49   meconium aspiration syndrome (MAS). Although foetal distress and subsequent sympathetic

50   stimulation have been considered as the primary upstream events of MAS, this clinical

51   complication sometimes occurs due to other pathologies, such as intraamniotic inflammation.

52   The aim of this study was to investigate whether the incidence of MAS is associated with the

53   presence of funisitis and chorioamnionitis in term neonates with meconium-stained amniotic

54   fluid.

55   Methods: Between April 2013 and March 2015, 95 term neonates with meconium-stained

56   amniotic fluid, who were hospitalized at a neonatal intensive care unit, were enrolled in the

57   study. The placenta and umbilical cord were histopathologically examined. Clinical variables

58   and histopathological findings associated with the incidence of MAS were studied.

59   Results: A total of 36 neonates developed MAS. Univariate logistic regression analysis revealed

60   that a heavier birth weight, male sex, 1-min Apgar score ≤7, funisitis (but not chorioamnionitis),

61   and elevated acute-phase inflammatory reaction score were associated with increased incidence

62   of MAS (all p<0.05). The multivariate model comprised funisitis (OR=5.03, 95%CI, 1.63-15.5),

63   1-min Apgar score ≤7 (OR=2.74, 95%CI, 1.06-7.09), and male sex (OR=3.4, 95%CI, 1.24-9.34).

64   Conclusion: In neonates with meconium-stained amniotic fluid, funisitis, as well as low 1-min

65   Apgar score and male sex, was identified as an independent variable for MAS development.

66   Intraamniotic inflammation might be involved in the pathological mechanisms of MAS.

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68

69   Introduction

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71 Meconium-stained amniotic fluid is observed in approximately 10-15% of all deliveries (Yoder

72 et al., 2002), which has been considered as the primary cause of meconium aspiration syndrome

73 (MAS). However, only 5% of neonates with meconium-stained amniotic fluid develop MAS

74 (Yoder et al., 2002; Dargaville et al., 2006; Swarnam et al., 2012). In addition, the use of

75 surfactant replacement, nitric oxide therapy and extracorporeal membrane oxygenation has led to

76 a marked decrease in mortality and morbidity associated with MAS in the recent times (Swarnam

77 et al. 2012). Nevertheless, these therapeutic options are usually unavailable for neonates born in

78 a low-resource setting. Even in a high-resource setting, advanced intervention is often delayed,

79 because the current diagnostic algorithms of MAS based on clinical symptoms and X-ray

80 patterns do not provide sufficient insight as to who are likely to develop severe respiratory

81 failure (Ahanya et al., 2005; Swarnam et al., 2012).

82   To establish early, reliable biomarkers of MAS, precise understanding of the pathological

83 mechanism is essential. Although asphyxia and subsequent sympathetic stimulation have been

84 considered to trigger foetal passage of meconium into amniotic fluid (Ahanya et al., 2005; van

85 Ierland et al., 2009; Swarnam et al., 2012; Lindenskov et al., 2015), MAS sometimes develops in

86 the absence of overt episodes suggestive of antenatal asphyxia; several previous studies did not

87 find an association between low Apgar scores, umbilical arterial acidosis, and incidence of MAS

88 (Trimmer et al., 1991; Ramin et al., 1993; Blackwell et al., 2001; Oyelese et al., 2006),

89 suggesting the presence of additional triggers of MAS, such as intraamniotic inflammation.

90   Studies suggested that meconium-stained amniotic fluid is more likely to be contaminated with

91 bacteria, endotoxin, and inflammatory mediators than clear amniotic fluid, and often

92 accompanied by clinical evidence of intraamniotic inflammation (Romero et al., 2014).

93 Meconium-stained amniotic fluid is also linked to findings suggestive of intraamniotic

94   inflammation, which is represented by funisitis and chorioamnionitis (Burgess et al., 1996;

95   Romero et al., 2014; Choi et al., 2015; Lee et al., 2016). While chorioamnionitis is suggestive of

96   maternal inflammatory response, funisitis is considered to represent foetal inflammatory

97   response (Redline et al., 2003). In preterm neonates with funisitis, elevated plasma interleukin-6

98   (IL-6) level in cord blood (Yoon et al., 2000) is associated with increased incidence of adverse

99   neonatal outcomes such as chronic lung disease, intracranial haemorrhage, and cerebral palsy

100   (Kim et al., 2001; Yoon et al., 2000). However, few studies in term neonates have investigated

101   the influence of intraamniotic inflammation to the development of MAS.

102   We performed a prospective observational study to investigate whether the incidence of MAS is

103   associated with the presence of funisitis and chorioamnionitis.

104
105   Methods

106   Patients and Methods

107   This study was conducted under the approval of the Ethics Committee of Nagoya West Medical

108   Center (approval reference number: 18-04-315-07). The internal review board advised that there

109   is no statutory requirement for parental consent for the use of clinical and histopathological data,

110   which are collected as a routine clinical care in the unit.

111

112   Study population

113   Between April 2013 and March 2015, 969 inborn neonates were hospitalized at a level-II

114   neonatal intensive care unit of Nagoya West Medical Center (Nagoya, Aichi, Japan), which

115   provides intensive care for virtually all sick neonates, except for those who require advanced life

116   support treatments, such as haemodialysis/filtration, cardiac surgery, and extracorporeal

117   membrane oxygenation. Of these, 239 inborn neonates ≥37 weeks of gestation were admitted

118   within 24 h of birth, whose blood samples on day 0 were available. Subsequently, 99 neonates

119   were enrolled in the study after excluding 10 neonates with major congenital anomalies and 130

120   neonates without meconium-stained amniotic fluid (Figure 1).

121

122   Clinical variables

123   Presence of meconium-stained amniotic fluid was evaluated at birth under visual observation as

124   previously described (Lee et al., 2016). MAS was defined as respiratory distress in neonates

125   though meconium-stained amniotic fluid whose symptoms cannot be otherwise explained,

126   requiring assisted mechanical ventilation or oxygen at a concentration of $\geq$40% for at least 48 h,

127   and radiographic findings were consistent with MAS (Fraser et al., 2005; Lee et al., 2016). X-ray

128   photographs were reviewed by an experienced staff neonatologist, and the placenta and umbilical

129   cord were histopathologically examined by a single pathologist. Chorioamnionitis was defined as

130   infiltration of neutrophils identified in chorionic membranes (Stage II by Blanc's classification),

131   whereas funisitis was defined as infiltration of neutrophils within the walls of umbilical vessels

132   or in Wharton's jelly (Blanc et al., 1979; Redline et al., 2003). Other clinical variables were

133   collected from patients' clinical record, including gestational age, birth weight and its standard

134   score calculated against the standard Japanese birth weight for parity and gestational age

135   (Itabashi K et al. 2010), sex, delivery mode, premature rupture of the membranes, Apgar score at

136   1 and 5 min, cord blood pH, and the duration of invasive/non-invasive positive-pressure

137   ventilation and oxygen supplementation.

138

139   Laboratory studies

140   In our unit, at the admission of neonates born through meconium-stained amniotic fluid, routine

141   blood tests are performed including blood gas analysis, cell counts, blood sugar level, and serum

142   inflammatory markers. For the inflammatory markers, C-reactive protein (CRP), α_1-acid

143   glycoprotein (α_1AG), and haptoglobin were measured using turbidimetric immunoassay (Quick

144   Turbo, Shino-Test Corporation, Japan). CRP >0.3 mg/dL, α_1AG >20 mg/dL, and haptoglobin

145   >13 mg/dl were regarded as positive (Speer et al., 1983; Ipek et al., 2010; Nakamura et al.,

146   2015); the acute-phase inflammatory reaction scores of 0 (none positive), 1 (one positive

147   biomarker), 2 (two positive biomarkers) and 3 (all three positive biomarkers) were given based

148   on the number of positive inflammatory biomarkers (Nakamura et al., 2015). Venous blood

149   culture was assessed for neonates with clinical signs suggestive of antenatal infection (e.g.

150   elevation of maternal white blood cell count and CRP, maternal pyrexia, and premature rupture

151   of the membranes) or with the acute-phase inflammatory reaction scores ≥1.

152

153   Data analysis

154   The clinical variables were compared between neonates with and without MAS. Apgar scores

155   (≤7) and cord blood pH (<7.1) were divided using clinically relevant thresholds. All statistical

156   analyses were performed using R (The R Foundation for Statistical Computing, Vienna, Austria,

157   version 1.22) and its Japanese interface (EZR; Saitama Medical Center, Jichi Medical

158   University, Saitama, Japan) (Kanda, 2013). Logistic regression analyses were performed to

159   develop a model explaining MAS development using funisitis and chorioamnionitis as

160   mandatory independent variables (only one of the two mandatory variables were tested at a time

161   because of the collinearity observed between each other). Statistical findings were not corrected

162   for multiple comparisons for univariate analysis, because of the exploratory nature of this study.

163

164   Results

165   Of 99 term neonates with meconium-stained amniotic fluid, histopathological examination

166   was not available in 4 neonates, whose data were not considered further. Of 95 remaining

167   neonates, 36 neonates (37.9%) developed MAS, none of whom showed positive blood culture.

168   Gestational age, caesarean delivery, and incidence of premature rupture of the membranes and

169   cord blood acidosis did not differ between neonates who developed and did not develop MAS

170   (Table 1). Univariate logistic regression analysis showed that a high birth weight, male sex, 1-

171   min Apgar score ≤ 7, funisitis, and a high acute-phase inflammatory reaction score of 3

172   (compared with 0) were associated with increased incidence of MAS (all $p<0.05$; Table 1). The

173   final model to explain the incidence of MAS in neonates with meconium-stained amniotic fluid

174   comprised funisitis (OR, 5.03; 95%CL, 1.63-15.5), low 1-minute Apgar score ≤ 7 (OR, 2.74;

175   95%CL, 1.06-7.09), and male sex (OR, 3.4; 95%CL, 1.24- 9.34), where chorioamnionitis was

176   not identified as an independent variable to explain MAS development (See Supplemental Table

177   1 for the multivariate model, which used chorioamnionitis instead of funisitis; and Supplemental

178   Tables 2 and 3 for the multivariate model, which used the duration of mechanical ventilation and

179   oxygen supplementation as alternative dependent variables).

180
181   Discussion

182   MAS is one of the most commonly recognized respiratory diseases in neonates, however, the

183   exact pathological mechanism leading to foetal meconium passage into the amniotic fluid and

184   its aspiration is still unknown. Our study suggested that, in neonates with meconium-stained

185   amniotic fluid, funisitis, as well as low 1-min Apgar scores and male sex, was associated with

186   increased incidence of MAS, pointing towards the role of inflammatory factors in the

187   pathophysiology of MAS. Further large-scale studies are required to delineate the role of

188   antenatal inflammation in the development of meconium-stained amniotic fluid and MAS.

189   Previous studies highlighted that MAS development is associated with low cord blood pH and

190   Apgar scores at 5 min (Dargaville et al., 2006; van Ierland et al., 2009; Karabayir et al., 2015),

191   supporting the hypothesis that hypoxic-ischemic events trigger foetal meconium passage into the

192   amniotic fluid and its subsequent aspiration. However, several studies failed to demonstrate the

193   association between cord blood pH, Apgar scores and MAS development (Trimmer et al., 1991;

194   Ramin et al., 1993; Blackwell et al., 2001; Oyelese et al., 2006). Our study also found that only

195   the 1-min Apgar scores, but not the 5-min Apgar scores and cord blood pH, were associated with

196   MAS development, suggesting that antenatal hypoxic-ischemia is not sufficient to explain MAS

197   development, and other control variables of MAS might exist.

198   In our study, funisitis was associated with an increased incidence of MAS, supporting a

199   hypothesis that intraamniotic inflammation might be an additional factor involved in the

200   development of MAS. A study that investigated autopsy cases with histopathologic evidence of

201   intrauterine meconium exposure found that pulmonary inflammation was associated with

202   inflammation of the umbilical cord, as well as meconium aspiration (Romero et al., 2014).

203   Thureen et al. showed that three of six placentas with MAS showed funisitis associated with

204   villus ischemic changes, which may have contributed to in utero foetal hypoxia (Thureen et al.,

205   1997). Lee et al. showed that neonates diagnosed with funisitis were at more than 4-fold risks of

206   developing MAS than those without funisitis, whereas Kim et al. observed that the incidence and

207   severity of funisitis were associated with those of MAS (Lee et al., 2016; Kim et al., 2017),

208   suggesting that intraamniotic inflammation with foetal systemic inflammation might be an

209   important upstream control variable in MAS (Lee et al., 2016). By performing placental

210   histopathological examination virtually for all NICU neonates, our study provided additional

211   evidence to support the impact of intraamniotic inflammation to MAS development.

212   In our study, MAS development was associated with funisitis, but not chorioamnionitis. It is

213   possible that chorioamnionitis might represent a maternal inflammatory response to

214   intraamniotic infection, whereas funisitis might be predominantly represent the foetal

215   inflammatory response, which is accompanied by elevation of inflammatory cytokine and

216   procytokine levels. Intraamniotic inflammation occurs more frequently in the presence of

217   meconium-stained amniotic fluid than that in clear amniotic fluid, as shown by elevated amniotic

218   fluid IL-6 levels in neonates with meconium-stained amniotic fluid (Romero et al., 2014). In

219   animal models, foetal swallowing of amniotic fluid containing bacteria, endotoxin and

220   proinflammatory mediator is suggested to evoke inflammation and subsequent bowel peristalsis

221   and meconium passage, which may explain both meconium passage in utero and its aspiration in

222   association with foetal infection and inflammation. A clinical study confirmed that meconium

223   with bacteria and inflammatory mediators may be aspirated in utero following stressful events

224   with or without acidaemia. It is possible to extrapolate that intraamniotic inflammation triggers a

225   range of foetal inflammatory response, which is represented by funisitis and elevation of

226   inflammatory biomarkers. Foetal ingestion of amniotic fluid containing inflammatory mediators

227   may ultimately induce the meconium passage into the amniotic fluid. Although our study did not

228   measure inflammatory cytokine levels, three inflammatory biomarkers, CRP, α_1AG, and

229   haptoglobin, were employed, which are produced in the liver in response to intrinsic/extrinsic

230   stimuli and subsequent elevation of IL-1ß and IL-6 (Ipek et al., 2010; Nakamura et al., 2015).

231   Although these inflammatory biomarkers and their composite scores were not involved within

232   the final model to explain the development of MAS, there was a consistent trend that an increase

233 in inflammatory biomarkers was associated with relatively higher incidence of MAS. Consistent

234 to our findings, Hofer et al. reported that elevated CRP levels and changes in white blood

235 cell/neutrophil counts were associated with the severity of MAS, although this study

236 simultaneously highlighted the potential impact of mechanical ventilation on the inflammatory

237 biomarkers (Hofer et al., 2016). Further accumulation of knowledge regarding the role of

238 inflammatory reactions may accelerate the establishment of biomarkers, which provide early,

239 precise prediction of MAS development in neonates, who are born through meconium-stained

240 amniotic fluid.

241   It is known that inflammatory responses are sex-dependent; for example, studies in vivo

242 demonstrated greater release of proinflammatory cytokines after in vivo lipopolysaccharide

243 stimulation in male foetuses than in female foetuses (Kim-Fine et al., 2012; Koch et al., 2014).

244 Lambermont and colleagues reported increased white blood count response in bronchoalveolar

245 fluid and worse lung compliance following exposure to chorioamnionitis in male than in female

246 neonates (Lambermont et al., 2012). In our study, male neonates had at a higher risk of

247 developing MAS than female neonates. However, the size of our study population was too small

248 to confirm the role of sex on MAS development.

249   Several limitations of our study were noted, which need to be considered when interpreting

250 our findings. Although we performed standard histopathological examinations of the placenta

251 and umbilical cord for all participating neonates, the study population is relatively small, which

252 did not allow the assessment of some important clinical cofounders. To minimize the

253 population bias, our study was conducted at a single level-II unit recruiting only inborn

254 neonates. However, we were not able to include neonates, who did not have meconium-stained

255 amniotic fluid, and those, who were not hospitalized at NICU, leaving uncertainly around the

256 role of intraamniotic inflammation in the entire birth cohort. Several important clinical variables

257 associated with antenatal inflammation and hypoxic-ischemic stress, such as maternal fever,

258 infection and finding from cardiotocography, were not incorporated within the analysis, leading

259 to a difficulty in discriminating acute chorioamnionitis and funisitis from chronic ones. A

260 prospective cohort study is currently underway to elucidate the role of intraamniotic

261 inflammation on MAS development in all neonates born at our hospital by involving extensive

262 maternal and neonatal variables.

263
264 Conclusions
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266 The incidence of MAS was associated with funisitis, as well as low 1-min Apgar scores and

267 male sex, suggesting that intraamniotic inflammation might also be involved in the pathological

268 mechanisms of MAS. Further large-scale prospective studies are needed to delineate the detailed

269 mechanism of MAS and allow its efficient prediction and prevention.

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372   an intra-amniotic inflammation and the development of cerebral

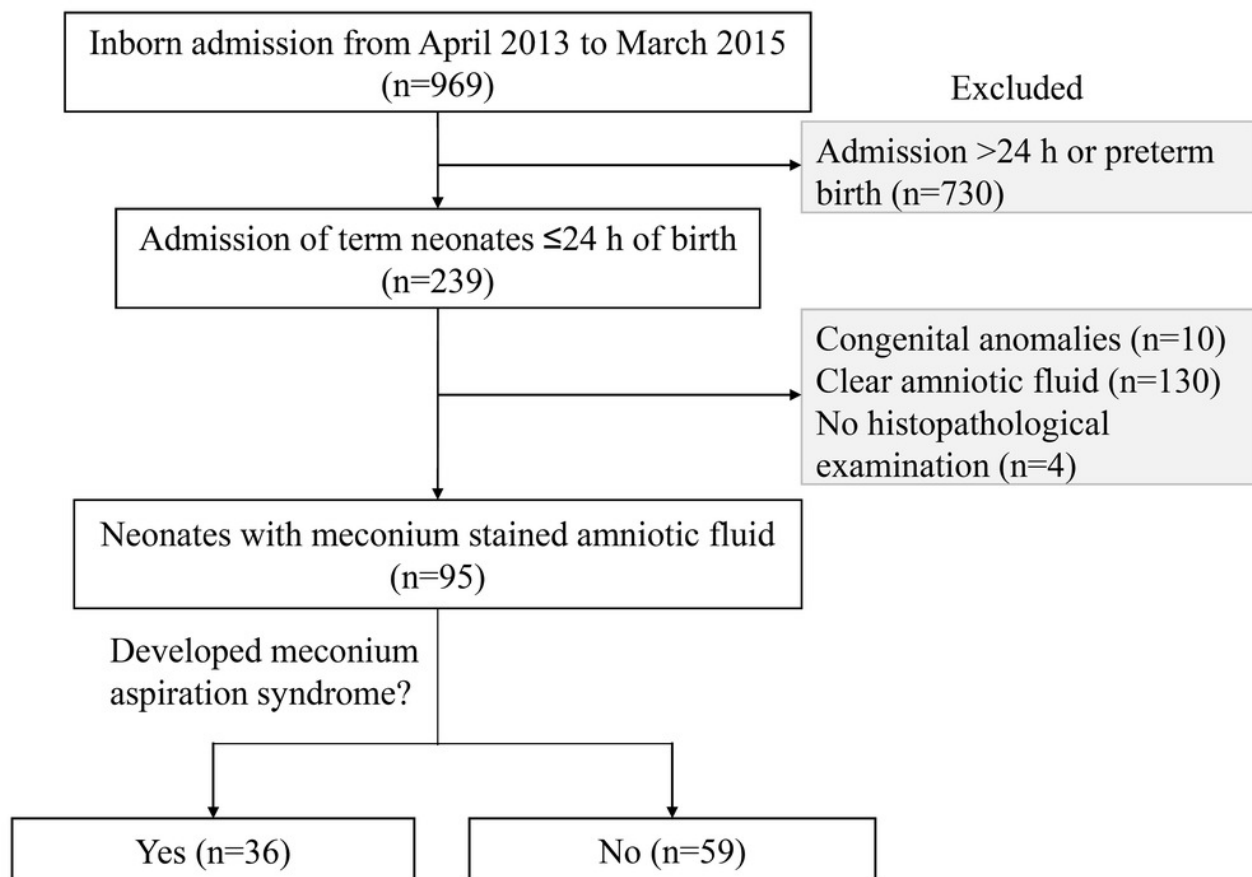
373   palsy at the age of three years. *Am J Obstet Gynecol* 182:675-681.

374

Figure 1

Profile of the study population

Ninety-five neonates, who were born through meconium-stained amniotic fluid and admitted to the neonatal intensive care unit within 24 hours of birth, were studied.

Table Independent variables of meconium aspiration syndrome development

1 **Table**

3 **Table 1: Independent variables of meconium aspiration syndrome development**

| | Meconium aspiration syndrome | | Odds ratio | | | |
| | | | | 95% CI | | p-value |
	No (n=59)	Yes (n=36)	Mean	Lower	Upper	
Univariate model						
Gestation (weeks)	40.3 ± 0.3	40.5 ± 0.4	1.11	0.74	1.66	0.610
Birth weight (kg)	3.02 ± 0.56	3.24 ± 0.45	2.38	1.00	5.66	0.050
Birth weight z-score	-0.32±1.48	0.21±1.25	1.32	0.97	1.81	0.080
SGA <2SD of norm	11(19)	2 (6)	0.26	0.03	1.31	0.259
Male sex	31 (54)	28 (78)	3.16	1.24	8.07	0.016
Delivery mode			1.45	0.89	2.34	0.136
Vaginal	34 (58)	16 (44)	0.56	0.24	1.29	0.171
Forceps/vacuum	11 (19)	7 (19)	0.91	0.32	2.58	0.865
Caesarean	12 (20)	13 (36)	2.15	0.87	5.33	0.097
PROM>24h	13 (22)	11 (31)	1.56	0.61	3.98	0.355
1-minute Apgar ≤7	20 (34)	21 (58)	2.44	1.04	5.70	0.040
5-minute Apgar ≤7	12 (20)	9 (25)	1.12	0.41	3.07	0.827
Cord blood pH <7.1	7 (12)	5 (14)	1.20	0.35	4.10	0.773
Cord blood pH <7.2	22 (37)	19 (53)	1.88	0.81	4.36	0.141
Chorioamnionitis	26 (44)	20 (56)	1.59	0.69	3.65	0.278
Funisitis	35 (59)	30 (83)	3.43	1.24	9.50	0.018
Inflammatory biomarkers at admission						
CRP (>0.3 mg/dl)	8 (14)	9 (25)	1.18	0.83	1.69	0.354
α1-AG (>20 mg/dl)	38 (64)	28 (78)	1.01	1.00	1.03	0.101
HP (>13 mg/dl)	8 (14)	10 (28)	1.02	1.00	1.04	0.060
Acute-phase inflammatory reaction score						
0	21 (36)	8 (22)		Reference		
1 or 2	33 (55)	19 (53)	1.59	0.59	4.26	0.356
3	5 (9)	9 (25)	4.72	1.21	18.5	0.026
Multivariate model						
Funisitis			5.03	1.63	15.5	0.005
1-minute Apgar ≤7			2.74	1.06	7.09	0.038
Male sex			3.4	1.24	9.34	0.018

4 **Notes:** Values are shown as mean ± standard deviation or number (%).

5 **Abbreviations:** CI, confidence interval. CRP, C-reactive protein. α_1AG, α_1-acid glycoprotein. HP,

6 haptoglobin. SD, standard deviation. SGA, small for gestational age.