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ORIGINAL ARTICLE

Comparison of amniotic fluid matrix metalloproteinase-8 and cathelicidin in the diagnosis of intra-amniotic infection

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OBJECTIVE: To evaluate the association of amniotic fluid (AF) matrix metalloproteinase-8 (MMP-8) and cathelicidin concentrations with microbial invasion of the amniotic cavity (MIAC) in pregnancies with preterm prelabor rupture of the membranes or intact membranes.

STUDY DESIGN: Amniocentesis was performed in 54 singleton pregnancies between 22^{+0} and 34^{+2} gestational weeks with suspected intra-amniotic infection. AF-MMP-8 was analysed by immunoassay and AF-cathelicidin by commercial ELISA. Standard biochemical methods, molecular microbiology and culture techniques were used.

RESULTS: MIAC was present in 18 (33%) women. The cutoff value for the diagnosis of MIAC was 41.5 ng ml^{-1} for AF-MMP-8, and 11.6 ng ml^{-1} for AF-cathelicidin. With these cutoff values AF-MMP-8 had a sensitivity of 100%, specificity of 69%, positive predictive value of 62% and negative predictive value of 100% for MIAC. The corresponding values for AF-cathelicidin were 89, 81, 70 and 94%.

CONCLUSION: The performance of AF-cathelicidin in the prediction of MIAC is comparable to AF-MMP-8.

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INTRODUCTION

Preterm birth is often associated with intra-amniotic infection (IAI).[1] IAI is defined as increased amniotic fluid (AF) inflammatory biomarkers with or without microbial invasion of the amniotic fluid (MIAC),[2] and often called as subclinical chorioamnionitis. IAI has an important role in neonatal morbidity and mortality.[3–6] When clear symptoms of clinical chorioamnionitis appear, the window of opportunity for the prevention of adverse neonatal outcomes has narrowed or even gone. Thus, a diagnosis in the subclinical stage of chorioamnionitis for proper timing of delivery is important and challenging.

Several AF biomarkers for IAI have been studied, but only a few of them are in clinical use.[6–10] One such biomarker is matrix metalloproteinase-8 (AF-MMP-8).[11,12] MMP-8 belongs to a proteolytic MMP enzyme family degrading collagen type-1, which provides strength to fetal membranes. In normal pregnancies it participates in the degradation of extracellular matrix at parturition.[13] MMP-8 is also able to degrade non-matrix bioactive substances such as chemokines, cytokines and growth factors, which modify immune reactions.[14,15] MMP-8 has a role in fetal inflammatory response to IAI[16] and is associated with MIAC in cases with or without preterm prelabor rupture of membranes (PPROM).[13,17,18]

Cathelicidin is a candidate biomarker for IAI diagnostics. Cathelicidin belongs to an antimicrobial peptide family participating in innate host–defense mechanisms.[19] The active form of human cathelicidin, LL-37, is expressed primarily by neutrophils and epithelial cells induced by microorganisms and it has also microbicidal effects.[20,21] Moreover, antimicrobial properties occur also in AF.[22] Cathelicidin is expressed also in cervical epithelial cells playing a role in blocking ascending infections.[23] Furthermore, cathelicidin can stimulate other immune mediators including interleukin-6, another biomarker for IAI.[9,10,24] In a previous study, women with PPROM and MIAC showed increased AF-cathelicidin (AF-cathelicidin) concentrations.[25]

We wanted to compare AF-MMP-8 and AF-cathelicidin concentrations in women with clinically suspected IAI, with or without PPROM, in order to explore whether AF-cathelicidin could augment the diagnosis of IAI.

METHODS

This prospective study was conducted at the University Hospital of Helsinki, Finland, between June 2012 and March 2015. Amniocentesis was performed in 57 women with singleton pregnancy and suspected IAI, between 22^{+0} and 35^{+0} weeks of gestation, with or without PPROM. Multiple pregnancies and pregnancies with fetal structural anomaly, or proven or suspected aneuploidy were not eligible. The gestational age was established by the first trimester ultrasonography screening. IAI was suspected in the presence of contractions with at least one of the following criteria: uterine tenderness, fetal tachycardia, infectious discharge from cervix, increased maternal plasma C-reactive protein >10 mg l^{-1}, total blood white cell count $>20\times10^{9}$ l^{-1} or sludge visible on ultrasound examination. PPROM was diagnosed clinically by speculum examination or by a positive insulin-like growth factor binding protein test (ActimProm, Medix Biochemica, Espoo, Finland) result.

AF-MMP-8 and AF-cathelicidin concentrations were also determined from 32 healthy controls at gestational age (weeks; median, range) 20.8 (15.0 to 36.89) in order to find out their concentrations in normal pregnancies. The indication for amniocentesis in these pregnancies was either the need for karyotyping ($n=24$) or determination of fetal lung maturity ($n=8$).

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Table 1. Microorganisms detected in amniotic fluid by biomarker concentrations and membrane status

		Detected microbes		*Biomarker concentrations*	
Case	*PPROM Yes/No*	*Culture*	*PCR*	*Cathelicidin ng ml^{-1}*	*MMP-8 ng ml^{-1}*
1	Yes	*Peptostreptococcus anaerobius* and *Streptococcus viridans*	*P. anaerobius* and *S. viridans*	28.3	4887
2	No	*Candida albicans*	*Peptostreptococcus* species	37.8	5431
3	No	*S. pneumoniae*	*S. pneumoniae*	3.2	69
4	No	*S. viridans*	*S. viridans*	14.9	1532
5	No	*Escherichia coli*	*Fusobacterium nucleatum*	42.9	4928
6	Yes	*C. albicans*	*Ureaplasma urealyticum/parvum*, *Campylobacter ureolyticus*	23.8	13 292
7	No	*S. agalactiae*	*S. agalactiae*	33.2	3552
8	Yes	*Gardnerella vaginalis*	Negative	14.7	2257
9	Yes	*C. albicans*	Negative	20.7	1483
10	No	*C. albicans*	Negative	14.5	3019
11	No	Negative	*Mycoplasma* species	35.9	6693
12	No	Negative	*C. ureolyticus*	41.7	5413
13	No	Negative	*C. ureolyticus*, *F. nucleatum*	32.2	5440
14	Yes	Negative	*S. viridans*	4.9	45
15	Yes	Negative	*U. urealyticum/parvum*	27.3	6290
16	No	Negative	*U. urealyticum/parvum*	12.6	144
17	Yes	Negative	*U. urealyticum/parvum*	12.1	5296
18	Yes	Negative	*U. urealyticum/parvum*	40.9	9753

Abbreviations: Culture, microbial culture; MMP-8, matrix metalloproteinase-8; PPROM, preterm prelabor rupture of membranes.

Women with PPROM were managed by our clinical protocol including routine administration of cefuroxime and azithromycin and antenatal steroids on admission. Women with intact membranes and imminent preterm labor before 35 gestational weeks received antenatal steroids, but not antibiotics.

Ultrasound-guided transabdominal amniocentesis was performed by our study group members. AF samples were retrieved. AF-MMP-8 and AF-cathelicidin concentrations, AF microbial culture and AF 16 S rRNA gene sequencing (AF-PCR) were determined.

MIAC was defined as a positive AF culture or AF-PCR. Three cases had no microbial analysis available and were excluded. Thus, the final analysis comprised 54 cases. Microbial culture result was available in 44 (81%) cases, AF-PCR in 53 (98%) cases and both in 43 (80%) cases. AF samples were cultured for aerobic and anaerobic bacteria on chocolate blood agar in 5% CO_2 and on Fastidious Anaerobe Agar in anaerobic conditions at 35 ± 1 °C. A thioglycolate broth enrichment was used. The samples were in culture for 7 days and were inspected after 1, 2 and 7 days. The culture methods used enabled also detection of common *Candida* species and *Mycoplasma hominis*, but not *Ureaplasma* species. For bacterial AF-PCR a minimum of 500 µl of AF was subjected to ceramic bead-beating cell lysis (Precellys 24 tissue homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France) followed by magnetic bead-based DNA extraction method (NucliSENS kit with easyMAG automatic nucleic acid purification platform, bioMérieux, Marcy l'Etoile, France) according to the manufacturer. The extracted DNA was amplified in duplicates by PCR using following primers: 5′-TTG GAG AGT TTG ATC MTG GCT C-3′ (forward) and 5′-GTA TTA CCG CGG CTG CTG-3′ (reverse). DNA of λ-phage served as an inhibition control in PCR. A positive PCR product was verified by gel electrophoresis, 5 µl of the PCR product was sequenced, and the obtained sequence was compared with NCBI BLAST sequence database (www.ncbi.nlm.nih.gov/blast). Mixed sequences were analysed by Ripseq mixed analysis tool (https://www.ripseq.com/), when appropriate.

Next the AF specimens were divided into aliquots, frozen and stored at − 20 °C until MMP-8 and cathelicidin were analysed (Medix Biochemica). AF-MMP-8 was quantitated with a solid-phase immunoenzymometric assay (MMP-8 IEMA, Medix Biochemica) and analysed according to the manufacturer. The absorbance was measured at 414 nm using a microplate reader (Multiskan, Thermo Fisher Scientific, Vantaa, Finland). AF-cathelicidin was analysed by a commercial enzyme-linked immunosorbent assay (HUMAN LL-37 [HK321] ELISA kit, Hycult biotech, Uden, The Netherlands) according to the manufacturer.

All calculations were carried out by Microsoft Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) for Windows v22.0. Data with continuous variables not following a normal distribution were compared by Mann–Whitney *U*-test. Spearman's correlation was used to determine the relation of two continuous variables. Receiver operating characteristics curves were constructed and area under the curve was determined. A *P*-value < 0.05 was considered significant.

All patients provided a written informed consent. The local Ethics Committee approved the protocol (75/13/03/03/2013).

RESULTS

A total of 54 women with singleton pregnancies were enrolled. Amniocentesis was performed between 22^{+0} and 34^{+2} gestational weeks. Of the 54 women 24 (44%) were primiparous. The mean maternal age was 29.7 (±5.8) years, and the mean body mass index was 27.2 (±6.8) kg m^{-2}. The median amniocentesis to delivery time interval was 3 days (range 0 to 124 days).

MIAC was present in 18 (33%) women. Table 1 shows the microbiologic findings in the MIAC cases. Of these cases, seven were positive by PCR and culture, three by culture only and eight by PCR only. Thus, different microorganisms were detected by culture or PCR (Table 1).

Both AF-MMP-8 and AF-cathelicidin concentrations were higher in cases with MIAC than in cases without MIAC (Figures 1a and b). In the reference group ($n = 32$) AF-MMP-8 concentration (median, range) was 4.0 ng ml^{-1} (1.0 to 16.0) and AF-cathelicidin concentration (median, range) was 1.2 ng ml^{-1} (0.0 to 4.3). A strong correlation between AF-cathelicidin and AF-MMP-8 ($r_s = 0.932$, $P < 0.001$) existed (Figure 2).

By receiver operating characteristics curve (Figure 3), the best cutoff value of AF-MMP-8 for the diagnosis of MIAC was 41.5 ng ml^{-1} (area under the curve = 0.900, 95% confidence interval = 0.82 to 0.98). The corresponding value for AF-cathelicidin was 11.6 ng ml^{-1} (area under the curve = 0.898, 95% confidence interval = 0.82 to 0.98). When these cutoff values were used for the diagnosis of MIAC the sensitivity was 100%, specificity was 69%, positive predictive value was 62% and negative predictive value was 100% for AF-MMP-8. The corresponding values for

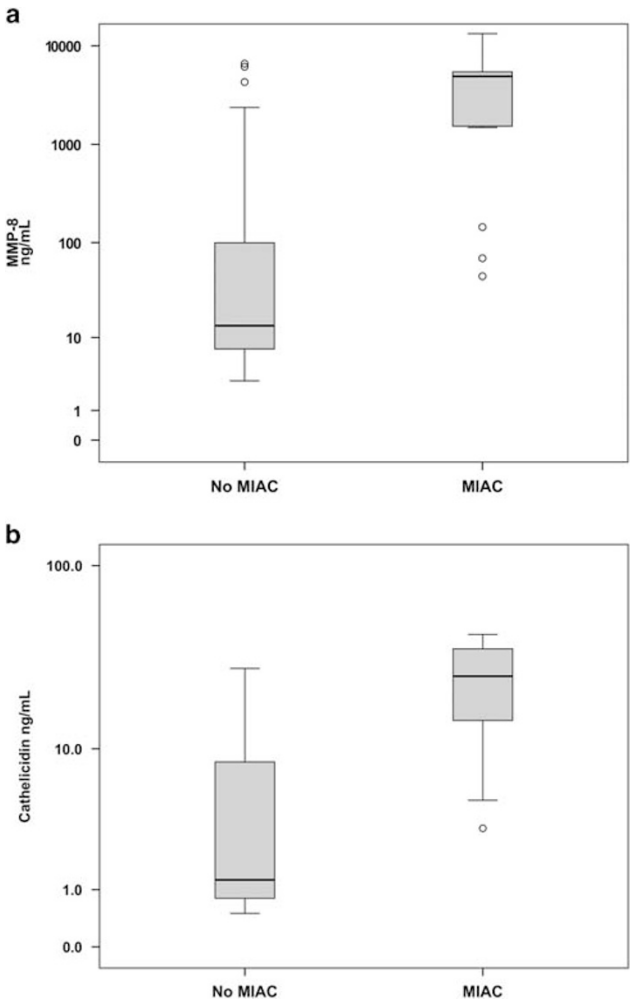

Figure 1. (**a** and **b**) Association of amniotic fluid matrix metalloproteinase-8 (AF-MMP-8) (**a**) and amniotic fluid cathelicidin (AF-cathelicidin) (**b**) with microbial invasion of amniotic cavity (MIAC). AF-MMP-8 had higher median concentrations with MIAC than without MIAC: 4907.5 ng ml^{-1} (range from 45 to 13 292) vs 13.5 ng ml^{-1} (range from 3 to 6591), $P < 0.001$. AF-cathelicidin had higher median concentrations with MIAC than without MIAC: 25.6 ng ml^{-1} (range from 3.2 to 42.9 ng ml^{-1}) vs 1.3 ng ml^{-1} (range from 0.5 to 28.1 ng ml^{-1}), $P < 0.001$.

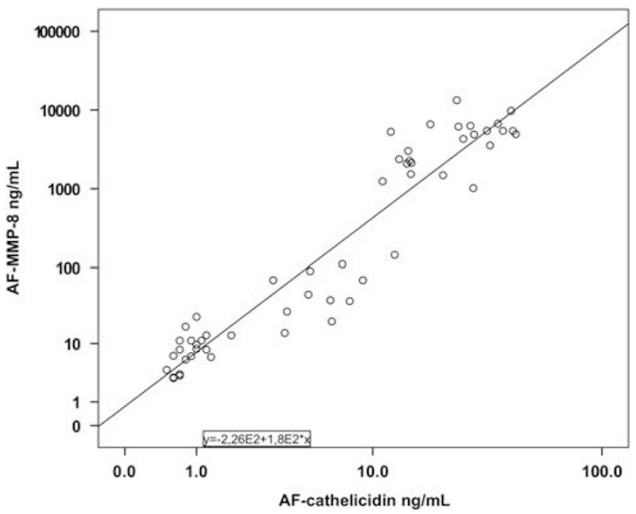

Figure 2. Correlation between amniotic fluid cathelicidin and amniotic fluid matrix metalloproteinase-8 (MMP-8), $r_s = 0.932$, $P < 0.001$.

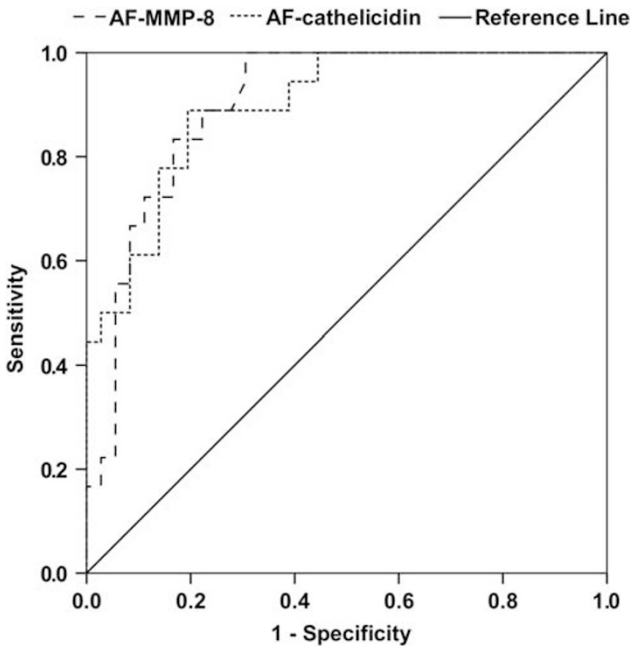

Figure 3. Receiver operating characteristic curves of amniotic fluid matrix metalloproteinase-8 (AF-MMP-8) (area under the curve = 0.900, (95% confidence interval = 0.820 to 0.981), $P < 0.001$) and amniotic fluid cathelicidin (AF-cathelicidin) (area under the curve = 0.898, (95% confidence interval = 0.816 to 0.980), $P < 0.001$) in the diagnosis of microbial invasion of the amniotic cavity.

AF-cathelicidin were 89, 81, 70 and 94%. For test combination the corresponding values were 89, 81, 70 and 94%. Thus, the combined use of AF-MMP-8 and AF-cathelicidin did not increase diagnostic performance (Tables 2 and 3). Table 2 also shows the accuracies to compare when specificities were set to 75 and 89%. MIAC occurred in eight cases with PPROM and in ten cases with intact membranes. AF-MMP-8 and AF-cathelicidin were associated with MIAC both in cases with PPROM and in cases without PPROM (Table 4).

The rates of infection (increased biomarker concentrations in the presence of MIAC), inflammation (increased biomarker concentrations in the absence of MIAC) and colonization (normal biomarker concentration in the presence of MIAC) by gestational age are shown in Figures 4a and b. Infection and inflammation occurred more often before 28 gestational weeks, whereas the rate of colonization showed no such trend. In cases with or without PPROM infection or inflammation were equally common (Figures 5a and b). AF-cathelicidin was associated with infection defined as increased AF-MMP-8 concentration (>41.5 ng ml^{-1}) in the presence of MIAC, $P < 0.001$.

DISCUSSION

AF-MMP-8 and AF-cathelicidin were both associated with MIAC in cases with or without PPROM. We found a strong correlation between these two biomarkers. However, the combined use of AF-MMP-8 and AF-cathelicidin did not improve the diagnostic performance. The infection and inflammation rates were equally common in women with or without PPROM, as reported also by

Kim *et al.*[2] An important clinical finding was negative predictive value of MMP-8 to be 100%. If MMP-8 was below the cutoff, none of the patients had MIAC, and in such cases prenatal interventions, that is, tocolysis can be commenced in cases of threatening preterm delivery.

Cathelicidin has various anti-inflammatory and antimicrobial effects.[26] Cathelicidin is expressed in various tissues and cell types, mostly in epithelial surfaces, for example in fetal membranes, which are naturally exposed to environmental microbes.[25] Moreover, cathelicidin is also expressed in myometrium after spontaneous delivery,[24] which is an inflammatory process by itself. Whether AF-cathelicidin is produced by maternal inflammatory cells or by fetal tissues or both is not known.[26,22] However, AF-cathelicidin levels were higher in cases with proven MIAC regardless of the membrane status. This biomarker may be upregulated in MIAC reflecting it's antimicrobial properties shown in previous studies.[19,22,24,25] Microorganisms activate the toll-like receptor system, which leads to elevated cathelicidin expression and release.[25] Interestingly, one case of *Streptococcus pneumoniae*, detected by both microbial culture and PCR, was not able to evoke cathelicidin response despite being a significant human pathogen. Cathelicidin response may therefore be, at least in part, bacterial species-specific.

Many studies of AF-MMP-8 in IAI exist.[3,12,27] In most studies MMP-8 cutoff value of 23 ng ml^{-1} is used. In the present clinical study, AF-MMP-8 cutoff value (41.5 ng ml^{-1}) between healthy subjects and MIAC cases was higher. This difference between studies may be partly explained by different antibodies of MMP-8 used in the immunoenzymometric assay of Medix Biochemica. There is a recent evidence that antibodies now used have higher affinity against the active form of MMP-8 and this will yield higher overall levels of MMP-8.

We found that the rate of IAI was higher in pregnancies with lower gestational age, as also reported by Combs *et al.*[28] Ureaplasma is a typical colonizing microbe in the lower genital tract and is associated with IAI.[29] Almost one-third of the detected microbes in our study were *Ureaplasma* species, which were not detectable by the present culture method. More than two-thirds of the microbes were detected by PCR, which advocates its use in the diagnosis of IAI. *Candida* species could not be detected by PCR as it detected only bacterial DNA. The main difference between the only published study on AF-cathelicidin[25] and our study is the microbiologic definition of MIAC. In that study, MIAC was defined by a positive-specific PCR for genital mycoplasmas (*Ureaplasma* species, *M. hominis)* or growth of any bacteria except *Staphylococcus epidermidis*. We instead defined MIAC based on any microorganisms detected by PCR (bacterial 16 S rRNA gene sequencing) or culture, which yielded more microbial findings than species-specific PCR.

Amniocentesis is an important tool to rule out IAI in preterm pregnancies and may give an opportunity to continue pregnancy. One previous study showed increased concentrations of AF-cathelicidin in PPROM cases with MIAC and histologic chorioamnionitis.[25] We now found that AF-MMP-8 and AF-cathelicidin were both strongly associated with MIAC regardless of the membrane status.

No randomized trials of the role of amniocentesis on neonatal outcome exist. Three observational studies suggest that amniocentesis may be beneficial.[30–32] In PPROM pregnancies amniocentesis may sometimes be challenging due to oligohydramnios. Unfortunately, vaginally obtained AF samples are not reliable in the diagnosis of IAI.[33]

The problem with the more commonly used biomarkers, AF lactate dehydrogenase and glucose, is poor performance in the diagnosis of MIAC and histologic chorioamnionitis[34–36] AF-MMP-8

Table 2. Performance and accuracy of AF-cathelicidin, AF-MMP-8 and their combination for MIAC

	MMP-8 cutoff (ng ml^{-1})	*Cathelicidin cutoff (ng ml^{-1})*	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*LR+*
AF-MMP-8	41.5		100	69	62	100	3.3
AF-cathelicidin		11.6	89	81	70	94	4.6
Combination	41.5	11.6	89	81	70	94	4.6
AF-MMP-8	100		89	75	64	93	3.6
AF-cathelicidin		8.4	89	75	64	93	3.6
AF-MMP-8	2194		72	89	76	86	6.5
AF-cathelicidin		16.6	61	89	73	82	5.5

Abbreviations: AF, amniotic fluid; LR+, positive likelihood ratio; MIAC, microbial invasion of the amniotic cavity; MMP-8, matrix metalloproteinase-8; NPV, negative predictive value; PPV, positive predictive value.

Table 3. Contingency table of AF-MMP-8 and AF-cathelicidin in the prediction of MIAC

	MIAC+ (n = 18)	*MIAC− (n = 36)*
	n *(%)*	n *(%)*
Cathelicidin >11.6 ng ml^{-1}	16 (89)	7 (19)
Cathelicidin <11.6 ng ml^{-1}	2 (11)	29 (81)
MMP-8 >41.5 ng ml^{-1}	18 (100)	11 (31)
MMP-8 <41.5 ng ml^{-1}	0 (0)	25 (69)
Cathelicidin >11.6 ng ml^{-1} and MMP-8 >41.5 ng ml^{-1}	16 (89)	7 (19)
One or both of them below the cutoff	2 (11)	29 (81)

Abbreviations: AF, amniotic fluid; MIAC, microbial invasion of the amniotic cavity; MMP-8, matrix metalloproteinase-8; ROC, receiver operating characteristics. Cutoff values are defined by the ROC curve.

Table 4. The association of amniotic fluid biomarkers with MIAC separately in PPROM and intact membranes groups

	PPROM+			*PPROM−*		
	MIAC+	*MIAC−*	P-*value*	*MIAC+*	*MIAC−*	P-*value*
n	8	17		10	19	
AF-MMP-8 ng ml^{-1}	5091.5 (45–13 292)	38 (8–6591)	0.003	4240 (69–6693)	9.6 (3–2372)	<0.001
AF-cathelicidin ng ml^{-1}	22.3 (4.9–40.9)	6.3 (0.7–28.1)	0.011	32.7 (3.2–42.9)	0.9 (0.5–15.0)	<0.001

Abbreviations: AF, amniotic fluid; MIAC, microbial invasion of the amniotic cavity; MMP-8, matrix metalloproteinase-8; PPROM, preterm prelabor rupture of membranes.

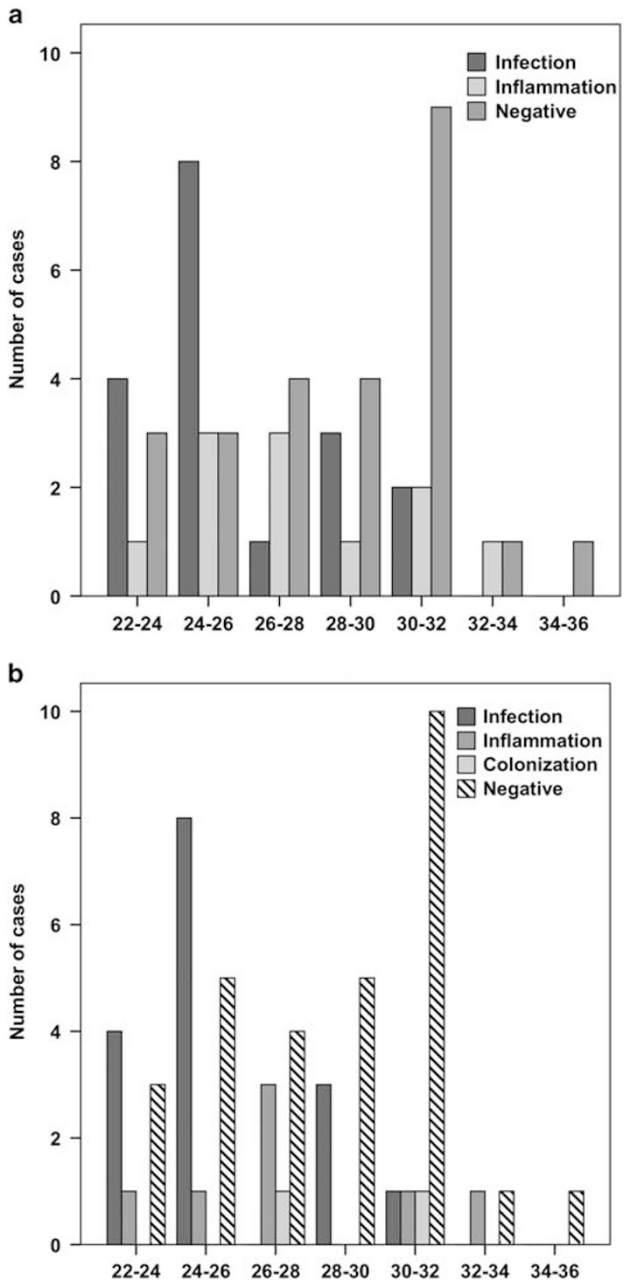

Figure 4. (**a**) Rates of infection (microbial invasion of the amniotic cavity+ (MIAC+), matrix metalloproteinase-8 (MMP-8) >41.5 ng ml^{-1}), inflammation (MIAC−, MMP-8>41.5 ng ml^{-1}), colonization (MIAC+, MMP-8 <41.5 ng ml^{-1}) and negative (MIAC−, MMP-8 <41.5 ng ml^{-1}) in amniotic fluid by MMP-8 and gestational age at amniocentesis. None had colonization (MIAC+, MMP-8 <41.5 ng ml^{-1}). (**b**). Rates of infection (MIAC+, cathelicidin >11.6 ng ml^{-1}), inflammation (MIAC−, cathelicidin >11.6 ng ml^{-1}), colonization (MIAC+, cathelicidin <11.6 ng ml^{-1}) and negative (MIAC–, cathelicidin <11.6 ng ml^{-1}) in amniotic fluid by cathelicidin and gestational age at amniocentesis.

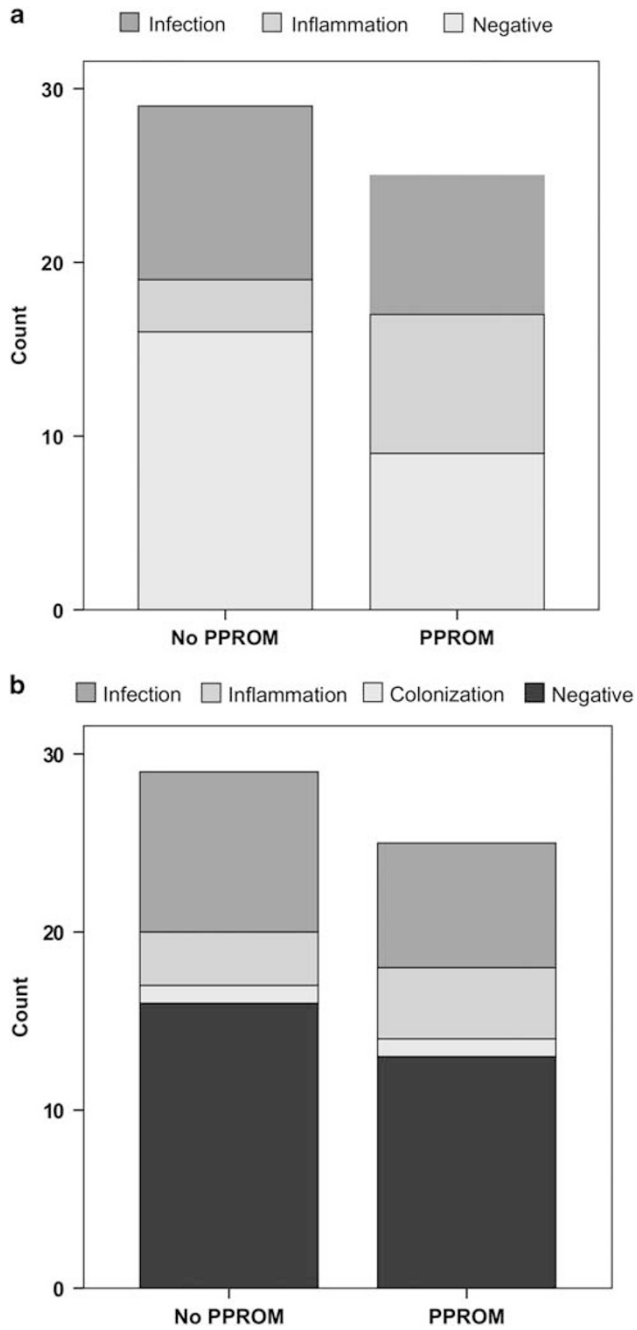

Figure 5. (**a**) Rates of infection (microbial invasion of the amniotic cavity+ (MIAC+), matrix metalloproteinase-8 (MMP-8) >41.5 ng ml^{-1}) $n=8$ vs $n=10$ (preterm prelabor rupture of membranes (PPROM) vs No PPROM), inflammation (MIAC–, MMP-8 >41.5 ng ml^{-1}) $n=3$ vs $n=8$, colonization (MIAC+, MMP-8 <41.5 ng ml^{-1}) $n=0$ vs $n=0$ and negative (MIAC–, MMP-8 <41.5 ng ml^{-1}) $n=16$ vs $n=9$ in amniotic fluid by membrane status. (**b**). Rates of infection (MIAC+, cathelicidin >11.6 ng ml^{-1}) $n=7$ vs $n=9$ (PPROM vs No PPROM), inflammation (MIAC–, cathelicidin >11.6 ng ml^{-1}) $n=4$ vs $n=3$, colonization (MIAC+, cathelicidin <11.6 ng ml^{-1}), $n=1$ vs $n=1$ and negative (MIAC–, cathelicidin <11.6 ng ml^{-1}) $n=13$ vs $n=16$ in amniotic fluid by membrane status.

and AF-cathelicidin may be more accurate and clinically useful. However, these new biomarkers are costly and not yet available in clinical practice. We hoped that the combination of AF-MMP-8 and AF-cathelicidin would improve the diagnostic accuracy for MIAC, but unfortunately this was not the case.

One weakness of our study was the number of cases enrolled. Thus, larger studies are needed to confirm our findings. Whether AF-cathelicidin is associated with adverse neonatal outcome also remains to be determined.

In summary, both AF-MMP-8 and AF-cathelicidin concentrations were increased in MIAC. Our data suggest that both are potential biomarkers for the diagnosis of IAI.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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