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Changes in amniotic fluid concentration of thrombin–antithrombin III complexes in patients with preterm labor: Evidence of an increased thrombin generation

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Abstract

Objective. Preterm labor is associated with excessive maternal thrombin generation, as evidenced by increased circulating thrombin–antithrombin (TAT) III complexes concentration. In addition to its hemostatic functions, thrombin has uterotonic properties that may participate in the mechanism leading to preterm birth in cases of intrauterine bleeding. Thrombin also has a proinflammatory role, and inflammation is associated with increased thrombin generation. The aim of this study was to determine whether intra-amniotic infection/inflammation (IAI) is associated with increased amniotic fluid (AF) thrombin generation in women with preterm and term deliveries.

Study design. This cross-sectional study included the following groups: (1) mid-trimester ($n = 74$); (2) term not in labor ($n = 39$); (3) term in labor ($n = 25$); (4) term in labor with IAI ($n = 22$); (5) spontaneous preterm labor (PTL) who delivered at term ($n = 62$); (6) PTL without IAI who delivered preterm ($n = 59$); (7) PTL with IAI ($n = 71$). The AF TAT III complexes concentration was measured by enzyme linked immunosorbent assay (ELISA). Non-parametric statistics were used for analysis.

Results. (1) TAT III complexes were identified in all AF samples; (2) patients with PTL who delivered preterm, with and without IAI, had a higher median AF TAT III complexes concentration than those with an episode of PTL who delivered at term ($p < 0.001$, $p = 0.03$, respectively); (3) among patients with PTL without IAI, elevated AF TAT III complexes concentration were independently associated with a shorter amniocentesis-to-delivery interval (hazard ratio, 1.5; 95% CI, 1.07–2.1); (4) among patients at term, those with IAI had a higher median AF TAT III complexes concentration than those without IAI, whether in labor or not in labor ($p = 0.02$); (5) there was no significant difference between the median AF TAT III complexes concentration of patients at term with and without labor; (6) patients who had a mid-trimester amniocentesis had a lower median AF TAT III complexes concentration than that of patients at term not in labor ($p < 0.001$).

Conclusions. We present herein a distinct difference in the pattern of intra-amniotic thrombin generation between term and preterm parturition. PTL leading to preterm delivery is associated with an increased intra-amniotic thrombin generation regardless of the presence of IAI. In contrast, term delivery is associated with an increased intra-amniotic thrombin generation only in patients with IAI.

Keywords: *Preterm parturition, delivery, inflammation, protease-activated receptors, survival curvel*

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Introduction

Thrombin plays a central role in the coagulation cascade and participates in transforming fibrinogen into fibrin and platelet activation [1,2], as well as the activation of the fibrinolytic and anticoagulation systems [3–6]. Other activities of thrombin include the activation of endothelial cells [7–9] and leukocytes (lymphocytes, monocytes and neutrophils) [10–17]. These activities are mediated at least in part by protease-activated receptors (PARs), which are G protein-coupled receptors activated through cleavage by thrombin and other coagulation factors [18–22].

The reaction of thrombin with its major inhibitor, antithrombin III, results in the formation of an inactive stable complex, the thrombin–antithrombin (TAT) III complex. In order to study the activation of the coagulation system, it is necessary to measure either peptides released from coagulation factor zymogens or complexes formed between activated coagulation factors and their natural inhibitors. This is required because activated coagulation factors have a short half-life and direct measurement of these factors during the activation of the coagulation cascade is difficult [23]. The presence and/or concentration of TAT III complexes is widely accepted as an index of thrombin generation *in vivo* [24–27].

During pregnancy, changes in the coagulation system are considered to be adaptive to prevent hemorrhage at the time of delivery [28–32]. Indeed, normal pregnancy has been associated with excessive maternal thrombin generation [31,33] and a tendency for platelets to aggregate in response to agonists [34,35]. In addition, increased thrombin generation in the maternal circulation has been reported in several obstetrical syndromes including preterm labor (PTL) [33,36], preeclampsia [37–45], fetal growth restriction [37,46,47] and preterm prelabor rupture of membranes (PROM) [33,48].

The administration of actively clotting blood, but not blood treated with heparin, into the uterine cavity has been associated with increased uterine contractility, and this has been attributed to a thrombin-specific uterotonic property, even at low concentrations [49]. This phenomenon has been implicated in the initiation of labor in cases of intrauterine bleeding, and perhaps infection [50]. In addition, thrombin may play a role in membrane rupture by activation of matrix metalloproteinase (MMP)-1 or interstitial collagenase [51,52], which can degrade type I and type III collagens, important components of the membranes. MMP-1 concentrations are elevated in the amniotic fluid of women with term as well as preterm PROM [53].

PTL is associated with an increased thrombin generation suggested by a higher median maternal plasma concentrations of TAT III complexes in patients with PTL compared to women with a normal pregnancy [33,36]. Moreover, high plasma concentrations of TAT III complexes are not associated with a history of vaginal bleeding during pregnancy [33,36] or the presence of intra-amniotic infection/inflammation (IAI) [33], suggesting that the activation of the maternal coagulation cascade is associated with an episode of PTL, regardless of the underlying mechanism responsible for the preterm parturition syndrome [54–64].

Amniotic fluid (AF) has procoagulant activity [65–68], which is higher than that of the maternal plasma, this activity has been attributed to tissue factor [69]. Indeed, the concentrations of tissue factor, are higher in AF than in maternal plasma [57]. However, the changes in AF thrombin generation during an episode of PTL have not been systematically studied. The aim of this study was to determine the changes in AF TAT III complexes concentration in women with preterm and term labor and delivery with and without IAI.

Material and methods

Study groups and inclusion criteria

A cross-sectional study was designed by searching our clinical database and bank of biologic samples, which included samples from pregnant women in the following groups: (1) women in the mid-trimester of pregnancy ($n = 74$) who underwent amniocentesis for genetic indications and delivered an appropriate-for-gestational age neonate at term; (2) women with a normal pregnancy at term not in labor ($n = 39$); (3) women with a normal pregnancy at term in labor ($n = 25$); (4) women with a normal pregnancy at term with IAI ($n = 22$). Patients with term gestation underwent amniocentesis for the determination of fetal lung maturity or to rule out IAI; (5) women with spontaneous PTL and intact membranes who delivered at term ($n = 62$); (6) patients with PTL without IAI who delivered preterm (< 37 weeks) ($n = 59$); and (7) PTL with IAI ($n = 71$).

All women provided written informed consent prior to the collection of AF. The collection of AF and its utilization for research purposes were approved by the Institutional Review Boards of the participating institutions and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH and DHHS. The patients included were recruited from the following centers: Hutzel Hospital, Detroit (women with PTL or mid-trimester), Pennsylvania Hospital, Philadelphia (mid-trimester) and Sotero del Rio Hospital, Santiago, Chile (women at term). Many of these samples have been used previously to study the biology of

inflammation, hemostasis, angiogenesis regulation, growth factor concentrations and other processes in normal pregnant women and those with pregnancy complications.

Clinical definitions

PTL was diagnosed by the presence of at least two regular uterine contractions every 10 min associated with cervical changes that required admission to the hospital before 37 weeks gestation. Small-for-gestational age (SGA) neonate was defined as birthweight below the 10th percentile [70]. Placental pathology was classified according to a previously described nomenclature [71]. Intra-amniotic infection was defined by the presence of positive AF cultures for microorganisms and intra-amniotic inflammation by an AF white blood cell (WBC) count $\geq$100 cells/ml and/or an AF IL-6 concentration $>$2.6 ng/ml [72–77].

Amniotic fluid collection

AF collection was performed by trans-abdominal amniocentesis as described in other publications by our group [78–85]. AF was transported to the laboratory in a capped plastic sterile syringe, and was cultured for aerobic and anaerobic bacteria, as well as for genital mycoplasmas. WBC count, glucose concentration and Gram stain for microorganisms were performed in AF shortly after collection. The results of the AF analyses were used for clinical management. The remainder of the AF was centrifuged at 4°C for 10 min to remove cellular and particulate debris. Aliquots of the supernatant were stored at (70°C until assay. Samples were not subject to repeat freeze–thaw cycles.

TAT III complex immunoassays

Samples of AF were assayed for TAT III complexes in duplicate using a commercially available immunoassay kit (Enzygnost TAT micro; Behring Diagnostics, Westwood, MA). This assay is a sandwich enzyme linked immunosorbent assay (ELISA). Prior to use on study samples, the assay system was validated for AF using spike and recovery experiments. The sensitivity of the assay was 1.06 μg/l, and the inter- and intra-assay coefficients of variation were 11.6 and 8.3%, respectively.

Statistical analysis

The normality of the data was tested by the Shapiro–Wilk and the Kolmogorov–Smirnov tests. As the AF TAT III complexes concentration were not normally distributed, the Mann–Whitney *U* and Kruskal–Wallis tests were used to analyze differences between groups. Spearman's rank correlation test was used to determine the relationship between AF TAT III complexes concentration and gestational age in women who had a normal pregnancy. A receiver operating characteristic (ROC) curve was constructed to describe the relationship between the sensitivity (true-positive rate) and the false-positive rate for different values of AF TAT III complexes concentration in the identification of patients at risk for preterm delivery. Survival analyses were performed by a Log rank test. Cox proportional hazard regression analysis was used to determine the association between an elevated TAT III complexes concentration and the amniocentesis-to-delivery interval in patients with PTL without IAI after adjusting for confounding variables. The statistical software package used was SPSS 12.0 (SPSS, Chicago, IL).

Results

Demographic and clinical characteristics

The clinical and demographic characteristics of the term and preterm groups are presented in Tables I and II. Among the PTL groups, patients with IAI had a lower gestational age at amniocentesis, gestational age at delivery and neonatal birthweight than patients without IAI who delivered preterm and than those who delivered at term (Table II).

Table I. Demographic and clinical characteristics of the study population.

	Term not in labor ($n=39$)	Term in labor ($n=25$)	Term labor with IAI ($n=22$)	p
Maternal age (years)	24 (21–30)	23 (20–27)	25 (21.8–29.3)	0.6
Gravidity	2 (2–4)	1.5 (1–2)	2 (1–3)	0.06
Gestational age at amniocentesis (weeks)	39 (39–40)	40 (39–40)	39 (38–40)	0.2
Gestational age at delivery (weeks)	39 (39–40)	40 (39–40)	39 (38–40)	0.2
Neonatal birthweight (g)	3260 (3085–3475)	3580 (3172.5–3760)	3320 (3100–3730)	0.2

Data is presented as median (iterquertile range).
PTL, preterm labor; IAI, intra-amniotic infection/inflammation.

Table II. Demographic and clinical characteristics of the preterm study population.

ss	PTL delivered at term ($n=62$)	PTL without IAI ($n=59$)	PTL with IAI ($n=71$)	p
Maternal age (years)	21.5 (19–25.7)	22 (19–28.2)	23 (19.5–27.5)	0.7
Gravidity	3 (2–4)	3 (2–5)	3 (2–5)	0.7
Gestational age at amniocentesis (weeks)	31.9 (29.9–33.1)	29.4 (26.3–31.7)	27.0 (25.1–31.9)	<0.001
Gestational age at delivery (weeks)	38.6 (38–39.4)	31.2 (29.0–33.8)	28.5 (25.0–32.3)	<0.001
Neonatal birthweight (g)	3000 (2720–3400)	1639.5 (1100–2262)	1060 (695–1735)	<0.001

Data is presented as median (iterquertile range).
PTL, preterm labor; IAI, intra-amniotic infection/inflammation.

Changes in amniotic fluid TAT III complexes concentration during normal pregnancy and labor

The AF concentration of TAT III was significantly higher in women at term not in labor than in the mid-trimester of pregnancy ($p < 0.001$, Figure 1). Labor at term was not associated with a significant change in the median AF TAT III complexes concentration ($p = 0.7$, Figure 2).

Patients with IAI at term had a higher median AF TAT III complexes concentration (median 118.5 μg/l, range 26.3–310.0) than women at term not in labor ($p = 0.006$, after Bonferroni correction) and than those in labor ($p = 0.02$, after Bonferroni correction) (Figure 2).

Changes in amniotic fluid TAT III complexes concentration in preterm labor

The median AF TAT III complexes concentration differed significantly among patients with PTL and intact membranes (Kruskal–Wallis, $p = 0.001$). Patients with PTL and IAI had a higher median AF TAT III complexes concentration than that of those with PTL who delivered at term ($p < 0.001$, Figure 3). Patients with PTL who delivered preterm without IAI had a higher median AF TAT III complexes concentration than that of those with PTL who delivered at term ($p = 0.03$ after Bonferroni correction). Although the median AF TAT III complexes concentration was higher in patients with PTL and IAI than in those with PTL without IAI, the difference did not reach statistical significance ($p = 0.2$, after Bonferroni correction) (Figure 3).

Among patients with PTL, a TAT III complexes concentration of 108.24 μg/l or more was associated with a sensitivity of 63.1% and a specificity of 72.6% for the identification of women who will deliver preterm. According to this cutoff for elevated AF TAT III complexes concentration, patients with PTL with elevated TAT III complexes concentration had an odds-ratio of 4.52 (95% CI, 2.2–9.3) to deliver preterm.

The effect of elevated TAT III complexes concentration on gestational age at delivery and amniocentesis-to-delivery interval among patients with

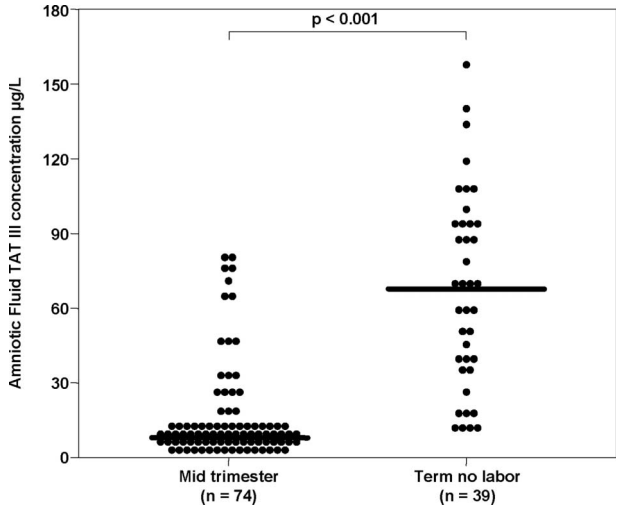

Figure 1. Amniotic fluid thrombin-antithrombin III complexes concentration in patients in the mid-trimester of pregnancy and women at term not in labor (mid-trimester: median 8.1 μg/L, range 2.1–160.0 vs. term no labor: median 66.9 μg/L, range 10.2–154).

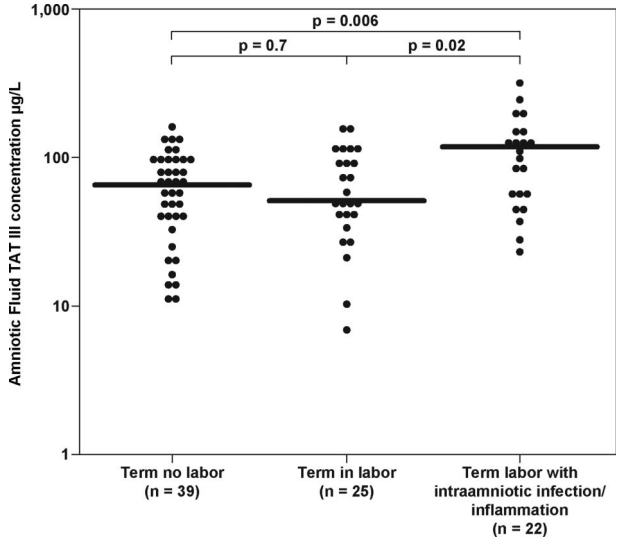

Figure 2. Amniotic fluid thrombin-antithrombin III complexes concentration in patients with term pregnancies not in labor, women at term in labor and patients with term labor and intra-amniotic infection/inflammation (term no labor: median 66.9 μg/L, range 10.2–154; term in labor: median 50.8 mg/L, range 6.8–150.0); term labor with IAI: median 118.5 μg/L, range 26.3–310.0.

PTL was studied further using survival analyses. An elevated AF TAT III complexes concentration was associated with an earlier gestational age at delivery [Log rank test, $p < 0.001$, Figure 4(a)] and a shorter amniocentesis-to-delivery interval [Log rank test, $p < 0.001$, Figure 4(b)]. When the cases were stratified according to the presence of IAI, patients with PTL without IAI but an elevated TAT III complexes concentration had an earlier gestational age at delivery [Log rank test, $p = 0.0002$, Figure 5(a)] and a shorter amniocentesis-to-delivery interval than patients without an elevated TAT III complexes concentration [Log rank test, $p = 0.0085$, Figure 5(b)]. However, among patients with PTL and IAI who delivered spontaneously, an elevated AF TAT III complexes concentration was not associated with an earlier gestational age at delivery ($p = 0.2$) or a shorter amniocentesis-to-delivery interval ($p = 0.5$). To further investigate the effect of an elevated TAT III complexes concentration among patients with PTL without IAI who delivered spontaneously, we conducted a survival analysis using Cox proportional hazard modeling. After correction for confounding factors (including gestational age and cervical dilatation at time of amniocentesis), an elevated AF TAT III complexes concentration was independently associated with a shorter amniocentesis-to-delivery interval (hazard ratio, 1.5; 95% CI 1.07–2.1) (Table III).

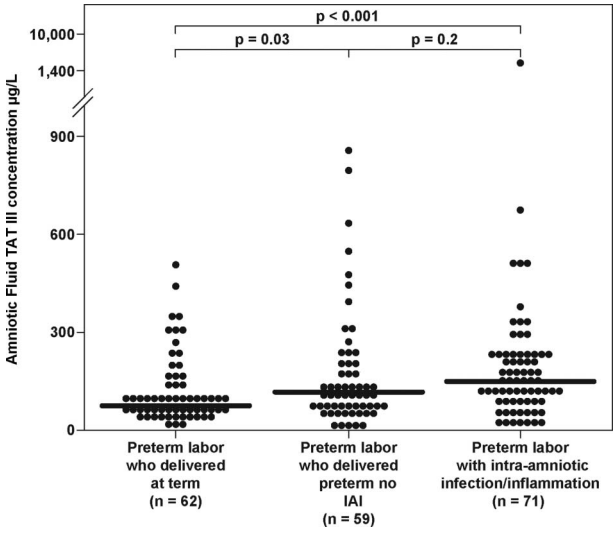

Figure 3. Amniotic fluid thrombin-antithrombin III complexes concentration in patients with preterm labor who delivered at term, women with preterm labor who delivered preterm without intra-amniotic infection/inflammation, and women with preterm labor and intra-amniotic infection/inflammation (PTL with IAI: median 147.7 μg/L, range 15.3–1424.8; PTL without IAI: median 116.0 μg/L, range 10.7–2073.9; PTL who delivered at term: median 73.4 μg/L, range 7.6–507.0).

Comments

Principal findings of the study

(1) TAT III complexes are normally present in AF at all gestational ages from the mid-trimester to term. (2) IAI at term is associated with an increased AF TAT III complexes concentration suggestive of an increase in thrombin generation within the amniotic cavity. (3) Among patients with PTL, those who delivered preterm had a higher median AF TAT III complexes concentration than those who delivered at term, regardless of the presence of IAI. (4) Among patients with PTL without IAI, elevated AF TAT III complexes concentration was associated with a

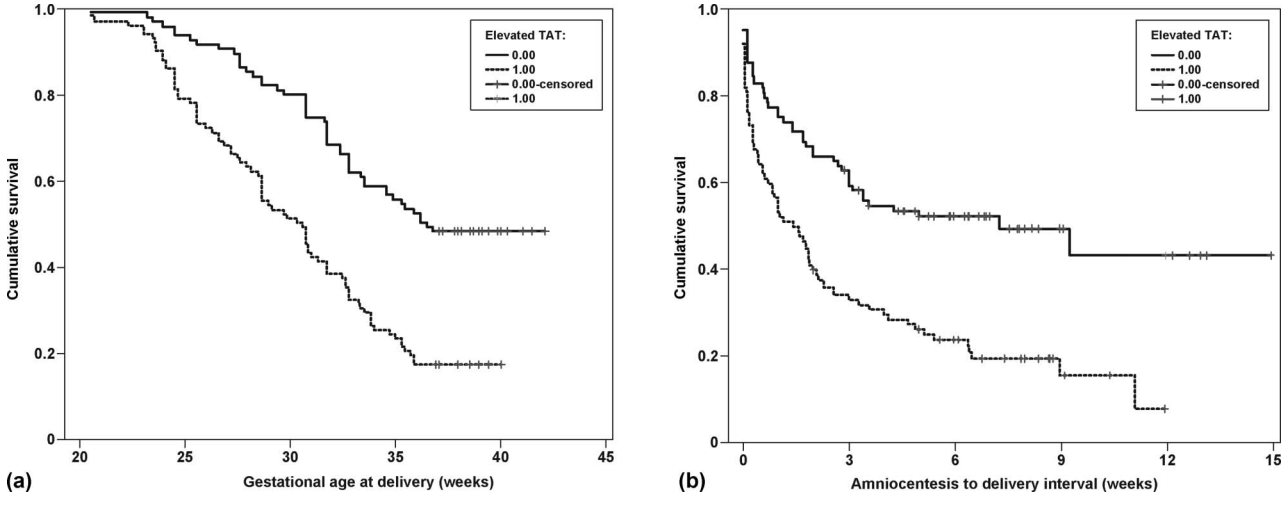

Figure 4. (a) The effect of an elevated amniotic fluid thrombin–antithrombin III complexes concentration on gestational age at delivery in patients with preterm labor. (b) The effect of an elevated amniotic fluid thrombin–antithrombin III complexes concentration on the amniocentesis-to-delivery interval in patients with preterm labor.

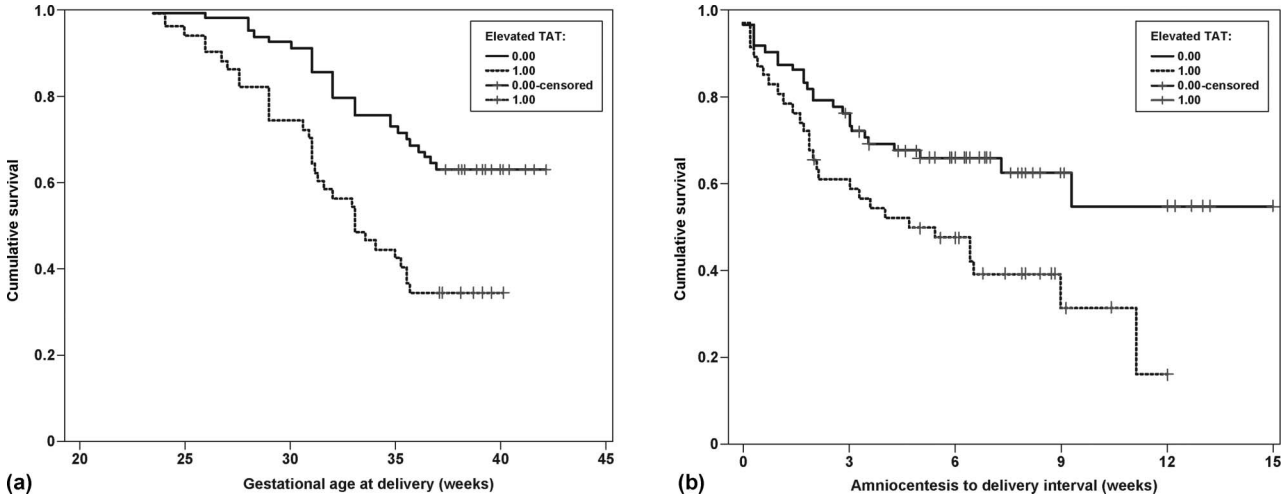

Figure 5. (a) The effect of an elevated amniotic fluid thrombin–antithrombin III complexes concentration on gestational age at delivery, among patients with preterm labor without intra-amniotic infection/inflammation. (b) The effect of an elevated amniotic fluid thrombin–antithrombin III complexes concentration on the amniocentesis-to-delivery interval of patients with preterm labor without intra-amniotic infection/inflammation.

Table III. Cox proportional hazards survival analysis in the prediction of amniocentesis-to-delivery interval.

Covariates	Hazard ratio	95% Confidence interval	*p*
Elevated amniotic fluid thrombin–antithrombin III complexes concentration*	1.5	1.07–2.1	0.02
Gestational age at amniocentesis (wk)	0.98	0.93–1.03	0.45
Cervical dilatation at amniocentesis (cm)	1.3	1.2–1.4	<0.001

*Variable was dichotomized: amniotic fluid thrombin–antithrombin III complex concentration (<108.24 μg/l *vs.* ≥ 108.24 μg/l).

higher risk for preterm delivery and a shorter amniocentesis-to-delivery interval.

Changes in thrombin during normal pregnancy and labor

Normal pregnancy is associated with an increased maternal thrombin generation as determined by the elevated concentrations of fibrinopeptide A, prothrombin fragments 1 and 2, and TAT III complexes [32,85–87]. In addition, the maternal plasma TAT III complexes concentration increases further during labor [88] as well as immediately after delivery [87,88] and decreases during the puerperium.

In contrast to maternal plasma, data about changes in AF TAT III complexes concentration is limited [67,89]. Our finding that the median AF TAT III complexes concentration increases from the mid-trimester to term is consistent with the report of Koh et al. [89] that examined the changes in AF TAT III complexes concentration in the second trimester and during the first stage of labor, and documented an increase in TAT III complexes concentration in the first stage of labor as compared to mid-trimester [89]. Uszynski et al. [67] examined TAT III complexes concentration in AF at term and concluded that they increase during labor [67]. The AF TAT III complexes concentration documented in the latter study [67] during the first stage of labor (54.2 ± 26.4 ng/ml) are in agreement with the concentrations we have found.

The sources and roles of thrombin in AF remain to be elucidated. The AF TAT III complexes concentration of patients with normal pregnancies is 2–4 times higher than that observed in maternal plasma [67]. Similarly, umbilical cord blood TAT III complexes concentration [66] is lower than in AF. These findings, along with the high molecular weight of thrombin (39,000 Da), suggest that thrombin is produced locally. Evidence in support of this view includes the following: (1) Prothrombin [91] and other coagulation factors from both the intrinsic and extrinsic pathways are present in AF [91,92]; (2) Tissue factor is present in AF in high concentrations and can directly convert prothrombin to thrombin [17,65,68,93–95]; and (3) The local production of thrombin is via activated factor X as it has been demonstrated that AF has direct factor X-activating properties [96]. Collectively, these observations provide a mechanism for the local generation of thrombin from prothrombin in the AF.

The association between thrombin generation and intra-amniotic infection/inflammation

The findings of an increased median AF TAT III complexes concentration among patients with PTL who delivered preterm and among patients at term

with IAI are novel. The interaction between coagulation and inflammation may be an explanation for the increased thrombin generation in patients with IAI during preterm and term parturition. A solid body of evidence supports the generation of thrombin during the course of inflammation [97–99], mainly through the tissue factor pathway [97,99–102]. Indeed, during inflammation, activated monocytes express tissue factor on their membrane [103–108] and shed micro-particles which contain tissue factor into the plasma [103,109–116]. An important mediator of tissue factor expression during inflammation are interleukin (IL)-6 [23,117], TNF-α [118,119], and IL-β [119–121]. This is noteworthy, since the AF concentration of these proinflammatory cytokines increases during IAI [122–145] and may be one of the mechanisms by which intra-amniotic inflammation activates the increase AF thrombin generation in these patients.

Activation of the AF complement system can be an additional mechanism by which intra-amniotic thrombin is generated during inflammation. Indeed, patients with PTL and intra-amniotic infection have a higher C5a concentration than patients with PTL who delivered preterm or at term [146]. The association between C5a and tissue factor activation and expression was previously reported [147,148]. C5a induces a 4.9-fold increase in tissue factor activity and a 3.8-fold increase in tissue factor mRNA expression by endothelial cells [147]. Furthermore, the administration of C5a to animals increases the procoagulant activity of alveolar macrophages 5- to 6-fold through tissue factor activation [148]. Thus, the increased concentrations of AF C5a in patients with PTL and intra-amniotic infection may activate AF tissue factor, leading to an increased thrombin generation that is reflected by the elevated AF TAT III complexes concentration in these patients.

Decidual bleeding is an additional mechanism that may be associated with an increased thrombin generation in patients with PTL and intra-amniotic infection/inflammation. Idiopathic vaginal bleeding is associated with IAI [149]. Moreover, patients with vaginal bleeding and microbial invasion of AF had a higher rate of early preterm delivery (before 32 weeks of gestation) than in women without bleeding [149]. Our group has proposed that IAI is associated with decidual bleeding that may be manifested as idiopathic vaginal bleeding. This concept is further supported by the findings that patients with PTL and IAI have a higher median AF total hemoglobin concentration than patients with PTL without IAI and than those who delivered at term [150]. Moreover, among patients with PTL, the fraction of fetal hemoglobin out of the total AF hemoglobin was lower in those with IAI than in those without it, suggesting a higher proportion of maternal hemoglobin in the AF of these patients [151]. Thus, occult decidual bleeding associated with IAI may contribute to activation of the cascade leading to an increased thrombin generation in the AF, as reflected by the elevated TAT III complexes concentration in these patients. Similar mechanisms may contribute to the higher median AF TAT III complexes detected in patients with IAI at term.

The association between thrombin generation and preterm birth in the absence of intra-amniotic infection/inflammation

The association between elevated median AF TAT III complexes concentration and preterm delivery among patients with PTL but without IAI is novel. This observation provides additional evidence to support the distinct underlying mechanisms of term and preterm parturition. Labor at term is a physiologic process that is not associated with a significant increase in AF thrombin generation. However, PTL leading to preterm parturition is a pathologic process that is associated with an increased AF thrombin generation, even in the absence of IAI, emphasizing the syndromic nature of PTL and delivery [54–56,152].

In the current study, among patients with PTL, those with an elevated AF TAT III complexes concentration had a higher risk for preterm delivery. Moreover, an elevated AF TAT III complexes concentration was associated with a shorter amniocentesis-to-delivery interval, only among those with PTL without intra-amniotic infection/inflammation. This observation suggests that the higher AF TAT III complexes concentration observed in cases of IAI (preterm and term) may be part of the inflammatory process in the AF. However, in the absence of infection, increased thrombin generation may be the mechanism actually leading to preterm birth. This assumption is based on the uterotonic properties of thrombin [36,49,50,117] and was proposed in the context of intrauterine bleeding mainly through the association with placental abruption [36,49,50].

The uterotonic effect of thrombin may be exerted by its receptor, the PAR-1, which mediates many of the effects of thrombin on platelet activation [153,154], proinflammatory cytokine secretion [17,155,156], local tissue remodeling after injury [157,158], fetal blood vessel development and stabilization [159–161], as well as uterine contractions [162–164]. Indeed, in a study on stem cells gene expression, PAR-2 and PAR-1 were among the top 25 up-regulated genes in stem cells of AF origin [165], suggesting that thrombin can exert its intracellular and proinflammatory effect via local mechanisms through its receptor. However, it is still not clear which underlying processes lead to an increased AF TAT

III complexes concentration in patients with PTL without IAI who delivered preterm.

In summary, the increased AF TAT III complexes concentration in patients with preterm and term IAI is probably mediated by intra-amniotic inflammatory processes. However, the association between elevated AF TAT III complexes concentration and preterm delivery, as well as a shorter amniocentesis-to-delivery interval even without IAI, suggests a role for thrombin in the pathogenesis of preterm parturition even in the absence of infection/inflammation. The precise pathophysiologic role of thrombin in AF remains to be determined because changes occur despite the absence of coagulation, suggesting that thrombin has other functions within the amniotic cavity, which may be unrelated to its role in the coagulation system.

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