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Evidence for complement activation in the amniotic fluid of women with spontaneous preterm labor and intra-amniotic infection

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Abstract

Objective. The complement system plays an important role in host defense against infection. Concentrations of complement split products or anaphylatoxins (C3a, C4a, and C5a) in biological fluids are considered to reflect complement activation. The purpose of this study was to determine if term and preterm parturition are associated with evidence of complement activation in the amniotic fluid.

Study design. Amniotic fluid (AF) samples were collected from 270 women in the following groups: (1) normal pregnant women in midtrimester ($n = 70$), (2) term not in labor ($n = 23$), (3) term in labor ($n = 48$), and (4) preterm labor (PTL) ($n = 129$). PTL was categorized into: (a) PTL without microbial invasion of the amniotic cavity (MIAC) who delivered at term ($n = 42$), (b) PTL who delivered preterm without MIAC ($n = 57$), and (c) PTL with MIAC ($n = 30$). C5a, C4a, and C3a concentrations in amniotic fluid were determined by ELISA. Nonparametric tests were used for statistical analysis.

Results. (1) The median AF C5a concentration was higher in women at term than that of those in the midtrimester ($p = 0.02$); (2) Spontaneous labor at term was not associated with changes in AF concentrations of anaphylatoxins C3a, C4a, and C5a (all $p > 0.05$); (3) Among patients with PTL who delivered preterm, those with MIAC had higher AF C4a and C5a concentrations than those without infection ($p < 0.01$); and (4) AF C3a, C4a, and C5a concentrations were higher in patients with PTL with MIAC than in those with PTL without MIAC who delivered at term.

Conclusion. Patients with spontaneous preterm labor and intact membranes with microbial invasion of the amniotic cavity had higher median amniotic fluid concentration of complement split products C3a, C4a, and C5a than patients without intra-amniotic infection. These findings suggest that preterm labor in the context of infection is associated with activation of the complement system.

Keywords: *C5a, C4a, C3a, anaphylatoxins, pregnancy, MIAC, chorioamnionitis, intra-amniotic inflammation, prematurity, complement*

Introduction

The immune system is composed by an innate and adaptive limb [1]. The innate limb is nonspecific, acts immediately and lacks immunological memory. A key component of innate immunity is the complement system [2,3], which is also involved in the regulation of the adaptive immune response [4]. The complement system is composed by a group of plasma proteins with catalytic properties that react in a sequential manner, yielding active biological mediators and lytic components to clear microorganisms and 'nonself' cells [2,3,5]. Activation of the complement system through 'the classical', 'the alternative',

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and 'the mannose-binding lectin' pathways [3] leads to the generation of complement split products C3a, C4a, and C5a [6]. These bioactive fragments, known as anaphylatoxins, can induce smooth muscle contraction [7–9], enhance vascular permeability [7,9,10], and attract white blood cells [11–13]. In addition to their role in host defense, uncontrolled or excessive production of anaphylatoxins have been implicated in the pathogenesis of inflammatory diseases including sepsis [14–17], asthma [18,19], rheumatoid arthritis [20], acute respiratory distress syndrome [21], ischemic/hypoxic injury [22,23], systemic lupus erythematosus [24], and pregnancy loss [25–28].

Preterm parturition is one of the leading causes of perinatal mortality and long-term neurologic handicap [29]. Intrauterine infection is a frequent and important mechanism of disease in preterm birth [30–37]. Infection triggers an inflammatory response in maternal and fetal tissues mediated by the production of proinflammatory cytokines and chemokines [37,38]. The maternal plasma concentrations of the complement split products C5a and C3a are higher in women with spontaneous preterm labor/delivery with microbial invasion of the amniotic cavity (MIAC) than in those who delivered preterm without MIAC [39]. These findings suggest that there is a systemic maternal immune response to either microbial products or proinflammatory mediators located in the uterine cavity. However, there is paucity of information regarding the changes in amniotic fluid anaphylatoxins during microbial invasion of the amniotic cavity. The purpose of this study was to determine whether term and preterm spontaneous labor and/or MIAC are associated with evidence of complement activation in the amniotic fluid.

Material and methods

Study population

A cross-sectional study was conducted by searching our clinical database and bank of biological samples, including 270 pregnant women classified into the following groups: (1) women in the midtrimester of pregnancy (14–18 weeks) who underwent amniocentesis for genetic indications and delivered a normal neonate at term ($n = 70$); (2) normal pregnant women at term ($\geq$37 weeks) not in labor ($n = 23$); (3) women with spontaneous labor at term ($n = 48$); and (4) women with spontaneous preterm labor and intact membranes (PTL, $n = 129$). Women with PTL were classified into: (a) PTL without MIAC who delivered at term ($n = 42$); (b) PTL without MIAC who delivered preterm ($n = 57$); and (c) PTL with MIAC who delivered preterm ($n = 30$).

All women provided written informed consent prior to the collection of amniotic fluid samples. The utilization of samples for research purposes was approved by the Institutional Review Boards of both Wayne State University and by the Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, DHHS. Many of these samples have been used in the previous studies of inflammatory mediators, growth factors, and other biological markers of disease.

Clinical definitions

Patients were considered to have a normal pregnancy outcome if they did not have any obstetrical, medical, or surgical complication of pregnancy and delivered a term neonate ($\geq$37 weeks) of appropriate birth weight for the gestational age [40]. Spontaneous preterm labor was defined by the presence of regular uterine contractions occurring at a frequency of at least two contractions every 10 min associated with cervical changes that required hospitalization before 37 weeks of gestation. Preterm delivery was defined as delivery before 37 weeks of gestation. Microbial invasion of the amniotic cavity was defined as a positive amniotic fluid culture for microorganisms.

Amniotic fluid samples were obtained from transabdominal amniocentesis performed for genetic indication, evaluation of microbial status of the amniotic cavity and/or assessment of fetal lung maturity in patients approaching term. Women at term in labor consisted of women who were admitted for suspected preterm labor because of uncertain dates and had an amniocentesis for the assessment of fetal lung maturity. The criteria for considering that these patients were at term in labor was derived retrospectively, if the following criteria were met: (1) spontaneous labor; (2) delivery within 24 h from amniocentesis; (3) analysis of amniotic fluid consistent with maturity; (4) birth weight $>$2500 g; (5) absence of respiratory distress syndrome or other complications of prematurity; and (6) physical examination of the newborn by pediatricians consistent with a term neonate. Immediately upon retrieval, amniotic fluid was transported to the laboratory in a capped plastic sterile syringe and cultured for aerobic/anaerobic bacteria and genital mycoplasmas (Ureaplasma urealyticum and Mycoplasma hominis), except in the midtrimester group. White blood cells count, glucose concentration, and Gram-stain were also performed shortly after collection except in the midtrimester group. The results of these tests were used for subsequent clinical management. Amniotic fluid not required for clinical purposes was centrifuged for 10 minutes at 4°C and stored at -70°C until analysis.

Complement C3a, C4a, and C5a immunoassays

The characteristics of the assays using this study have been previously described in publications by our group [39,41]. The calculated inter- and intra-assay coefficients of variation for C3a, C4a, and C5a immunoassays in our laboratory were 4.7, 6.4, and 4.1%, and 4.9, 5.8, and 2.5%, respectively. The sensitivity was 0.13 ng/ml for C3a assay, 0.27 ng/ml for C4a assay, and 0.06 ng/ml for C5a assay.

Statistical analysis

The Shapiro–Wilk test was used to test for normal distribution of the data. Kruskal–Wallis with *post hoc* Mann–Whitney *U* tests was performed when indicated to determine the difference of the median among groups, and Bonferroni correction was applied to adjust for multiple comparisons. χ^2 test was used for comparison of proportions. The statistical package used was SPSS 12 (SPSS; Chicago, IL). A probability value of <0.05 was considered significant.

Results

Tables I and II display the demographic and clinical characteristics of pregnant women in the midtrimester and term groups, and of those with spontaneous preterm labor, respectively. There were no significant differences in the maternal age, nulliparity gestational age at delivery, gestational age at amniocentesis, and birth weight between women at term in labor and those not in labor (Table I).

The gestational age at amniocentesis and delivery, as well as the birth weight, were significantly higher among women who had an episode of preterm labor and delivered at term when compared with those who delivered preterm with or without MIAC (Table II). Among patients with spontaneous preterm labor and delivery without MIAC, the gestational age at delivery, and birth weight were significantly higher than that of those who delivered preterm with MIAC (Table II).

Complement split products C3a, C4a, and C5a were detected in all amniotic fluid samples. Women at term not in labor had a significantly higher median C5a amniotic fluid concentration than those in the midtrimester (see Figure 1C). In contrast, no differences were observed in the median amniotic fluid concentrations of C3a and C4a (see Figures 1A and 1B, respectively).

Among normal pregnancies at term, there was no difference in the median amniotic fluid concentration of C3a, C4a, and C5a between women not in labor and those in labor (see Figure 2).

Table I. Demographic and clinical characteristics of women who underwent amniocentesis at midtrimester and term gestation not in labor and in spontaneous labor.

	Midtrimester, $n=70$	Term; no labor, $n=23$	Term; in labor, $n=48$
Maternal age (years)	37 (35–38)*	27 (21–32)	23 (20–27.5)
Nulliparity	12 (17.1)**	5 (23.8)†	23 (47.9)
Gestational age at amniocentesis (weeks)	16 (16–17)*	39.7 (38.7–40)	39.1 (38–40.2)
Gestational age at delivery (weeks)	39.5 (38–40)	39.5 (38.5–40)	39.1 (38–40.1)
Birth weight (g)	3345 (3103–3626.5)‡	3430 (3130–3790)†	3265 (3092–3695)

Values are expressed as median (interquartile range) or number (percent).
*$p < 0.05$ compared with term not in labor and in labor.
**$p < 0.05$ compared with term in labor.
†$n=21$.
‡$n=69$.

Table II. Demographic and clinical characteristics of women with spontaneous preterm labor and intact membranes.

	Preterm labor; No MIAC; Term delivery, $n=42$	Preterm labor; No MIAC; Preterm delivery, $n=57$	Preterm labor; MIAC; Preterm delivery, $n=30$
Maternal age (years)	21.5 (19–27)	23 (20–27)	25 (20–29.2)
Nulliparity	10 (23.8)	21 (36.8)	14 (46.7)**
Gestational age at amniocentesis (weeks)	30.6 (28.7–32.2)	27.2 (25–29.6)*	25.9 (23.6–29.2)**
Gestational age at delivery (weeks)	39 (37.6–40.1)	30.5 (26.1–33.4)*	26.1 (24–30.2)**,†
Birth weight (g)	3035 (2773–3296)	1300 (850–1958)*	844 (515–1395)**,†

MIAC, microbial invasion of the amniotic cavity.
Values are expressed as median (interquartile range) or number (percent).
*$p < 0.05$ compared with preterm labor no MIAC with term delivery.
**$p < 0.05$ compared with preterm labor no MIAC with term delivery.
†$p < 0.05$ compared with preterm labor no MIAC with preterm delivery.

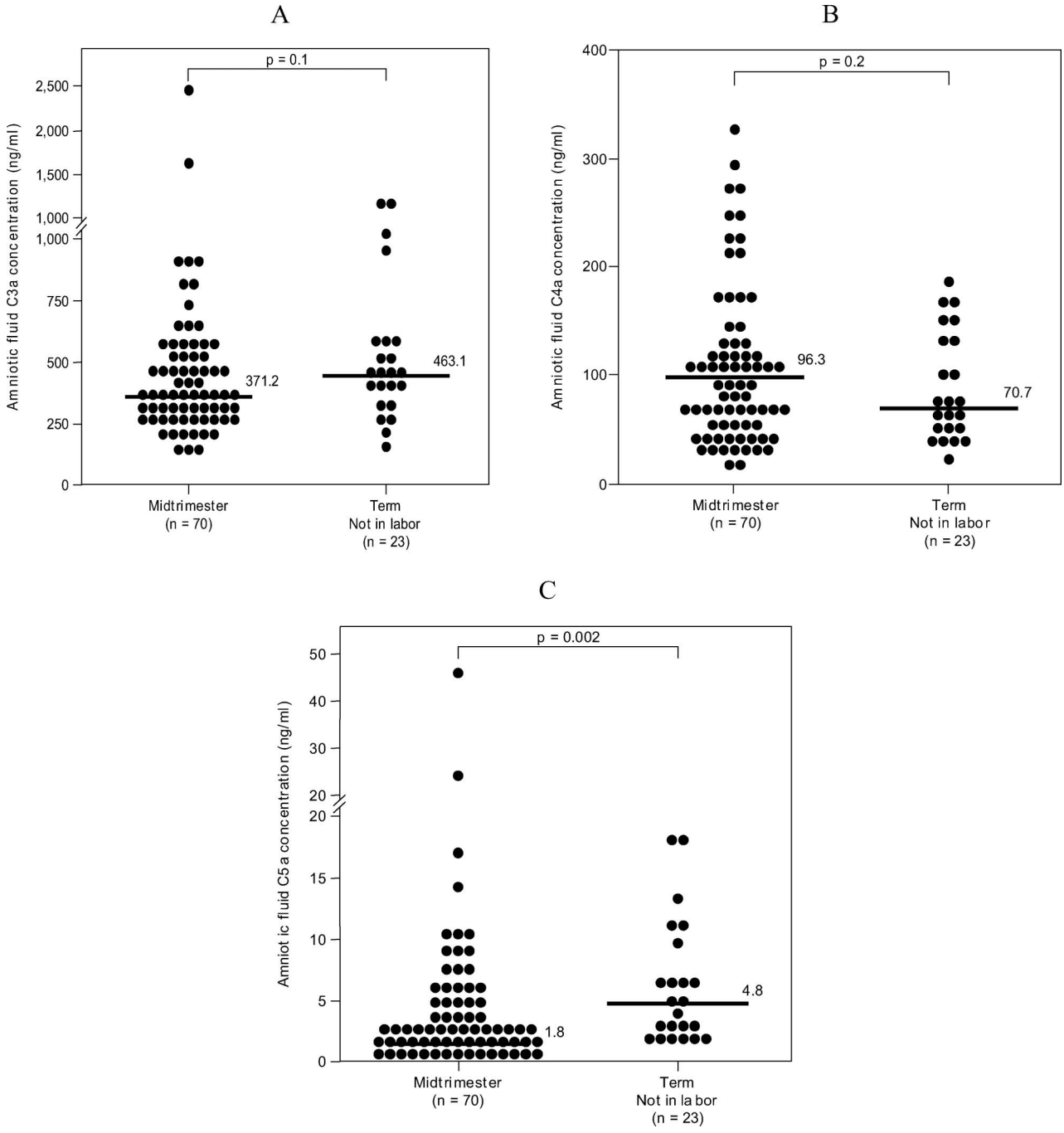

Figure 1. Amniotic fluid anaphylatoxins concentrations from women in the midtrimester (14–18 weeks of gestation) who delivered a normal neonate at term and those at term not in labor. A and B: There were no differences in the median amniotic fluid concentrations of C3a and C4a between pregnant women in the midtrimester and those at term not in labor [C3a: median 371.2 ng/ml, (range 130.4–2468.2) *vs.* median 463.1 ng/ml, (range 161.9–1131.3); $p = 0.1$] and [C4a: median 96.3 ng/ml, (range 13.8–326.2) *vs.* median 70.7 ng/ml, (range 21.4–184.4); $p = 0.2$]; C: In contrast, the median amniotic fluid concentration of C5a was significantly higher in women at term not in labor than in those in the midtrimester [median 4.8 ng/ml, (range 1.7–18.1) *vs.* median 1.8 ng/ml, (range 0.07–46.3); $p = 0.002$].

Among patients with spontaneous preterm labor, those with MIAC had a significantly higher median amniotic fluid concentration of C3a, C4a, and C5a than those with preterm labor without MIAC who delivered at term (all $p < 0.05$) (Figures 3A–3C). Similarly, women with spontaneous preterm labor and MIAC had a significantly higher median amniotic fluid concentration of C4a and C5a, but not of C3a, than those with spontaneous preterm labor without MIAC who delivered preterm (C4a and C5a: $p < 0.05$); (C3a: $p > 0.05$) (Figures 3A–3C). There were no differences in the median C3a, C4a, and C5a amniotic fluid concentrations between women with spontaneous preterm labor without MIAC who

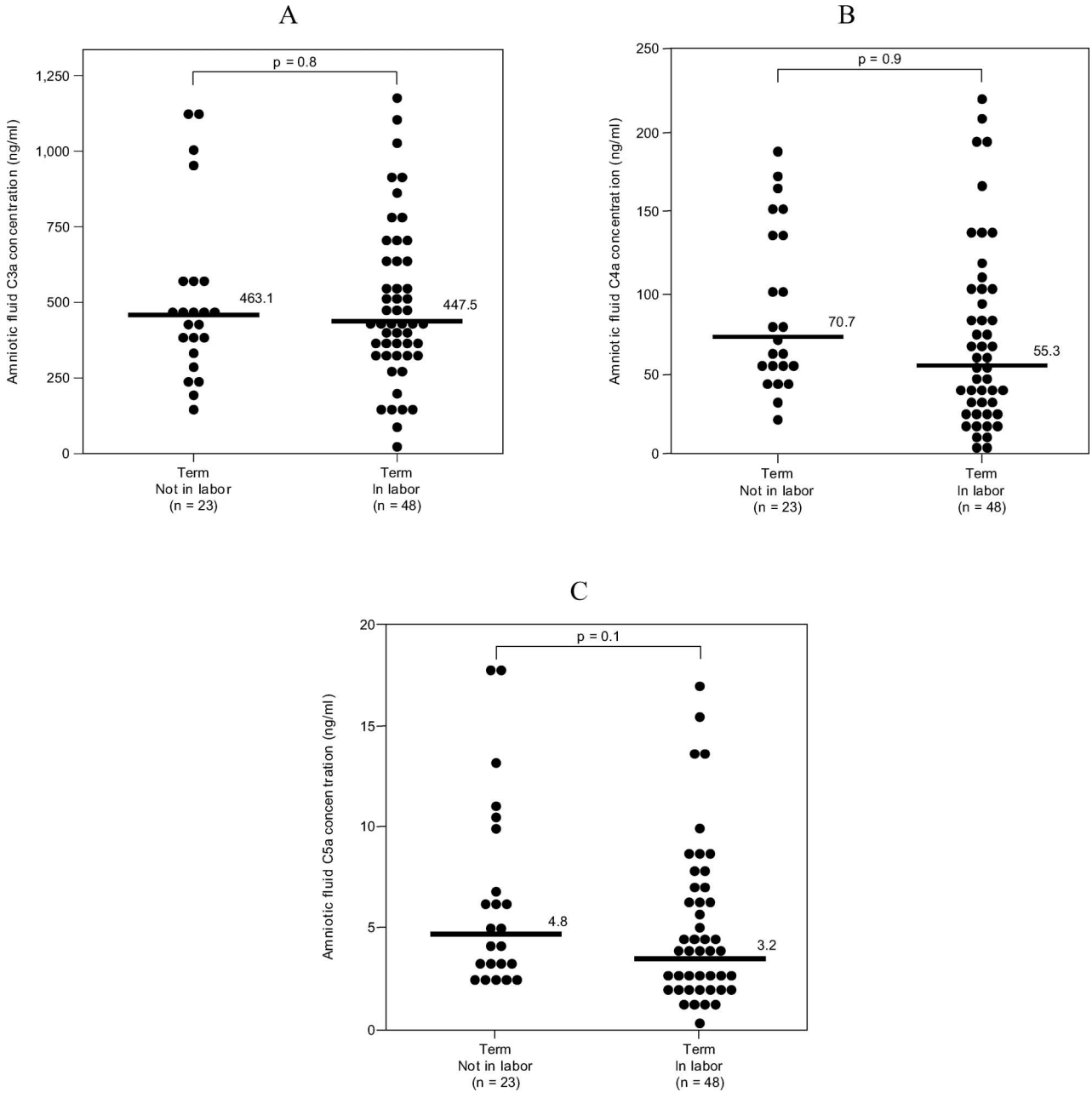

Figure 2. Amniotic fluid anaphylatoxins concentrations of normal pregnant women at term. A, B, C: There were no significant differences in the median amniotic fluid concentrations of C3a, C4a, and C5a between women at term in labor and those not in labor [C3a: median 463.1 ng/ml (range 161.9–1131.3) *vs.* median 447.5 ng/ml (range 12.3–1196.3; $p = 0.8$]; [C4a: median 70.7 ng/ml (range 21.4–184.4) *vs.* median 55.3 ng/ml (range 0.6–217.1; $p = 0.9$]; and [C5a: median 4.8 ng/ml (range 1.7–18.1) *vs.* median 3.2 ng/ml (range 0.07–17.1); $p = 0.1$].

delivered preterm and those who had an episode of spontaneous preterm labor and delivered at term (all p-values > 0.05) (Figures 3A–3C).

Discussion

Principal findings of the study

(1) Among patients with spontaneous preterm labor, those with microbial invasion of the amniotic cavity have significantly higher median amniotic fluid concentrations of C3a, C4a, and C5a than those with a negative amniotic fluid culture; (2) the amniotic fluid concentration of C5a, but not that of C3a and C4a, increases with advancing gestational age; and (3) spontaneous labor at term is not associated with changes in amniotic fluid C3a, C4a, and C5a concentrations.

What are anaphylatoxins C3a, C4a, and C5a?

These complement split products are known as anaphylatoxins because of their properties to induce edema, increase vascular permeability [10], and to

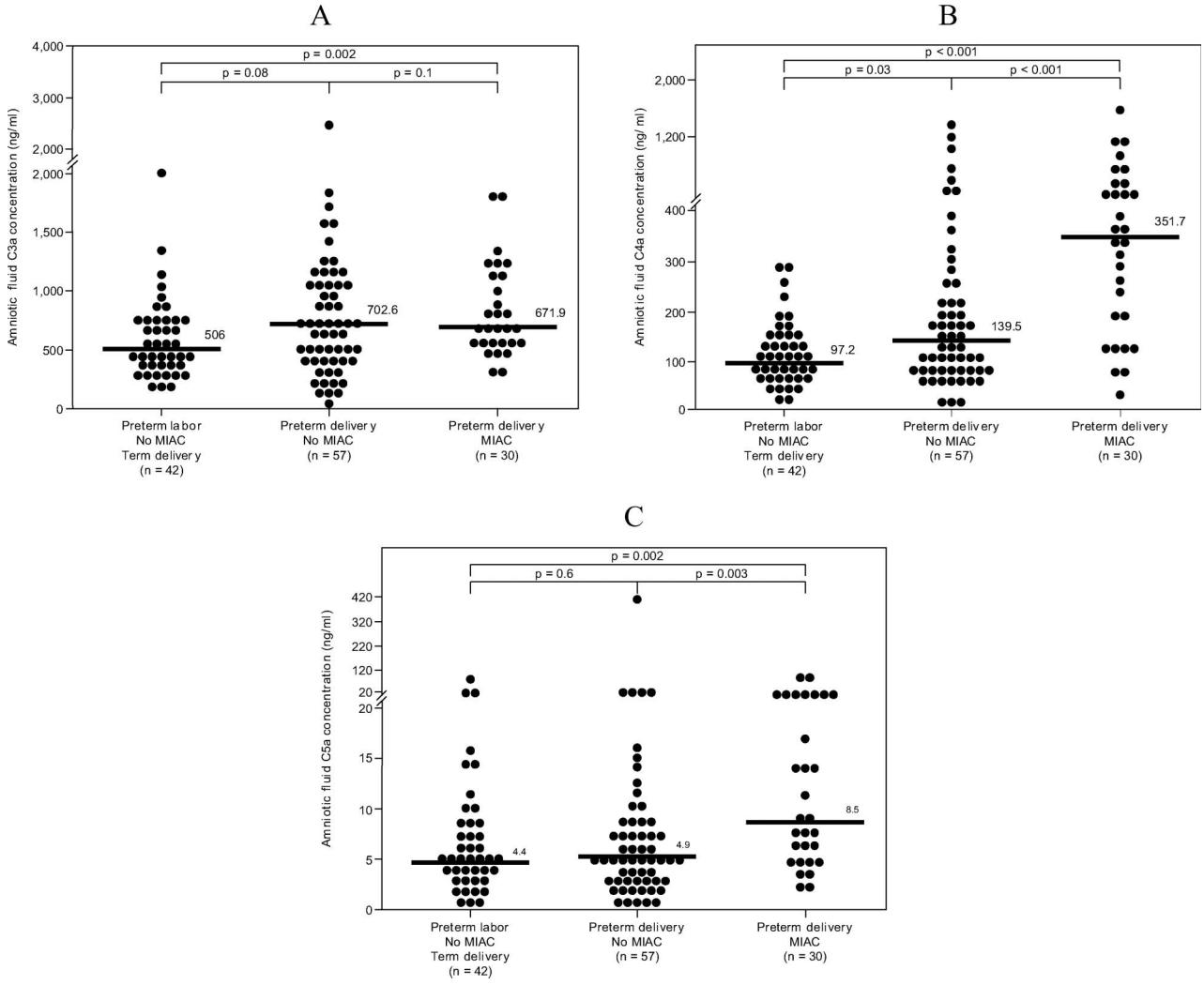

Figure 3. Amniotic fluid anaphylatoxins concentrations in patients with preterm labor. A: Patients with preterm delivery and MIAC had a median amniotic fluid C3a concentration higher than those who in preterm labor and delivered at term [median: 671.9 ng/ml (range 312.8–3552.9) vs. median: 506 ng/ml (range 184.5–1992.8)]. There was no difference in the median amniotic fluid C3a concentration between patients in preterm labor who delivered at term and those who delivered preterm without MIAC. Similarly, there was no difference in the median amniotic fluid C3a concentration between patients with preterm delivery and MIAC and those without MIAC. B, Patients with preterm delivery and MIAC had a higher median amniotic fluid C4a concentration than those who had a preterm delivery without MIAC [median: 351.7 ng/ml (range 24.8–1640.4) vs. median: 139.5 ng/ml (range 0.6–1377.4)]. Similarly, patients with preterm delivery and MIAC had a higher median amniotic fluid C4a concentration than those who had preterm labor a delivered at term [median: 351.7 ng/ml (range 24.8–1640.4) vs. median: 97.2 ng/ml (range 130.4–2468.2)]. In contrast, there was no difference between the median amniotic fluid C4a concentration of women who had a preterm delivery without MIAC and those who had preterm labor and delivery at term. C: Patients with preterm delivery and MIAC had a higher median amniotic fluid C5a concentration than those who had a preterm delivery without MIAC [median: 8.5 ng/ml (range 2.2–83.2) vs. median: 4.9 ng/ml (range 0.07–400)]. Similarly, patients with preterm delivery and MIAC had a higher median amniotic fluid C5a concentration than those who had preterm labor a delivered at term [median: 8.5 ng/ml (range 2.2–83.2) vs. median: 4.4 ng/ml (range 0.5–83.5)]. In contrast, there was no difference between the median amniotic fluid C5a concentration in women who had a preterm delivery without MIAC and those who had preterm labor and delivery at term.

stimulate smooth muscle contractions [7]. C3a biological effects are predominantly on mast cells and eosinophils, and include: (1) chemotaxis [12,13]; (2) granule release [12,42]; (3) expression and shedding of adhesion molecules [43]; (4) increased oxidative burst in neutrophils and eosinophils [44,45]; and (5) immunomodulation of IL-1, IL-6, and TNF-α [46,47]. C4a is the weakest of all anaphylatoxins inducing vascular permeability and smooth muscle contraction [9]. Nonetheless, C4a may modulate the inflammatory response because it can inhibit monocytes chemotaxis [48]. The biological activities of C5a depend on the target cell including: (1) chemotaxis [11–13] and degranulation of inflammatory cells [12,42,49]; (2) enhancement of the respiratory burst and consequent generation of reactive oxygen species in leukocytes [50–52]; and (3) delayed neutrophil apoptosis [53]. Other

important functions of C5a are the induction and/or release of inflammatory cytokines such as IL-1 [54–56], IL-6 [57–59], IL-8 [60], and TNF- α [55,56] from neutrophils, mononuclear and endothelial cells.

The complement system in the fetus and amniotic fluid

Complement proteins has been detected in cord blood [61–67], placenta [68–70], and chorioamniotic membranes [70–72]. Interestingly, the presence of complement proteins in amniotic fluid has been previously reported [64,73–75]. Stabile et al. [64] used rocket immunoelectrophoresis and detected complement factors C3, C4, C5, Factor B, H, and I in amniotic fluid and cord blood of normal pregnancies between 15 and 28 weeks of gestation. The authors reported that the concentrations of these complement proteins were 10 times higher in cord blood than in amniotic fluid, and that the concentrations of some of these proteins (C3 and Factor B) increased with gestational age in amniotic fluid. Similarly, Sharma et al. [73] reported that amniotic fluid concentration of C3 increased from the first to the second trimester, but not in the third trimester.

C3a, C4a, and C5a were detected in all amniotic fluid samples included in this study, suggesting that these anaphylatoxins are physiologic constituents of the amniotic fluid. Haeger et al. [76] were the first to describe the presence of complement anaphylatoxins C3a and C5a in amniotic fluid collected in patients with preeclampsia and in those with uncomplicated pregnancies. The concentration of C3a and C5a did not differ between the two groups. Interestingly, the authors performed and in vitro study incubating amniotic fluid with fresh plasma, and a dose-dependent release of C3a and C5a in the plasma was noted. The authors concluded that amniotic fluid can activate the complement cascade. Our results indicate that the amniotic fluid concentration of C5a changes during gestation, because women at term not in labor had a higher median amniotic fluid concentration of C5a than those in the midtrimester. In contrast, no changes were observed in the amniotic fluid concentrations of C3a and C4a (direct split product of C3 and C4, respectively) with advancing gestational age.

Amniotic fluid complement split products and term gestation

In this study, labor at term was not associated with changes in the amniotic fluid concentration of anaphylatoxins. This is not consistent with the conventional view that spontaneous labor at term is an inflammatory process [77,78] characterized by increased maternal neutrophil count [79] and increased maternal serum/plasma concentrations of proinflammatory cytokines (IL-1β, IL-6, IL-8, TNF α) [77,80–82] and chemokines (growth-related oncogene α, granulocyte colony stimulating factor, granulocyte macrophage colony stimulating factor, neutrophil attractant/activating peptide-1, etc.) [77,83]. However, it is possible that the low-grade inflammatory state involved in the process of labor may not require the activation of the complement system. Alternatively, if the fetus is the main source of the complement system in the amniotic fluid, it is possible that inflammation during normal labor at term may not be enough to activate the complement system in the amniotic cavity.

Complement and preterm delivery

The observation that MIAC is associated with complement activation in patients with preterm labor, as indicated by elevated amniotic fluid concentrations of C3a, C4a, and C5a, is novel. Elimian et al. [74] reported that among patients with preterm labor and intact membranes, those with positive amniotic fluid cultures had a higher amniotic fluid concentration of C3 (total protein) than those with negative amniotic fluid cultures. Moreover, the authors reported that the amniotic fluid concentration of C3 had similar diagnostic performance as other markers of intra-amniotic infection [74], such as amniotic fluid white blood cell count [84], glucose concentration [85], Gram stain [86], and LDH [87].

In a previous study, the maternal serum complement hemolytic activity (CH50) in women with preterm labor was similar between those who delivered preterm and those who delivered at term [75]. However, the CH50 assay is an insensitive marker of complement activation [88]. Recently, Lynch et al. [89] proposed that complement activation in early pregnancy may lead to preterm delivery. In a prospective study, the authors determined that elevated plasma concentrations of factor Bb (primarily part of the alternative pathway) was predictive of delivery at less than 34 weeks of gestation. Indeed, women with factor Bb plasma concentrations in the top quartile prior to 20 weeks of gestation were 4.7 times more likely to have an spontaneous preterm delivery before 34 weeks compared with women who had factor Bb plasma concentrations in the lower three quartiles (95% confidence interval 1.5–14). Our group reported that patients with intra-amniotic infection/inflammation (regardless of the membranes status) had higher median amniotic fluid fragment Bb concentrations than that of those without IAI (in press) [90]. In another study, maternal plasma C5a concentrations were higher in patients with spontaneous preterm labor with MIAC than that of those with preterm labor without MIAC delivering

preterm or at term [39]. This finding suggests that the maternal immune system is responding to either microbial products or proinflammatory mediators produced in the amniotic cavity, and that such response can be detected in the maternal compartment.

In conclusion, this study demonstrates that patients with spontaneous preterm labor and intact membranes with microbial invasion of the amniotic cavity had increased amniotic fluid concentrations of complement split products C3a, C4a, and C5a.

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