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Clinicopathologic implication of necrotizing funisitis as an indicator of severe intra-amniotic inflammatory response and frequent amnionitis

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Abstract

Objective: Necrotizing funisitis (NF) is defined as the presence of an arc (i.e., crescent/band/ring/halos) of infiltrated neutrophils and/or associated debris in Wharton's jelly (WJ) of umbilical-cord (UC). However, no information exists about the comparison in intra-amniotic inflammatory-response (IAIR) and inflammation in extra-placental membranes between the presence and absence of NF in the context of inflammation in WJ among spontaneous preterm births (PTBs). The objective of current study is to examine this issue.

Methods: We examined IAIR and the frequency of amnionitis according to the progression of inflammation in UC (i.e. stage-1,

umbilical phlebitis [inflammation in umbilical-vein(UV)] only; stage-2, involvement of at least one umbilical-artery[UA] and either the other UA or UV without extension into WJ; stage-3, the extension of inflammation into WJ without NF; stage-4, the extension of inflammation into WJ with NF) in 120singleton spontaneous PTBs (<37weeks). IAIR was gauged by AF MMP-8 (ng/ml) within 3days before birth.

Results: 1) Stage-1, stage-2, stage-3, and stage-4 were present in 20%(24/120), 6%(7/120), 61%(73/120), and 13%(16/120) of cases respectively; 2) AF MMP-8 continuously increased (stage-1 vs. stage-2 vs. stage-3 vs. stage-4; median[ng/ml], range[ng/ml]; 207.2[16.8-1196.5] vs. 444.1[8.5-2608.0] vs. 458.8[0.4-3116.7] vs. 1859.7[912.3-5304.8]; Spearman's rank correlation-test, α=0.454, P=0.006), and the frequency of increased AF MMP-8 (≥854.1ng/ml) elevated (stage-1 vs. stage-2 vs. stage-3 vs. stage-4; 13%[1/8] vs. 33%[1/3] vs. 32%[6/19] vs. 100%[5/5]; Linear-by-linear-association, P=0.012) with the progression of inflammation in UC; 3) Moreover, there was a stepwise increase in the frequency of amnionitis according to the progression of inflammation in UC (stage-1, 33%[8/24]; stage-2, 43%[3/7]; stage-3, 62%[45/73]; stage-4, 81%[13/16]; Linear-by-linear-association, P=0.001).

Conclusion: NF is an indicator that IAIR is more severe and amnionitis is more frequent in the context of the extension of inflammation into WJ. Therefore, current study confirms that NF is the most advanced stage in the progression of inflammation within UC.

Keywords: necrotizing funisitis, intra-amniotic inflammatory response, amnionitis, inflammation into Wharton's jelly

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Introduction

Ascending intra-uterine infection (AIUI) is one of the important pathophysiologies for preterm birth (PTB) due to either preterm labor and intact membranes (PTL) or preterm premature rupture of membranes (preterm-PROM) [1, 2]. AIUI sequentially progresses as follows: 1) firstly, vaginal-cervical canal (i.e., vaginitis and cervicitis); 2) chorio-decidua (CD) of extra-placental membranes (EPM) (i.e., choriodeciduitis); 3) amnion of EPM (i.e., amnionitis) and intra-amniotic cavity (i.e., intra-amniotic inflammatory response [IAIR]); 4) finally, fetus (i.e., inflammation in umbilical cord [UC]) [1, 2]. Inflammation in UC called as funisitis is the most advanced stage of AIUI and the histologic hallmark of fetal inflammatory syndrome (FIRS) defined as an elevated UC blood IL-6 (>11 pg/mL) or CRP (≥200 ng/mL) [3, 4]. Of note, among the progression of inflammation in UC, necrotizing funisitis (NF) is defined as the presence of an arc (i.e., band, ring, halos) of infiltrated neutrophils and/or associated debris in Wharton's jelly (WJ) around one or more umbilical vessels, while the extension of inflammation into WJ is diagnosed as the presence of infiltrated neutrophils in perivascular WJ and further extending deep into WJ [5, 6]. Neutrophils in amniotic fluid (AF) could be considered to be a signature of fetal inflammatory response (FIR) [4, 7]. Indeed, neutrophils in AF were reported to be of fetal origin in about two-thirds of cases with intra-amniotic infection or inflammation [8]. Of note, neutrophils in AF can release matrix metalloproteinase-8 (MMP-8) into AF [8, 9] thereby suggesting that IAIR gauged by AF MMP-8 can be a surrogate marker for FIR. Moreover,

amnionitis in the progression of inflammation in EPM may be another surrogate marker for FIR, considering that amnionitis is an independent risk factor for early onset neonatal sepsis and an indicator that FIR and IAIR are more likely and severe [10].

Up to now, there are various classifications for the progression of inflammation in UC (Table 1) [8, 10–28]. However, as shown in the Table 1, we cannot find any classification including both ‘the extension of inflammation into WJ’ and ‘NF’ in the progression of inflammation in UC. Indeed, '2015 Amsterdam placental workshop group consensus statement' [5, 6] did not consider the presence or absence of inflammation in WJ, while 'modified Salafia's criteria' [29] did not examine NF as in the following: 1) 2015 Amsterdam placental workshop group consensus statement; stage 1, neutrophil infiltration within the umbilical vein (UV) wall with or without extension into WJ: stage 2, neutrophil infiltration within at least one umbilical artery (UA) and either the other UA or UV with or without extension into WJ: stage 3, the presence of NF (Fig. 1a), and 2) modified Salafia's criteria; stage 0, inflammation free UC; stage 1, umbilical phlebitis; stage 2, involvement of at least one UA and either the other UA or UV without extension into WJ; stage 3, the extension of inflammation into WJ (Fig. 1b). Moreover, although two previous studies examined surrogate markers for FIR such as IAIR [17] and amnionitis [13] according to the progression of inflammation in UC, they did not include NF (Table 1). Given that the density of fetal neutrophils extending into WJ is generally higher in cases with NF than in those without NF, it is plausible that NF is associated with more severe IAIR and more frequent

amnionitis in the context of inflammation in WJ. Our hypothesis was that IAIR and amnionitis would be more severe and frequent in cases with NF than in those without NF in the context of inflammation in WJ. The objective of this study is to examine this issue.

Methods

1.1. Study design and patient population

The study population included one hundred and twenty pregnant women who delivered at Seoul National University Hospital (SNUH) from December 1993 to March 2013 and met the following criteria: 1) singleton pregnancy; 2) gestational age (GA) at delivery: 20.0–36.6 weeks, 3) PTB due to either PTL (45 cases) or preterm–PROM (75 cases), 4) placental pathologic slides available, and 5) the presence of inflammation in UC. At our institution, transabdominal amniocentesis for the identification of intra–amniotic infection/inflammation and placental pathologic examination after delivery were routinely recommended and performed on all pregnant women hospitalized with either PTL or preterm–PROM. The relationship between AF MMP–8 concentrations and the progression of inflammation in UC was examined in 41 patients delivered within 3 days of amniocentesis. The criterion of amniocentesis–to–delivery interval was applied to preserve a meaningful temporal relationship between the results of AF studies and the pathologic findings of placenta obtained at delivery. PTL and preterm–PROM were diagnosed with previously published criteria [29]. Written informed consent was gained from the study population. The Institutional Review Board of our institute (SNUH) specifically approved current study.

1.2. Clinical characteristics and pregnancy outcomes

Clinical characteristics and pregnancy outcomes were investigated from the medical records. Data included maternal age, parity, GA at amniocentesis, cause of preterm delivery, gender of newborn, delivery mode, GA at delivery, birth weight, 1 min and 5 min Apgar scores, antenatal use of corticosteroids, antenatal use of antibiotics, and antenatal use of tocolytics.

1.3. Diagnosis of inflammation in umbilical cord (UC) and amnionitis

Placental tissue samples for pathologic examination included EPM (i.e., CD and amnion), chorionic plate, and UC. These samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of prepared tissue blocks were stained with hematoxylin and eosin (H&E). Clinical information regarding the placental tissues was not disclosed to pathologists. Inflammation in UC was diagnosed in the presence of neutrophil infiltration into the umbilical vessel walls or WJ according to previously published criteria [26], and NF was defined neutrophils with or without associated debris in concentric bands-rings-halos around one or more umbilical vessels according to previously reported criteria [5]. The progression of inflammation in UC was classified into four groups as follows; 1) stage-1: neutrophil infiltration within the UV wall; 2) stage-2: neutrophil infiltration within at least one UA and either the other UA or UV without extension into WJ; 3) stage-3: the extension of neutrophil infiltration into WJ without NF; 4) stage-4: the extension of neutrophil infiltration into WJ with NF. Amnionitis was diagnosed

in the presence of one or more focus of at least five neutrophils in amnion according to the previously published criteria [26].

1.4. Amniotic fluid (AF) studies

AF was centrifuged and stored in polypropylene tubes at -70℃ [9]. MMP-8 concentrations in stored AF were measured with a commercially available enzyme-linked immunosorbent assay (Amersham Pharmacia Biotech, Inc., Little Chalfont, Bucks). Details about this assay and its performance have been previously reported [9]. The intensity of IAIR was gauged by AF MMP-8 level (ng/ml) as previously described [9]. AF MMP-8 results were available in 35 patients because IAIR was not examined in 6 patients due to the limited amount of remained AF amount.

1.5. Statistical analysis

We used the Kruskal-Wallis test and Pearson's chi-square test for the comparison of continuous and categorical variables respectively. Multiple comparisons of continuous and categorical variables between the groups according to the progression of inflammation in UC were performed with 1-way ANOVA with posthoc Tukey test and Fisher's exact test with Bonferroni's correction, respectively. Spearman's rank correlation test was used to examine the correlation between AF MMP-8 concentrations and the progression of inflammation in UC. The receiver operating characteristics (ROC) curve was used to estimate the best cut-off values (maximum sum of

sensitivity and specificity) at which to identify AF MMP-8 as being raised or not raised for the detection of NF. Using these cut-off values, we compared the frequency of increased AF MMP-8 and amnionitis according to the progression of inflammation in UC with Pearson's chi-square test. Moreover, linear by linear association was used to investigate the trend about the frequency of increased AF MMP-8 and amnionitis according to the progression of inflammation in UC. Diagnostics indices (sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio) were determined for AF MMP-8 and amnionitis to identify NF. Multiple logistic regression analysis was performed for the exploration of the relationship between various variables and NF. Statistical significance was defined as $P<0.05$.

Results

1.1. Clinical characteristics and pregnancy outcomes according to the progression of inflammation in umbilical cord (UC)

Table 2 demonstrated clinical characteristics and pregnancy outcomes according to the progression of inflammation in UC. Stage-1 (umbilical phlebitis only), stage-2 (involvement of at least one UA and either the other UA or UV without extension into WJ), stage-3 (the extension of inflammation into WJ without NF), and stage-4 (the extension of inflammation into WJ with NF) were present in 20.0% (24/120), 5.8% (7/120), 60.8% (73/120), and 13.3% (16/120) of cases respectively. There was no significant difference in GA at delivery and other variables except antenatal antibiotics use among the groups according to the progression of inflammation in UC.

1.2. Amniotic fluid (AF) MMP-8 concentrations according to the progression of inflammation in umbilical cord (UC)

Fig. 2 shows AF MMP-8 concentrations (ng/ml) according to the progression of inflammation in UC. AF MMP-8 concentrations significantly and continuously increased according to the progression of inflammation in UC (Kruskal-Wallis test, P=0.0031 and Spearman's rank correlation, P=0.006).

1.3. Diagnostic indices, predictive values, and likelihood ratios of increased AF MMP-8 concentration and amnionitis for the identification of necrotizing funisitis (NF)

A ROC curve was constructed to select the cut-off values at which to identify AF MMP-8 (area under curve, 0.887; standard error, 0.062; P=0.006) as being raised or not raised for the identification of NF, and a cut-off values of 854.1 for AF MMP-8 (ng/ml) was chosen respectively. Table 3 describes diagnostic indices, predictive values and likelihood ratios of AF MMP-8 concentration (≥854.1 ng/ml) and amnionitis to identify pregnant women with NF.

1.4. The frequency of increased AF MMP-8 concentration (≥854.1 ng/ml) and amnionitis according to the progression of inflammation in umbilical cord (UC)

Figs. 3a and 3b demonstrated a significant and gradual increase in the frequency of increased AF MMP-8 concentration (≥854.1 ng/ml) (Fig. 3a) and amnionitis (Fig. 3b) according to the progression of inflammation in UC (increased AF MMP-8 concentration [≥854.1 ng/ml], each for P<0.05 in Pearson's chi-square test and linear-by-linear association; and amnionitis, each for P<0.05 in Pearson's chi-square test and linear-by-linear association). Moreover, Table 4 demonstrated increased AF MMP-8 concentration (≥854.1 ng/ml) was a significantly independent risk factor for NF even after the correction for potential confounding variables including GA at delivery.

1.5. The progression of inflammation in umbilical cord (UC)

Fig. 4 shows representative images for umbilical phlebitis only (Fig. 4a, stage-1), involvement of at least one UA and either the other UA or UV without extension into WJ (Fig. 4b, stage-2), the extension of inflammation into WJ without NF (Fig. 4c, stage-3), and the extension of inflammation into WJ with NF (Fig. 4d, stage-4) in H&E stained histologic sections of UC.

Discussion

1.1. Principal findings of the study

The principal finding of this study is that the IAIR and amnionitis, the surrogate markers for FIR, are more severe and frequent in cases with NF than in those without NF in the context of inflammation in WJ among spontaneous PTBs.

1.2. The limitations of previous studies reporting the relationship between surrogate markers for fetal inflammatory response (FIR) and the progression of inflammation in umbilical cord (UC) with or without necrotizing funisitis (NF)

Almost all studies except Buhimschi's report [17] did not examine the relationship between IAIR and the progression of the inflammation in UC with or without NF although substantial numbers of studies examined the association between IAIR and the presence of either funisitis or NF [8, 10, 16, 17, 19, 21, 24] (Table 1). Moreover, several studies only reported the relationship between amnionitis and the presence of either funisitis or NF [10−13], but not the progression of inflammation in UC regardless of the inclusion of NF. Although only one study reported that umbilical arteritis was more frequent with increasing stages of membrane inflammation, it did not include NF in the progression of inflammation in UC [13]. However, current study firstly demonstrated that surrogate markers for FIR (i.e., IAIR and amnionits as an advanced stage of inflammation in EPM) continuously increased according to the progression of inflammation

in UC including both ‘the extension of inflammation into WJ’ and ‘NF’.

1.3. Intra-amniotic inflammatory response (IAIR), amnionitis and a more dense neutrophil infiltration into Wharton’s jelly (WJ) in cases with necrotizing funisitis (NF)

Neutrophils recruitment and infiltration develop in the CD of EPM, and subsequently activate and operate the pro-survival program (i.e., anti-apoptosis) leading to the more accumulation of neutrophils in the context of intrauterine infection and/or inflammation [30]. In general, the recruitment, infiltration, or activation of neutrophils is known to be increased according to the severity of the infection and/or inflammation [31]. Based on the representative images of stage-3 (the extension of neutrophil infiltration into WJ without NF) and stage-4 (the extension of neutrophil infiltration into WJ with NF) in Fig. 4, the density of neutrophil infiltration in WJ is much more in stage-4 with NF than in stage-3 without NF. Moreover, it should be noted that IAIR elevates according to the density of neutrophil infiltration in CD in cases with inflammation restricted to CD among spontaneous PTBs [32]. Given that the density of neutrophil infiltration in WJ is much more in stage-4 with NF than in stage-3 without NF and IAIR increases according to the density of neutrophil infiltration in the CD of EPM, one can expect that IAIR is more severe in stage-4 with NF than in stage-3 without NF in the context of inflammation into the WJ of UC. Indeed, cases with stage-4 (inflammation in WJ [+] and NF [+]) had a more severe IAIR than stage-3

(inflammation in WJ [+] and NF [−]) in cases with inflammation of UC. Moreover, we also found that amnionitis, as another surrogate marker for FIR, is more frequent in cases with stage−4 with NF than in those with stage−3 without NF among cases with inflammation of UC.

1.4. The effect of necrosis on inflammatory response

We should explain the reason why NF is associated with more intense surrogate markers for FIR (i.e., IAIR and amnionitis). Our explanation is as follows. Firstly, some neutrophils are reported to undergo necrosis in the tissue if the infection is serious enough, while neutrophils generally die in apoptosis [33]. In the setting of in vitro study, neutrophils are destined to be apoptotic or necrotic according to the intensity of infection as in the following: 1) High doses E. coli induced necrosis of neutrophils although stimulation with low dose E. coli resulted in apoptosis [34]; 2) Exposure of neutrophils to viable pneumococci caused necrosis of the cells while heat−killed pneumococci accelerated the process of apoptosis observed in cultivated non−stimulated neutrophils in vitro [35]. Indeed, our study demonstrated that although not being statistically significant, intra−amniotic infection was more frequent in stage−4 with NF (inflammation in WJ [+] and NF [+]) than in stage−3 without NF (inflammation in WJ [+] and NF [−]) (stage−4 vs. stage−3; 60% vs. 42.9%). Secondly, viable neutrophils enhance the control of infection [36] and apoptosis typically minimizes inflammation through sequestration and inactivation of intracellular danger−associated molecular−patterns (DAMPs) as a consequence

of early recognition and removal of apoptotic cells via local phagocytes [37]. However, necrotic cell death promotes the liberation of intracellular DAMPs (i.e., HMGB1, heat-shock proteins, DNA-chromatin complexes and members of the extended IL-1 cytokine family) to induce the generation of cytokines and chemokines on neighboring cells or tissues ultimately leading to neutrophils recruitment [34, 37-40]. Therefore, the presence of necrosis is likely to be associated with more intense inflammatory responses in the neighboring compartments (i.e., IAIR in intra-amniotic cavity, and amnionitis as an advanced stage of inflammatory response in EPM) as compared with the absence of necrosis in the context of the extension of inflammation into WJ.

1.5. Objective criteria for inflammation in Wharton's jelly (WJ) and necrotizing funisitis (NF)

There is no exact explanation for stage 3 of umbilical cord (UC) inflammation. According to the Salafia's criteria, stage 3 of UC inflammation is defined as polymorphonuclear leukocytes (PMNs) in the perivascular Wharton's jelly [WJ] [26]. Therefore, it is reasonable to consider as stage 3 when there are at least two PMNs in WJ.

Meanwhile, necrotizing funisitis (NF) is defined as bands of degenerating neutrophils and cellular debris forming concentric arcs partially surrounding one of more umbilical blood vessels, sometimes with calcification [6]. This definition may be taken obscurely because there is no quantitative concept of infiltrated neutrophils or immunologic markers of NF. Therefore, further

studies are needed about the definition of NF with the use of quantitative evaluation criteria or immunologic markers.

1.6. Necessity and limitation of stage-3 subdivision

According to our data, there are much more cases of stage−3 (PMNs in WJ but not NF) compared to those of stage−1, 2, and 4 (stage−1 vs. stage−2 vs. stage−3 vs. stage−4: 20% [24/120] vs. 5.8% [7/120] vs. 60.8% [73/120] vs. 13.3% [16/120]). Our result is consistent with the previous study's findings. Indeed, the previous study shows that WJ inflammation was present in 69.4% (75/108) of cases with funisitis. However, subdividing stages result in a smaller number of each case leading to difficulty in statistical analysis.

1.7. Strength of this study

Unfortunately, although previous several classifications [5, 6, 11, 15] included NF, these studies had limitations in the classification for the progression of inflammation in UC as follows: 1) the presence or absence of inflammation in WJ was not considered [5, 6]; and 2) only NF, but not inflammation in umbilical vessels and WJ, was focused [11, 15]. In addition to these limitations, they did not examine IAIR [5, 6, 11, 15]. Notably, our current study demonstrated that NF is the most advanced stage in the inflammation of UC based on the finding that the surrogate markers for FIR (i.e., IAIR and amnionitis) are continuously increased with the progression of inflammation in the whole subdivisions of UC

(i.e., stage−1, umbilical phlebitis only; stage−2, involvement of at least one UA and either the other UA or UV without extension into WJ; stage−3, the extension of inflammation into WJ without NF; and stage−4, the extension of inflammation into WJ with NF).

1.8. Significance of this study

To the best of our knowledge, this is the first study reporting that the presence of NF is associated with more severe and frequent surrogate markers for FIR (i.e., IAIR and amnionitis) than the absence of NF in the context of inflammation in WJ. One can expect that necrosis in UC easily provokes an intense inflammatory response in both AF and amnion as adjacent biologic fluid and placental compartment. Indeed, our current study demonstrated surrogate markers for FIR (i.e., IAIR and amnionitis) are continuously increased with the progression of inflammation in UC. Therefore, the progression of inflammation in UC should be revisited as classified by current study.

1.9. Unanswered questions and proposals for future research

To confirm that NF is the most advanced stage in the progression of inflammation in UC, we should show the molecular signatures about the positive relationship among NF, DAMPs (i.e., HMGB1) and neutrophil infiltration in UC. Moreover, the comparison in the inflammatory responses between narrow and wide bands of NF should be examined to ascertain the impact of NF on inflammatory

responses.

Conclusion

NF is the most advanced stage in the progression of inflammation within UC based on IAIR and outside in neutrophil migration in EPM. This finding suggests that inflammation in WJ should be divided into the presence and absence of NF in the progression of inflammation within UC.

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Table 1. Previous studies reporting the relationship among intra-amniotic inflammatory response (IAIR), advanced stage of inflammation in extra-placental membranes (EPM) (i.e., amnionitis) and the progression of inflammation in umbilical cord (UC)

Primary Author (year) [Reference number]	**n**	**GA at delivery**	**Cause of PTB**	**Other inclusion criteria**	**Diagnostic criteria of inflammation in UC**	**Inclusion of either NF or inflammation of WJ**	**The relationship of IAIR with either funisitis or NF**		**The relationship of IAIR with the progression of inflammation in UC**		**The relationship of advanced stage of inflammation in EPM (i.e., amnionitis) with either funisitis or NF**		**The relationship of advanced stage of inflammation in EPM (i.e., amnionitis) with the progression of inflammation in UC**	
							Funisitis	**NF**	**Regardless of the inclusion of NF**	**Including NF**	**Funisitis**	**NF**	**Regardless of the inclusion of NF**	**Including NF**
Jacques et al., (1992)[11]	45	18-40 wks	NA	NF (+)	1) mild; narrow, but still distinct, bands of neutrophils that usually surrounded only one or two of the blood vessels; 2) moderate; no information; 3) severe; typically marked calcification or wide bands of necrosis filled with neutrophils and cellular debris surrounding at least two blood vessels	NF (+) No comment about inflammation in WJ	NA	Not done	NA	NA	NA	Acute HCA was present in 100% of NF cases.	NA	NA (The severity of acute HCA did not correlate with the degree of NF.)
Romero R et al., (1992) [12]	92	22-36 wks	PTL	Delivery within 48h after amniocentesis	ref. [26]‡	NF (-) WJ (+)	Not done	NA	Not done (AF cul[+]; [grade of inflammation in UC] : grade 0, 17.6% grade 1, 75% grade 2, 83.3% grade 3, 66.7% grade 4, 90% : P<0.001 in chi-square for trend)	NA	The values indicate excellent agreement for readings from UC and amnion.	NA	Not done	NA
K. H. Van Hoeven et al., (1996)[13]	2899	NA	NA	-	Acute funisitis: stage 1; limited to the vein alone; stage 2; involving one or more arteries	NF (-) WJ (-)	Not done	NA	Not done	NA	¶ Acute funisitis was more frequent with increasing stages of membrane inflammation.	N	¶¶ Acute funisitis stage 2 was more frequent with increasing stages of membrane inflammation.	NA

Yoon BH et al., (2000)[14]	315	20-35 wks	PTL, preterm-PROM, MFI	Delivery within 3 days of amniocentesis (n=106)	ref. [27]‡‡	NF (-) WJ (+)	Not done (AF cul[+]; funisitis[-] < [+])	NA	NA	NA	Not done†	NA	NA	NA
Ohyama et al., (2002)[15]	383	23-32 wks	NA	Clinically ill infant or grossly abnormal placenta	ref. [28]∫	NF (+) WJ (-)	NA	Not done	NA	NA	NA	Not done	NA	NA
SE Lee et al., (2006)[16]	832	>37 wks	NA	Delivery within 72h of amniocentesis	ref. [27]‡‡	NF (-) WJ (+)	AF WBC; funisitis(-) < (+)	NA	NA	NA	Not done†	NA	NA	NA
CW Park et al., (2008)[8]	139	<35 wks	PTL, preterm-PROM, MFI	Delivery within 72h of amniocentesis	ref. [27]‡‡	NF (-) WJ (+)	AF MMP-8 kit(+), AF IL-6 & AF WBC; funisitis(-) < (+)	NA	NA	NA	Not done†	NA	NA	NA
Buhimschi et al., (2009)[17]	132	≥23.1 wks	PTL, preterm-PROM	-	ref. [26]‡	NF (-) WJ (+)	§funisitis; MR 0 < MR 1-2 < MR 3-4	NA	Increase in funisitis grade; §MR 0 < MR 1-2 < MR 3-4	NA	Not done	NA	NA	NA
CW Park et al., (2009)[10]	290	<36 wks	PTL, preterm-PROM, MFI	HCA	ref. [27]‡‡	NF (-) WJ (+)	AF MMP-8 & WBC; funisitis(-) < (+)	NA	NA	NA	Amnionitis: funisitis(-) < (+)	NA	NA	NA
J. Lee et al., (2011)[18]	301	24-32 wks	iPTB, sPTB	-	ref. [27]‡‡	NF (-) WJ (+)	Not done	NA	NA	NA	Not done†	NA	NA	NA
T. Cobo et al., (2012)[19]	107	23-36 wks	preterm-PROM	-	ref. [26]‡	NF (-) WJ (+)	AF IL-8, IL-6, IL-10 sIL-6r, sTNF- RI, etc; funisitis(-) < (+)	NA	NA	NA	NA	NA	NA	NA
Lee SY et al., (2012)[20]	306	23-35 wks	PTL, preterm-PROM	Delivery within 72h of maternal CRP measurement	ref. [27]‡‡	NF (-) WJ (+)	Not done	NA	NA	NA	Not done†	NA	NA	NA
M. Kacerovsky et al., (2012)[21]	115	24-37 wks	preterm-PROM	-	NA	NA	1) 24-32 wks funisitis; ¥MIAC(-) < (+) 2) 32-37 wks funisitis; ¥MIAC(-) = (+)	NA	NA	NA	NA	NA	NA	NA
S. M. Kim et al., (2015)[22]	412	24-35 wks	NA	Delivery within 120h of amniocentesis	ref. [27]‡‡	NF (-) WJ (+)	Not done	NA	NA	NA	Not done	NA	NA	NA
JW Park et al.,	153	20-34 wks	PTL, preterm-	Delivery within 48h of	ref. [27]‡‡	NF (-) WJ (+)	Not done	NA	NA	NA	Not done	NA	NA	NA

(2017)[23]			PROM	Amniocentesis										
Musilova et al., (2018)[24]	159	34-37 wks	preterm-PROM	-	ref. [26]‡	NF (-) WJ (+)	funisitis; [MIAC-IAI] : All (+) = IAI alone = MIAC alone = All (-)	NA	NA	NA	NA	NA	NA	NA
RJ Martinez-Portilla et al., (2019)[25]	47	26-37 wks	preterm-PROM	-	NA	NA	Not done	NA	NA	NA	Not done††	NA	NA	NA

AF, amniotic fluid*; EPM*, extra-placental membrane*; GA*, gestational age*; HCA*, histologic chorioamnionitis*; IAI*, intra-amniotic inflammatory*; IAIR*, intra-amniotic inflammatory response*; IL-8*, interleukin-8*; iPTB*, indicated PTB*; MFI*, maternal-fetal indication*; MIAC*, Microbial invasion of the amniotic cavity*; MR score*, mass-restricted sc*ore; NA*, not applicable*; NF*, necrotizing funisitis*; PMN*, polymorphonuclear leukocyte*; preterm-PROM*, preterm premature rupture of membranes*; PTB*, preterm birth*; PTL*, preterm labor*; SNF*, subacute necrotic funisitis*; sIL-6r*, soluble IL-6 receptor*; sPTB*, spontaneous PTB*; sTNF-R1*, soluble TNF receptor-1*; UC*, umbilical cord*; WJ*, Wharton's jelly

‡ Grade of inflammation in UC, grade 1: PMN within inner third of umbilical vein wall; grade 2: PMN within inner third of at least two umbilical vessel walls; grade 3: PMN in perivascular WJ; and grade 4: panvasculitis and funisitis extending deep into WJ
‡‡ Grade of inflammation in UC, grade 1: PMN infiltration confined to umbilical vessel walls; and grade 2: extension of PMN infiltration into WJ
ʃ SNF: degenerating neutrophils, lymphocytes, monocytes, occasional plasma cells; and cord stripes (rings or crescents on cross section)

¶ There is no exact data about the frequency of acute funisitis according to the stage of membrane inflammation.
¶¶ There is no exact data about the frequency of acute funisitis stage 2 according to the stage of membrane inflammation.
§ MR score: a proteomic profile that is highly characteristic of intra-amniotic inflammation. (MR 0: no inflammation, MR 1-2: minimal inflammation, and MR 3-4: severe inflammation)
¥ MIAC was defined as a positive polymerase chain reaction analyses for genital mycoplasmas and/or *C. trachomatis* and/or the growth of any bacteria in the amniotic fluid.
† Inflammation in at least one placental compartment was more frequent in cases with funisitis than in those without funisitis.
†† Funisitis was more frequent in cases with inflammation in membranes than in those without inflammation in membranes.

Table 2. Clinical characteristics and pregnancy outcomes according to the progression of inflammation in umbilical cord (UC)

	Stage-1[†] (n=24)	Stage-2[†] (n=7)	Stage-3[†] (n=73)	Stage-4[†] (n=16)	P value[a]
	20.0% (24/120)	5.8% (7/120)	60.8% (73/120)	13.3% (16/120)	
Maternal age, year (mean ± SD)	32.8 ± 3.8	29.0 ± 3.2	32.1 ± 4.9	31.4 ± 4.3	NS (0.218)
Parity (≥ 1)	75.0% (18/24)	57.1% (4/7)	53.4% (39/73)	62.5% (10/16)	NS (0.312)
Median GA at amniocentesis, wks [range][‡]	32.7 [27.9-35.7]	29.0 [25.3-32.3]	29.1 [23.7-36.9]	27.5 [25.6-36.0]	NS (0.067)
Cause of preterm birth					NS (0.443)
Preterm-PROM	37.5% (9/24)	14.3% (1/7)	37.0% (27/73)	50.0% (8/16)	
PTL	62.5% (25/24)	85.7% (6/7)	63.0% (46/73)	50.0% (8/16)	
Male Newborn	50.0% (12/24)	85.7% (6/7)	45.2% (33/73)	43.8% (7/16)	NS (0.225)
Cesarean delivery	54.2% (13/24)	28.6% (2/7)	38.4% (28/73)	25.0% (4/16)	NS (0.265)
Median GA at delivery, wks [range]	33.5 [21.0-36.7]	29.1 [24.3-35.0]	30.1 [23.3-36.9]	29.4 [24.3-36.3]	NS (0.133)
Birth weight, g (mean ± SD)	1945 ± 705	1369 ± 606	1618 ± 683	1463 ± 611	NS (0.054)
1 min Apgar score of <7	33.3% (8/24)	71.4% (5/7)	61.6% (45/73)	50.0% (8/16)	NS (0.079)
5 min Apgar score of <7	20.8% (5/24)	28.6% (2/7)	31.5% (23/73)	31.2% (5/16)	NS (0.793)
Antenatal corticosteroids use[b]	52.2% (12/23)	57.1% (4/7)	70.8% (51/72)	75.0% (12/16)	NS (0.317)
Antenatal antibiotics use[c]	56.5% (13/23)	100% (7/7)	84.3% (59/70)	87.5% (14/16)	**0.011[e]**
Antenatal tocolytics use[d]	39.1% (9/23)	57.1% (4/7)	60.0% (42/70)	68.8% (11/16)	NS (0.246)

GA, gestational age; *NS*, not significant; *preterm-PROM*: preterm premature rupture of membranes; *PTL*, preterm labor and intact membranes; *SD*, standard deviation.

[†] Stage-1, umbilical phlebitis [inflammation in umbilical vein (UV)] only;
[†] Stage-2, involvement of at least one umbilical artery (UA) and either the other UA or UV without extension into Wharton's jelly (WJ);
[†] Stage-3, the extension of inflammation into WJ without necrotizing funisitis (NF);

† Stage-4, the extension of inflammation into WJ with NF.

‡ Forty-one patients who underwent amniocentesis within 3 days of birth were included in this analysis to preserve a meaningful temporal relationship among the results of amniotic fluid studies and those of placental histologic examination and perinatal outcome.

[a], Intergroup difference by Chi-square test (categorical variables) and Kruskal-Wallis test (continuous variables)
[b], Of 120 cases, 118 patients were included in this analysis, because the information about antenatal corticosteroids use in the medical records was omitted in 2 patients.
[c], Of 120 cases, 116 patients were included in this analysis, because the information about antenatal antibiotics use in the medical records was omitted in 4 patients.
[d], Of 120 cases, 116 patients were included in this analysis, because the information about antenatal tocolytics use in the medical records was omitted in 4 patients.
[e], There was no significant difference in the frequency of antenatal antibiotics use between each group with the use of Fisher's exact test with Bonferroni's correction.

Table 3. Diagnostic indices, predictive values, and likelihood ratios of amniotic fluid (AF) MMP-8 concentration ≥854.1 ng/ml and amnionitis for the identification of necrotizing funisitis (NF) among cases with either preterm labor and intact membranes (PTL) or preterm premature rupture of membranes (preterm-PROM) (Prevalence of NF is 13.3% [16/120])

		Sensitivity	Specificity	Positive predictive value	Negative predictive value	Positive LR (95% CI)	Negative LR (95% CI)
AF MMP-8	≥854.1 ng/ml	100.0% (5/5)	73.3% (22/30)	38.5% (5/13)	100% (22/22)	NC	0.2677 [0.1473-0.4827]
Amnionitis		81.2% (13/16)	46.2% (48/104)	18.8% (13/69)	94.1% (48/51)	2.4615 [0.8693-6.9705]	0.6627 [0.4934-0.8902]

AF, amniotic fluid; *CI*, confidence interval; *LR*, likelihood ratio; *NC*, not calculable; *MMP-8*, matrix metalloproteinase-8

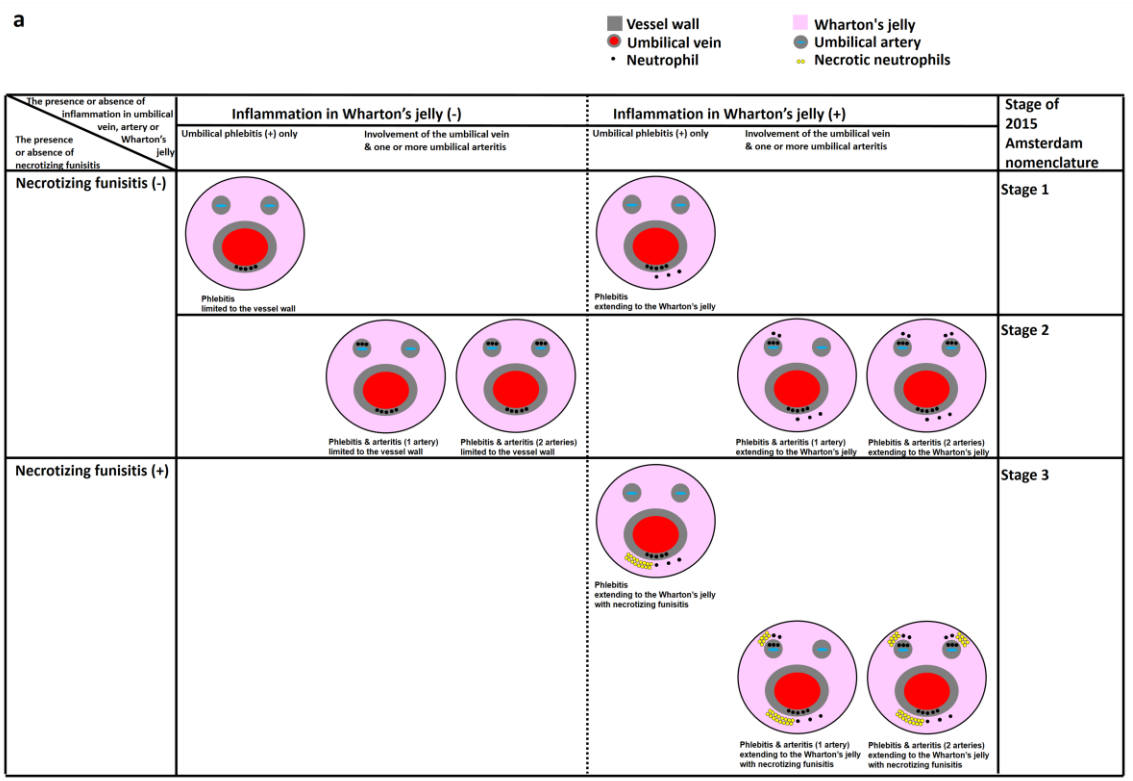
Table 4. The relationship of various variables with necrotizing funisitis (NF) by multiple logistic regression analysis

	OR	95% CI	P value
AF MMP-8 ≥854.1 ng/ml	26.348	[2.465, 281.604]	0.007
GA at delivery	1.182	[0.825, 1.694]	NS (0.362)
Antenatal corticosteroids use	0.616	[0.048, 7.961]	NS (0.710)
Antenatal antibiotics use	139584812.9	[0.000,]	NS (0.999)
Preterm-PROM as a cause of preterm delivery	3.454	[0.250, 47.666]	NS (0.355)
Apgar score < 7 at 5 min	8.761	[0.487, 157.731]	NS (0.141)
Male gender of newborn	0.890	[0.089, 8.939]	NS (0.921)
Vaginal delivery	3.066	[0.234, 40.097]	NS (0.393)

AF, amniotic fluid; *CI*, confidence interval; *GA*, gestational age; *MMP-8*, matrix metalloproteinase-8; *NS*, not significant; *OR*, odd ratio*; Preterm-PROM*, preterm premature rupture of membranes

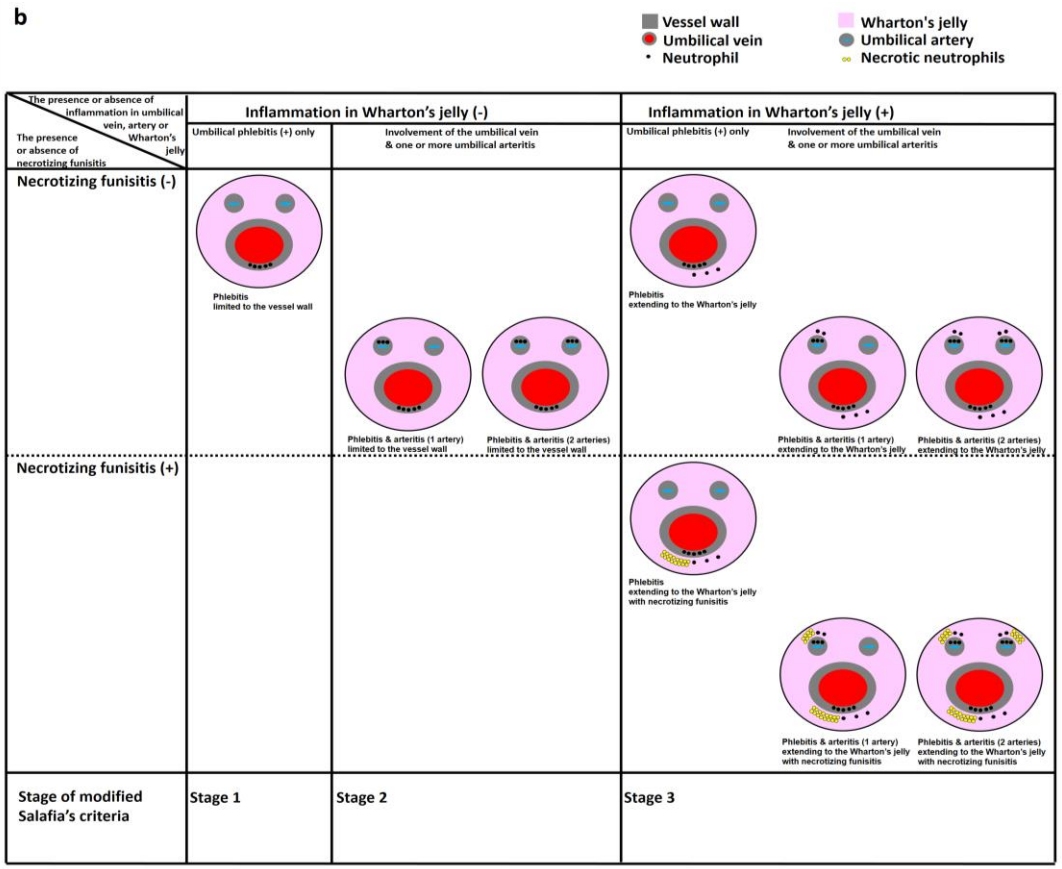
Figure legends

Fig. 1. Schema of classifications for the progression of inflammation in umbilical cord (UC) in previous studies and current study.



Khong TY, Mooney EE, Ariel I, et al. Arch Pathol Lab Med. 2016;140:698-713.

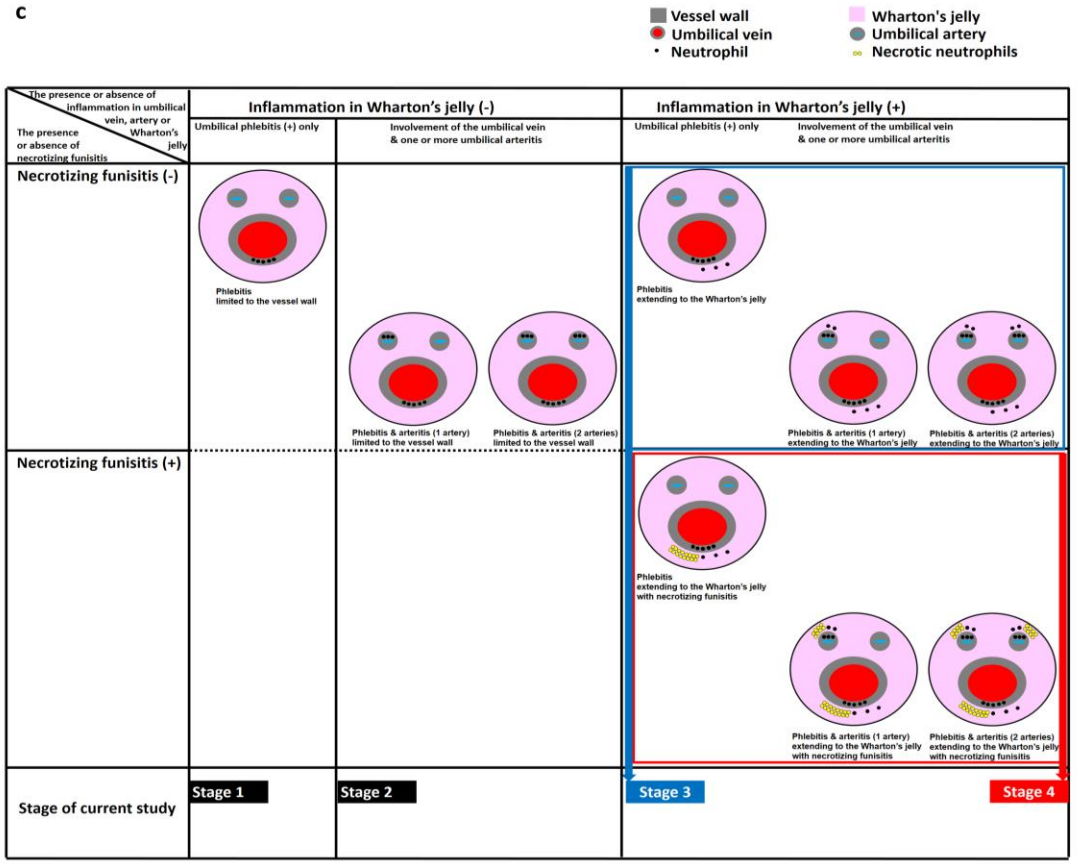
Limitation
: This classification did not consider the presence or absence of neutrophil infiltration (inflammation) into Wharton's jelly.



Oh JW, Park CW, Moon KC, Park JS, Jun JK. PLoS One. 2019;14:e0225328.

Limitation

: This classification did not differentiate between the presence or absence of necrotic neutrophil infiltration (necrotizing funisitis) into Wharton's jelly in stage 3.

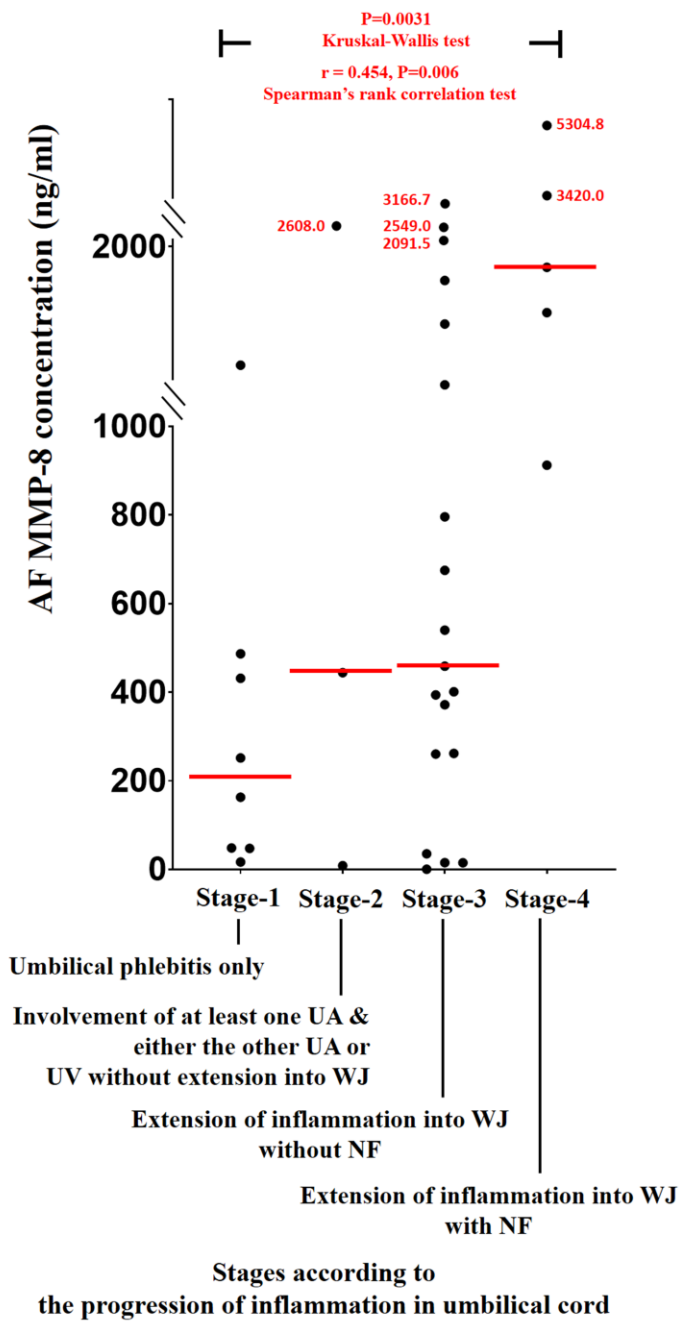


Strength

: This classification differentiated between the presence or absence of necrotic neutrophil infiltration (necrotizing funisitis) in the context of neutrophil infiltration (inflammation) into Wharton's jelly.

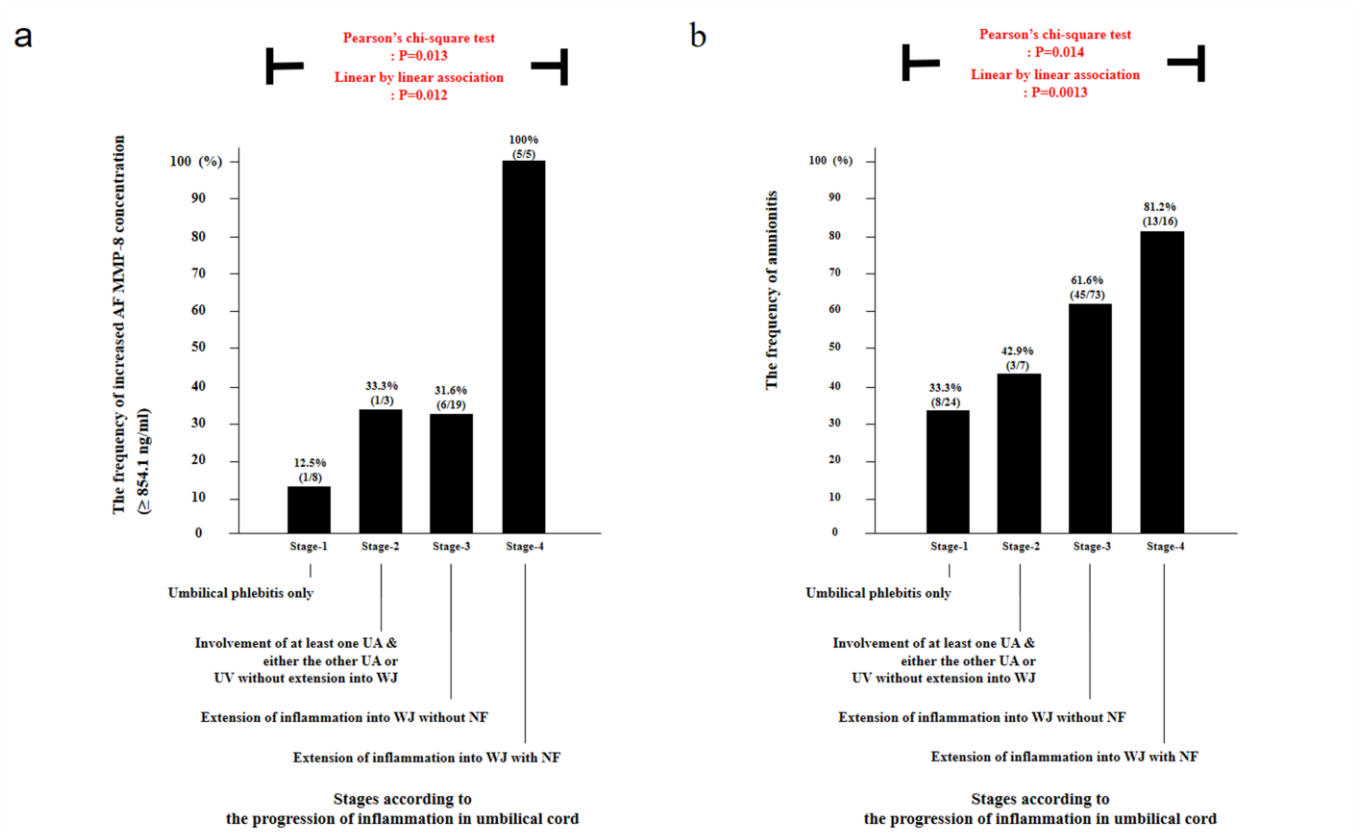
1) 2015 Amsterdam placental workshop group consensus statement (7, 8); stage 1, neutrophil infiltration within the umbilical vein (UV) wall with or without extension into Wharton’ s jelly (WJ): stage 2, neutrophil infiltration within at least one umbilical artery (UA) and either the other UA or UV with or without extension into WJ: stage 3, the presence of necrotizing funisitis (NF) (a); 2) modified Salafia's criteria (32); stage 0, inflammation free UC; stage 1, umbilical phlebitis [inflammation in UV] only; stage 2, involvement of at least one UA and either the other UA or UV without extension into WJ; stage 3, the extension of inflammation into WJ (b); and 3) the classification of current study; stage−1, umbilical phlebitis only; stage−2, involvement of at least one UA and either the other UA or UV without extension into WJ; stage−3, the extension of inflammation into WJ without NF; stage−4, the extension of inflammation into WJ with NF (c).

Fig. 2. Amniotic fluid (AF) MMP-8 concentrations (ng/ml) according to the progression of inflammation in umbilical cord (UC).



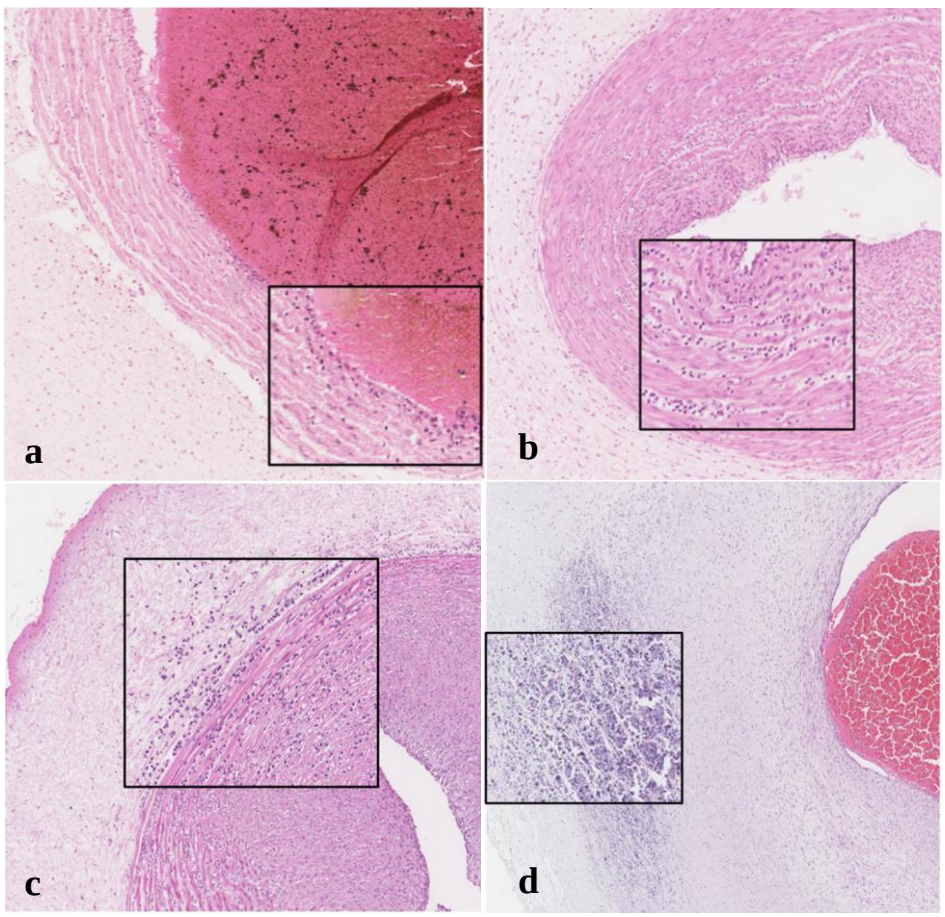
(i.e., stage−1, umbilical phlebitis [inflammation in umbilical vein (UV)] only; stage−2, involvement of at least one umbilical artery [UA] and either the other UA or umbilical vein [UV] without extension into Wharton's jelly [WJ]; stage−3, the extension of inflammation into WJ without necrotizing funisitis [NF]; and stage−4, the extension of inflammation into WJ with NF). AF MMP−8 concentrations continuously increased according to the progression of inflammation in UC (stage−1 vs. stage−2 vs. stage−3 vs. stage−4; median [ng/ml], range [ng/ml]; 207.2 [16.8−1196.5] vs. 444.1 [8.5−2608.0] vs. 458.8 [0.4−3116.7] vs. 1859.7 [912.3−5304.8]). Each P value is shown in the graph. AF MMP−8 concentration results were available in 35 patients, and those were not examined in 6 patients due to the limited amount of remained AF amount.

Fig. 3. The frequency of increased amniotic fluid (AF) MMP-8 concentration (≥854.1 ng/ml) (a) and amnionitis (b) according to the progression of inflammation in umbilical cord (UC).



(i.e., stage−1, umbilical phlebitis [inflammation in umbilical vein (UV)] only; stage−2, involvement of at least one umbilical artery (UA) and either the other UA or UV without extension into Wharton's jelly [WJ]; stage−3, the extension of inflammation into WJ without necrotizing funisitis [NF]; and stage−4, the extension of inflammation into WJ with NF). Each frequency and P value is shown in the graphs. AF MMP−8 concentration results wereavailable in 35 patients, and those were not examined in 6 patients due to the limited amount of remained AF amount.

Fig. 4. Histopathology of the progression of inflammation in umbilical cord (UC).



Hematoxylin and eosin stained histologic sections of UC are shown as follows: neutrophil infiltration within the umbilical vein (UV) wall (a), neutrophil infiltration within at one umbilical artery (UA) without extension into Wharton's jelly (WJ) (b), neutrophil infiltration into WJ without necrotizing funisitis (NF) (c), neutrophil infiltration into WJ with NF (d). These images are based on the magnification setting x200, and the insets of panels are based on the magnification setting x400.

요약(국문초록)

목표: 괴사성 제대염은 제대의 워튼 젤리에 침윤된 호중구 및/또는 관련 파편의 원호(즉, 초승달/띠/고리/광륜)의 존재로 정의된다. 그러나, 자연 조산 중 워튼 젤리의 염증 상황에서, 괴사성 제대염의 유무에 따라 양수 내 염증성 반응 및 태반 외막의 염증을 비교한 연구는 없다. 이 연구의 목적은 이것에 대해 조사하는 것이다.

방법: 자연 조산한 120명의 단태임신에서 제대에서 염증의 진행(즉, 1단계: 오직 제대 정맥염[제대 정맥에 염증]; 2단계: 최소 하나 이상의 제대 동맥염과 워튼 젤리까지의 염증없이 다른 제대 동맥 또는 제대 정맥염 침범; 3단계: 괴사성 제대염 없이 워튼 젤리로의 염증; 4단계: 괴사성 제대염 동반한 워튼 젤리의 염증)에 따라 양수 내 염증성 반응과 양막염이 더 자주 발생하는지 조사하였다.

결과: 1) 1단계, 2단계, 3단계, 4단계는 각각 20%(24/120), 6%(7/120), 61%(73/120) 및 13%(16/120)를 차지하였다. 2) 제대 염증의 진행에 따라, 양수 MMP-8이 지속적으로 증가하였고(1단계 vs. 2단계 vs. 3단계 vs. 4단계; 중앙값[ng/ml], 범위[ng/ml]; 207.2[16.8-1196.5] vs. 444.1[8.5-2608.0] vs. 458.8[0.4-3116.7] vs. 1859.7[912.3-5304.8]; Spearman's rank correlation-test, α=0.454, P=0.006), 증가된 양수 MMP-8(≥854.1ng/ml)의 빈도도 상승하였다. 3) 또한, 제대 염증의 진행에 따라 양막염의 빈도가 단계적으로 증가하였다.

결론: 괴사성 제대염은 워튼 젤리로 염증이 확장되는 상황에서, 양수 내 염증성 반응이 더 심하고 양막염이 더 자주 발생한다는 지표이다. 따라서, 이 연구의 결과는 괴사성 제대염이 제대 내 염증에서 가장 진행된 단계임을 시사한다.

주요어 : 괴사성 제대염, 양수 내 염증 반응, 양막염, 워튼 젤리로 염증

학번 : 2019-20196