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The implication of second-trimester amniotic fluid TNF-alpha, cytochrome C and cell death nucleosomes in the prediction of preterm labor and/or premature rupture of membranes

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Abstract
Aim The multifactorial pathway leading to preterm labor possibly includes the implication of apoptosis. This study aimed to clarify the role of amniotic fluid apoptotic molecules (TNF-alpha, cytochrome C and cell death nucleosomes) at midtrimester as possible predictors of preterm labor (PTL) and/or premature rupture of membranes (PROM).
Method In this case–control study, comprising 360 women undergoing genetic amniocentesis and out of whom 38 delivered preterm and 18 out of the latter after PROM, the above apoptotic molecules were determined by ELISA. The 38 cases with PTL and 18 cases with PROM were matched for age with 38 and 18 respective controls delivering at term, and the levels of apoptotic molecules were compared.
Results Cell death nucleosome levels were found to be significantly associated with preterm delivery. Specifically, for every unit increase in nucleosomes, women were on average 0.2% more likely to deliver preterm (OR: 1.002, CI: 1.0–1.003, $p = 0.018$). In contrast, such an association was not found concerning the other two apoptotic molecules (TNF-a and Cytochrome C).
Conclusion Second-trimester amniotic fluid cell death nucleosomes' levels are significantly associated with preterm delivery and could possibly serve as predicting markers.

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Introduction

The etiology of preterm delivery remains still unknown [1]. Several theories have been developed to explain its pathophysiology. Among them subclinical intraamniotic infection activating the cytokine signaling system [2–9], as well as the 'placental clock' theory [10–12] have been suggested. Another pathogenetic mechanism of preterm delivery implicates the pathway of apoptosis [13, 14].

Apoptosis is defined as the process of programmed cell death characterized by cell membrane changes, as well as nuclear and chromosomal DNA fragmentation [15]. Cell signals such as toxins, nitric oxide and cytokines are involved in apoptosis controlling functions (e.g. homeostasis and ischemia). Intracellular signaling is a common response to stress. As preterm labor is considered to result either from inflammation or stress processes, apoptosis could be implicated in PTL pathway. Moreover, programmed cell death of the fetal membranes might explain PROM. In this respect, it has been shown that fetal membranes from term vaginal deliveries present a zone of weakness exhibiting characteristics of apoptosis and remodeling [16]. More specifically, such a weak zone presents either paracervically in cases with artificial rupture of membranes or adjacent to the tear line in cases with spontaneous rupture of membranes showing characteristic remodeling and apoptosis findings including elevated matrix metalloproteinase 9, elevated (ADP-ribose) polymerase cleavage and decreased tissue inhibitor of matrix metalloproteinase 3 [16].

Tumor necrosis factor (TNF-alpha) is a cytokine involved in systemic inflammation and stimulation of acute

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phase reaction. It regulates immune cells and induces apoptotic cell death [17, 18]. TNF-alpha is a product of both maternal and fetal tissues [19–22], and recently it was shown that TNF-alpha and TNF-alpha-308-gene polymorphism could be used as markers for early prediction of PTL and PROM [23, 24].

Cytochrome C is a heme protein of the inner mitochondrial membrane [25]. It controls hydroxylation and aromatic oxidation. It is released by mitochondria—in response to proapoptotic stimuli—and causes an interaction with IP3 receptor of the endoplasmic reticulum, leading to calcium release implicated in the initiation of labor [26].

Cell death nucleosomes are the fundamental repeating units of eukaryotic chromatin [27]. Their function is to allow the binding of basal transcription factors [28, 29]. They modulate eukaryotic gene regulation by affecting the accessibility of other proteins to the DNA, which can impact gene activation and repression [30].

We posed the question whether midtrimester amniotic fluid levels of the above molecules (implicated in the apoptotic pathway) might be elevated in cases complicated with PTL and/or PROM as compared with controls delivering at term. Therefore, we determined their levels at the time point of genetic amniocentesis.

Methods

A prospective matched case control study was performed during a 16-month period (September 2005–December 2006). The study population consisted of 360 consecutive women with singleton pregnancies who presented for genetic amniocentesis in the majority at 16–18 weeks of gestation. Amniocentesis was performed for advanced maternal age, and/or increased risk for aneuploidy during nuchal translucency ultrasound. Exclusion criteria were twin pregnancy, known history of uterine abnormalities, cone biopsy, significant vaginal bleeding and fetal malformations. Cases with pathological results (e.g. pathological karyotype) were not included in the study (10 cases).

Preterm labor and consequent delivery was defined as labor before 37 weeks of gestation with regular uterine contractions (at least two uterine contractions/10 min during 30 min) in combination with characteristic cervical changes [31–33]. Preterm premature rupture of the fetal membranes was defined as the rupture of the amniotic membranes with release of the amniotic fluid more than 1 h before the onset of preterm labor [34]. Gestational age was calculated from the last menstruation and was confirmed during routine ultrasound in the second trimester ([16–19] weeks of gestation).

In this study, the criteria for microbial invasion of the amniotic fluid were positive PCR for *Mycoplasma hominis* and *Chlamydia trachomatis* and/or growth of any bacteria (aerobic or anaerobic) in the amniotic fluid cultures except for coagulase-negative Staphylococcus, which was considered to be a skin contamination. All patients were followed until delivery for the occurrence of pregnancy complications by their doctors. This is the reason why we can provide data regarding only pregnancies with preterm labor or premature rupture of membranes, delivering preterm, but not for pregnancies with preterm labor delivering at term, as our results were related to the time of birth. The ethics committee of our teaching hospital approved the study, and informed consent was acquired from each woman before enrolment. A questionnaire regarding personal and family history was completed, and maternal medical and perinatal data were kept into a database.

Ultrasound-guided transabdominal amniocentesis was performed with a 21-gauge needle under aseptic conditions. The first 0.5 ml of collected amniotic fluid was discarded to avoid maternal contamination. Twenty ml of amniotic fluid was aspirated from each woman (15 ml were used for genetic diagnosis). One ml of the uncentrifuged amniotic fluid was then immediately transported to the microbiological laboratory and was cultured for aerobic and anaerobic bacteria. Another 1 ml of the uncentrifuged amniotic fluid was also tested by polymerase chain reaction (PCR) for *Mycoplasma hominis* and/or *Chlamydia trachomatis* detection. The remaining 3 ml of amniotic fluid was immediately placed in a refrigerator (+4°C) and centrifuged within the next 6 h at 3,000*g* and +4°C for 10 min. The supernatant was stored in polypropylene tubes at −80°C until analysis.

Amniotic fluid TNF-alpha, cytochrome-C, and cell death nucleosomes determined with enzyme-linked immunosorbent assays ELISA by Phoenix Pharmaceuticals INC (Burlingame, California, 94010)]. The amniotic fluid samples ran in duplicates. Laboratory personnel were blinded to the clinical history of the involved women.

Statistical analysis

All data except for chronological age, gestational age at delivery and gestational age at amniocentesis followed normal distribution (Kolmogorov–Smirnov test). Student's *t* test was applied to detect differences between groups where continuous variables were normally distributed. Otherwise, Mann–Whitney *U* test was applied. Pearson's Chi square test was used to detect differences between categorical variables.

Conditional logistic regression analysis was used to examine the possible associations of human TNFa, Cytochrome C and Nucleosomes with preterm labor. Women with a preterm labor were defined as cases ($N = 38$) while for each case a woman matched for age

Table 1 Demographic data for women with preterm labor who delivered before 37 weeks of gestation (cases, $N = 38$) and women at term (controls, $N = 38$)

Groups	Preterm labor and delivery ($N = 38$) mean ± SD/median (range)	Matched control ($N = 38$) mean ± SD/median (range)	p value
Gestational age at delivery (weeks)	35.43 (22–37.0)	38.28 (37–40)	<0.001
Gestational age at amniocentesis (weeks)	17.86 (15.14–25.43)	17.43 (15.86–19.71)	NS
Birth weight (g)	2447.2 ± 590.5	3232.8 ± 308.8	<0.001
Age (years)	37 (27–43)	37 (27–44)	NS
Parity			NS
0 *N* (%)	27 (71.0)	25 (65.8)	
≥1 *N* (%)	11 (29.0)	13 (34.2)	
Smoking before pregnancy *N*(%)	14 (36.8)	12 (31.6)	NS
Smoking during pregnancy *N*(%)	6 (15.8)	9 (23.7)	NS
Alcohol consumption during pregnancy *N*(%)	1 (2.6)	4 (10.5)	NS
Medication	17 (44.7)	21 (55.3)	NS
Mode of delivery			NS
Vaginal	6 (15.8)	15 (39.5)	
Cesarean	32 (84.2)	23 (60.5)	
Gender			
Male	26 (68.4)	14 (36.8)	0.011
Female	12 (31.6)	24 (63.2)	

and parity who delivered at term served as control ($N = 38$). Furthermore, subgroup analyses were conducted in order to examine any possible association of TNF-alpha, Cytochrome C and Nucleosomes in women with preterm labor who delivered before 32 weeks of gestation ($N = 8$). Finally, we evaluated a subgroup of women who had preterm labor and premature rupture of membranes in order to clarify whether TNF-alpha, Cytochrome C and Nucleosomes could be predictors of PROM ($N = 18$ in each group). Results were presented as odds ratios (OR) and 95% confidence intervals (CI). Statistical analysis was performed using SPSS 11.5 edition. A p value of <0.05 was considered statistically significant.

Results

Out of 360 women who were included in the study, 38 had spontaneous preterm delivery (incidence: 10.56%) and of those, 18 delivered preterm with premature rupture of membranes (incidence: 5%). Amniocentesis was performed in the majority of cases due to advanced maternal age (66.5%), whereas other reasons were previous history of fetal malformations or syndromes (13.2%), increased nuchal translucency (2.7%), pathologic results of PAPP-A (5.5%) and suspicious ultrasound findings (4.1%). Out of the initial 360 women, ten were excluded after amniocentesis in the presence of fetal chromosomal abnormalities (two with trisomy 18, two with trisomy 21, one with Turner syndrome, one with Klinefelter syndrome and four others with less common pathological karyotypes). Eight women were lost to follow-up. Four infants were delivered by cesarean section before the onset of labor for maternal (severe preeclampsia) or fetal reasons (compromised fetal growth or umbilical Doppler flow abnormalities). Two women, who delivered within 30 days following amniocentesis, were excluded from the study as their delivery was considered to be related to the procedure of amniocentesis [35–39].

The demographic data of the study population are presented in Tables 1 and 2. No statistical significant differences were found in mean maternal age, gestational age at amniotic fluid sampling, or parity between the groups of women delivering preterm or at term. All preterm and term neonates were appropriate for gestational age. Regarding the results of the microbiological examinations of the patients, amniotic fluid cultures for common bacteria in the participating mothers were negative, while *M. hominis* and *C. trachomatis* were identified in 2/360 and 2/360, respectively. However, one of the two women with Mycoplasma and one of the two women with Chlamydia delivered preterm. No correlation was found between PCR detection of *M. hominis* and *C. trachomatis* with preterm labor.

Levels of TNF-alpha and Cytochrome C were not found to be significantly associated with preterm delivery

Table 2 Demographic data for women with preterm labor and premature rupture of membranes (cases, $N = 18$) and women delivering at term (controls, $N = 18$)

Groups	Preterm labor and premature rupture of membranes ($N = 18$) mean ± SD/median (range)	Matched control ($N = 18$) mean ± SD/median (range)	p value
Gestational age at delivery (weeks)	33.58 ± 4.23	38.59 ± 0.86	<0.001
Gestational age at amniocentesis (weeks)	19.0 ± 2.64	17.85 ± 1.0	NS
Birth weight (g)	2,483.5 ± 669.2	3252.4 ± 353.5	<0.001
Age (years)	34.44 ± 9.4	36.67 ± 3.94	NS
Parity			NS
0 *N* (%)	11 (61.1)	8 (44.4)	
≥1 *N* (%)	7 (38.9)	10 (55.6)	
Smoking before pregnancy *N*(%)	6 (33.3)	6 (33.3)	NS
Smoking during pregnancy *N*(%)	3 (16.7)	4 (22.2)	NS
Alcohol consumption during pregnancy *N*(%)	1 (5.6)	2 (11.1)	NS
Medication	6 (33.3)	9 (50)	NS
Mode of delivery			NS
Vaginal	4 (22.2)	8 (44.4)	
Cesarean	14 (77.8)	10 (55.6)	
Gender			0.038
Male	15 (83.3)	9 (50)	
Female	3 (16.7)	9 (50)	

(Table 3). On the other hand, cell death nucleosomes levels, as well as gender were found to be significant independent predictors for preterm delivery. Specifically, for every unit increase in cell death nucleosomes, women were on average 0.2% more likely to have a preterm delivery (OR: 1.002, CI: 1.0–1.003, $p = 0.018$). Furthermore, women who delivered female neonates were 89.3% less likely to have a preterm delivery as compared with women who delivered males (OR: 0.107, CI: 0.02–0.58, $p = 0.009$).

In a subanalysis, levels of TNF-alpha, Cytochrome C and cell death nucleosomes in women with preterm delivery after premature rupture of membranes were not found to be statistically significant (Table 4). Moreover, in another subanalysis, levels of TNF-a, Cytochrome C and cell death nucleosomes in women delivering before 32 weeks of gestation (8 women) were not found to be significantly associated with preterm delivery. Levels of cell death nucleosomes in women with PTL and in those with PTL and PROM are shown in Figs 1 and 2.

Table 3 TNFalpha, Cytochrome C and Nucleosomes concentrations in cases with preterm labor ($N = 38$) and controls ($N = 38$)

Groups	Preterm labor ($N = 38$) mean ± SD/median (range)	Matched control ($N = 38$) mean ± SD/median (range)
TNF-alpha (pg/ml)	1.36 ± 1.03	1.90 ± 1.07
Cytochrome C (pg/ml)	363.37 (133.25–1850)	366.82 (195.42–966)
Nucleosomes (AU)	574.60 (207.14–3106)	471.36 (78.57–1334.38)

Table 4 Levels of TNF-alpha, Cytochrome C and cell death nucleosomes in cases with preterm delivery after premature rupture of membranes ($N = 18$) and controls ($N = 18$)

Groups	Preterm delivery after premature rupture of membranes ($N = 18$) mean ± SD/median (range)	Matched control ($N = 18$) mean ± SD/median (range)
TNF-a (pg/ml)	1.55 (0.22–182.50)	1.76 (0.67–4.19)
Cytochrome C (pg/ml)	506.76 ± 314.18	381.72 ± 156.37
Nucleosomes (AU)	668.96 ± 532.10	505.26 ± 208.61

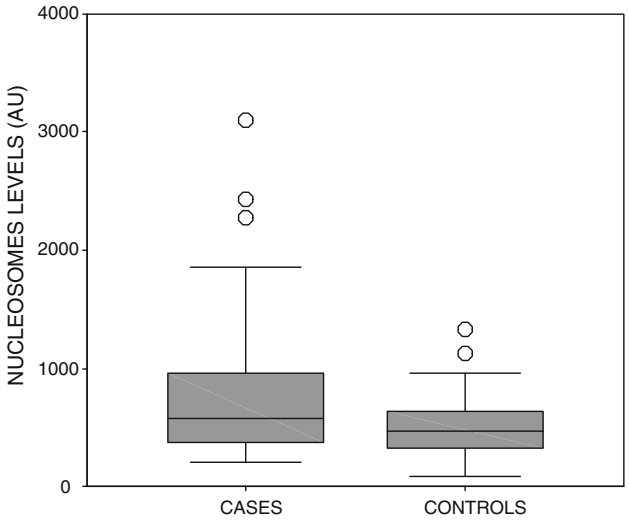

Fig. 1 *Box* and *whiskers* plots of the concentrations of nucleosomes from women with spontaneous preterm delivery who delivered before 37 weeks of gestation (cases) and women with normal (fullterm) pregnancies. Each *box* represents the median concentration with the interquartile range (25th and 75th percentiles)

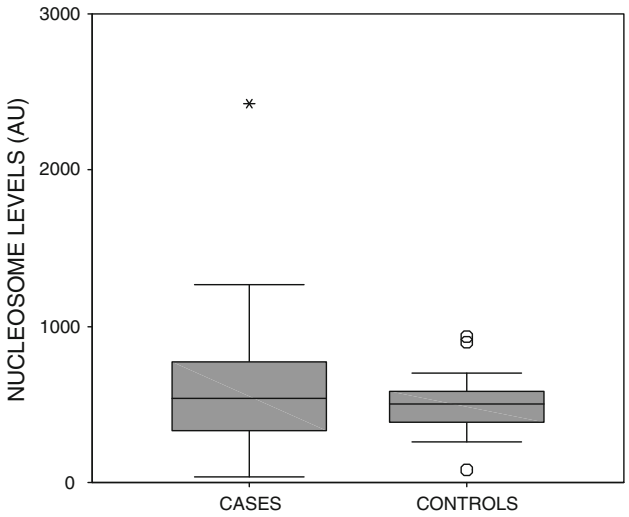

Fig. 2 *Box* and *whiskers* plots of the concentrations of nucleosomes from women with spontaneous preterm delivery who delivered before 37 weeks of gestation (cases) ($N = 18$) and had membrane rupture and women with normal (fullterm) pregnancies ($n = 18$). Each *box* represents the median concentration with the interquartile range (25th and 75th percentiles)

Discussion

The significant impact of preterm delivery to neonatal mortality and morbidity (e.g. blindness, deafness, developmental delay, cerebral palsy and chronic lung disease) urges for its early recognition and treatment; thus, the identification of possible predictors, among which molecules identified in midtrimester amniotic fluid during genetic amniocentesis is of outmost importance [40–42].

The role of apoptosis in the pathogenesis of preterm delivery is still under investigation. Thus, apoptosis is implicated in several mechanisms, regulating myometrial differentiation during pregnancy [43].

More specifically, the presence of the signal docking proteins TNF-alpha receptor-associated death domain, as well as Fas-associated death domain and the induction of caspase cascade by lipopolysaccharide and TNF-alpha, suggest that TNF-alpha-mediated apoptosis may occur in the human fetal membranes [44]. Furthermore, the complex of TNF-alpha-TNF-alpha-receptor-2 has an antiapoptotic but proinflammatory action which activates NFKB, cytokines and prostaglandins production [45]. It also increases proapoptotic p53 levels and caspase activities in fetal membranes [46].

Several authors have investigated the role of TNF-alpha; however, in the majority of the studies TNF-alpha was investigated as a proinflammatory factor leading to preterm labor pathway. Some studies tried to clarify the role of TNF-alpha as an apoptotic molecule; however, the results were contradictory. Romero et al. [20] showed that amniotic fluid from pregnant women in the second trimester, who were not in labor, did not contain TNF-alpha. In contrast, in our study TNF-alpha levels were measurable at the time point of genetic amniocentesis. Maymon et al. [18] proposed that TNF-alpha levels decrease with advancing gestational age. However, other studies have shown elevated amniotic fluid TNF-alpha levels during preterm delivery [18, 20, 47–49]. Our study did not find a correlation between TNF-alpha and either preterm labor and delivery or premature rupture of membranes. The failure to detect association between TNF-alpha and preterm labor and delivery may be due to gene-environment interacting as some SNPs may differ as a function of specific environmental factors [50].

To our knowledge, our study is the first to correlate amniotic fluid cytochrome C levels during genetic amniocentesis with preterm labor and delivery and/or premature rupture of membranes. However, no significant association between these two parameters could be identified. On the other hand, in a previous study soluble cell death nucleosome levels in amniotic fluid of pregnant women with and without intra-amniotic infection were found to be significantly higher in women with intraamniotic infection and could be used as their possible predictors [51]. Again, to our knowledge, our study is the first to correlate amniotic fluid levels of cell death nucleosomes during genetic amniocentesis with occurence of preterm labor and delivery, and a positive correlation was found.

Cytochrome C is released to the systematic circulation from the mitochondria of the apoptotic cells and for this

reason acts as a marker of apoptotic homeostasis in the circulation [52]. On the other hand, nucleosomes are chromatin units that are usually identified in the apoptotic cells and are packed into apoptotic bodies which are engulfed by macrophages leading to their release into the circulation in cases of high rates of cellular turnover and cell death [53, 54]. In our study, elevation of nucleosomes, but not cytochrome C in cases with preterm rupture of membranes might be explained based on the hypothesis that rupture of membranes is an evolutionary process of non mitochondrial type correlated with apoptosis rather than necrosis. It is known that apoptosis could be initiated via an extrinsic pathway that is triggered by engagement of death receptors on the cell surface (better expressed by nucleosomes) or via an intrinsic pathway induced by mitochondrial injury (better expressed by cytochrome C) [55]. Nevertheless, such a correlation was not identified in the preterm labor and delivery group as onset of labor could be correlated with necrotic rather than apoptotic mechanisms. Cytochrome C, as well as nucleosomes, could be used as apoptotic markers in systematic circulation, and for this reason further investigation regarding their blood levels could clarify their role in preterm labor and delivery.

Conclusion

Increased amniotic fluid cell death nucleosome levels during genetic amniocentesis are positively correlated with preterm delivery prediction. Although our study did not find a predictive role of apoptotic molecules such as TNF-alpha and cytochrome C in the second trimester of pregnancy for preterm labor, the possible increase of their levels with advancing gestation cannot be excluded on the basis of a programmed cell death of the membranes. Further studies are needed to elucidate the possible role of TNF-alpha, cytochrome C and cell death nucleosomes as well as other apoptotic molecules in human parturition.

Conflict of interest None.

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