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RESEARCH ARTICLE

Isoprostanes 8-iPF$_{2\alpha}$-III: risk markers of premature rupture of fetal membranes?

Sebastian Kwiatkowski[1], Andrzej Torbé[1], Barbara Dołęgowska[2], Wojciech Błogowski[3], Ryszard Czajka[1], Dariusz Chlubek[2], and Rafał Rzepka[1]

[1]Department of Obstetrics and Gynecology, Pomeranian Medical University, Szczecin, Poland, [2]Department of Biochemistry and Medical Chemistry, Pomeranian Medical University, Szczecin, Poland, and [3]Department of Laboratory Diagnostics and Molecular Medicine, Pomeranian Medical University, Szczecin, Poland

Abstract

Aims: Isoprostanes may serve as sensitive and specific markers of *in vivo* oxidative stress intensity. We wanted to determine, whether or not isoprostane concentration may be considered as a risk marker of premature rupture of fetal membranes (PROM).

Methods: On the basis of the presence of PROM and gestational maturity, a total of 128 patients were divided into: (1) preterm PROM (pPROM) group; (2) PROM at term group; (3) control preterm (C1) group and (4) control at term (C2) group. The concentrations of 8-iPF$_{2\alpha}$-III were determined using the enzyme-linked immunosorbent assay method.

Results: The mean free isoprostane concentrations, examined in amniotic fluid and maternal plasma in the PROM at term patients were significantly higher than in C2 individuals (p <0.01). The mean concentrations of free 8-iPF$_{2\alpha}$-III measured in blood plasma from women in the C1 group were significantly lower than in patients from the pPROM, PROM at term and C2 groups (p <0.001, p <0.00001 and p <0.00001, respectively).

Conclusion: The measurement of free isoprostane concentration in maternal plasma and amniotic fluid may be considered as a laboratory marker of a PROM-risk pregnancy.

Keywords: *Isoprostane; oxidative stress; premature rupture of membranes*

Introduction

The amniotic sac creates an optimal environment, which not only protects the thriving foetus, but also contains amniotic fluid that is indispensable for proper prenatal development. Therefore, rupture of the amniotic sac (rupture of membranes, ROM) disturbs pregnancy homeostasis. Physiologically this situation occurs at the beginning of the second stage of labour.

One of the most common obstetric complications is the premature rupture of membranes (PROM). The incidence of PROM is reported to be between 6 and 10% (McCaul et al. 1997). It refers to a patient, who is beyond 37 weeks' gestation, and has presented with ROM prior to the onset of labour. Moreover, in the medical literature another term is highlighted: the preterm premature rupture of membranes (pPROM), which defines ROM prior to 37 weeks' gestation. When pPROM occurs remote from term, significant risks of morbidity and mortality are present for both the foetus and the mother. According to various studies, pPROM is associated with around 40% of preterm deliveries (Shubert et al. 1992).

The aetiology of PROM is multifactorial. Generally, ROM is a consequence of processes that lead to a reduction of collagen synthesis in the membranes, as well as to an intensified degradation of collagen (Longini et al. 2007). Among several pathogenic factors, the inflammatory process, higher activity of proteolytic enzymes that

Address for Correspondence: Andrzej Torbé, Department of Obstetrics and Gynecology Pomeranian Medical University, Al. Powstancow Wlkp. 72, 70-111 Szczecin, Poland. Tel.: +48 91 466 1350. Fax: +48 91 466 1350. E-mail: torbea@wp.pl

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are 'released' by neutrophils (i.e. metalloproteinases, elastases) (Sibille et al. 1986, Winterbourn & Essman 1990, Panasenko et al. 1995) and cellular apoptosis and oxidative stress should be especially emphasized (Monboisse et al. 1993, Ikekuchi et al. 1991, Kuroda et al. 1995).

Pregnancy is a state in which the intensified generation of free radicals occurs (Little & Gladen 1999). F_2- isoprostanes, chemically stable prostaglandin isomers, are mostly generated in the process of non-enzymatic peroxidation of arachidonic acid (which is associated with cellular membrane phospholipids), and of lipoproteins caused by reactive oxygen species (ROS) (Rotschild et al. 1990). According to several authors (Dolegowska et al. 2007, Bengston et al. 1989, Kilbride et al.1996, Wiswedel et al. 2005), the concentration of these cellular membrane phospholipid and lipoprotein isoprostanes may serve as universal, sensitive and specific markers of *in vivo* oxidative stress intensity. The esterified forms of isoprostanes are 'released' into the circulatory system via phospholipase A_2 (PLA2) and/or platelet-activating factor acetyl hydrolase activity; nevertheless, free forms of isoprostanes demonstrate strong biological activity via prostanoid or similar receptors (Rokach et al. 1997). Interestingly, the fact, that despite acting via the same receptor, isoprostanes may activate pathways other than the prostaglandin signalling pathways. Therefore, their effect on cells can diversify from the physiological activity of receptor ligands (Pratico et al. 1997, Kunapuli et al. 1998, Makino et al. 2002).

Among several F_2-isoprostanes, the 8-iPF$_{2\alpha}$-III isomer is the most intensively generated, and, more interestingly, not only possesses strong vasoconstrictive and mitogenic activity in smooth muscle cells (Rokach et al. 1997, Takahashi et al. 1992, Fukunaga et al. 1993, Romero & Reckelhoff 2000), but also modulates platelet function (Minuz et al. 1998).

The problem of a possible influence of oxidative stress on the induction of preterm labour receives comparatively less attention in the literature. Therefore, in our research we wanted to examine the levels of oxidative stress in term and preterm patients presenting with or without PROM. This comparison enabled us to estimate the role of oxidative stress in PROM induction, and also helped us to obtain novel molecular insights into the pathophysiology of ROM. Moreover, we wanted to determine, whether or not isoprostane concentration may be considered as a clinical marker of a PROM-risk pregnancy.

Materials and methods

Patients

A total of 128 patients, hospitalized between January 2004 and June 2007 in the Department of Obstetrics and Gynecology of Pomeranian Medical University in Szczecin, Poland, were included in the study, after providing written informed consent. The study protocol was approved by the University Bioethical Committee (BN-001/63/04). The patients were divided into two study groups and two control groups on the basis of PROM presence and gestational maturity. PROM was diagnosed when pooling of fluid in the vagina or leakage of fluid from the cervix was observed during speculum vaginal examination of the cervix and vaginal cavity. Depending on the gestational maturity, the patients were qualified as preterm or at term. All patients were non-smokers, well-fed and generally healthy. The general characteristics of the four examined groups are summarized in Table 1.

Thirty women between 24 and 36 gestational weeks, in whom PROM was diagnosed, qualified for the pPROM group. The mean age of patients was 29.07 years (17–44), and the mean gestational week was 31.57 (24–36). The inclusion criteria in this group were: leakage of clear amniotic fluid for not longer than 6 h, gestational age between 24 and 36 weeks, and lack of hysterospasm activity confirmed by cardiotocography. Before collection of maternal blood and amniotic fluid for examination, no antibiotics, steroids or tocolytics were administered.

The second study group (PROM at term) comprised 38 term patients, in whom PROM was also diagnosed. The mean age of the examined women was 28.97 years (18–40) and the mean gestational age at the moment of PROM was 38.63 (38–41) weeks. The inclusion criteria were the same as for the pPROM group with the exception that in this group the gestational age was between 38 and 42 weeks.

Table 1. General characteristics of women in the study and control groups (means ± SD).

Parameters	pPROM	PROM at term	Control 1	Control 2
Number of women	30	38	30	30
Age (years)	29.07 ± 6.32	28.97 ± 5.86	27.29 ± 6.22	29.07 ± 4.41
Pregnancy	5 ± 1.81	1.74 ± 1.18	1.66 ± 0.97	2.03 ± 1.10
Gestational age (weeks)	31.57 ± 2.96*	38.63 ± 1.13	29.24 ± 3.23*	39.67 ± 0.71

pPROM, preterm premature rupture of membranes; PROM, premature rupture of membranes; *p <0.00001, level of significance for difference between: mean pPROM compared with PROM at term; mean Control 1 compared with Control 2; and mean Control 1 compared with pPROM.

In the third group (C1), 30 preterm healthy patients (between 24 and 36 gestational weeks) without a diagnosis of PROM were included. The mean age of the patients was 27.29 years (20–39). In these patients blood samples were collected during routine ambulatory visits.

Thirty term patients (between 38 and 42 gestational weeks), in whom a caesarean section before labour initiation was performed, were assigned to the C2 group. In this group the amniotic sac was punctured with a needle through the uterine wall, and then the fluid was drawn.

Clinical symptoms of infection (maternal fever and tachycardia, foetal tachycardia), presence of hysterospasm activity, multiple pregnancy, foetal abnormalities, and pregnancy associated diseases such as hypertension, diabetes, cholestasis, as well as, renal diseases were considered as excluding criteria.

Examinations

Peripheral maternal blood

Peripheral maternal blood, collected from the ulnaris vein, was treated with dipotassium ethylenediaminetetraacetic acid (EDTA-K_2). The haematological parameters were measured in whole blood. After centrifugation of the whole blood (10 min; 5000 rpm) plasma C-reactive protein levels were determined. Afterwards, antioxidant 0.05% butylated hydroxytoluene (BHT; Sigma, St Louis, MO, USA) was added, and plasma samples were stored at –80°C until the isoprostane analysis was performed.

Amniotic fluid

The amniotic fluid (8– 10 ml) was collected during speculum vaginal examination of the cervix and vaginal cavity when PROM was diagnosed (pPROM and PROM at term groups), or during intraoperative amniopunction (C2 group). The amniotic fluid samples, were treated with EDTA-K_2, and antioxidant 0.05% BHT was added. Plasma samples were stored at –80°C until the isoprostane analysis was performed.

Umbilical blood

The umbilical blood samples from patients assigned to the pPROM, PROM at term and C2 groups, were collected from the umbilical vein directly after labour, and were treated with anticoagulant (EDTA-K_2). Afterwards, antioxidant 0.05% BHT was added to the plasma and samples were stored at –80°C until the isoprostane analysis was performed.

Methods

Amniotic fluid, maternal and cord blood plasma free 8-iP$F_{2\alpha}$-III isoprostane measurements

Samples of 0.5 ml of peripheral maternal blood, cord blood and amniotic fluid were mixed with 1 ml of cold (4°C) 100% acetonitrile (Sigma). The mixture was thoroughly vortexed for 3 min, cooled down to –20°C and centrifuged (3824*g*, 10 min, 4°C). The supernatant was then transferred to another test tube and 1 mM HCl and 1 M HCL was added in order to acidify to pH 3. Next, the samples were purified and condensed in Bakerbond spe™ RP-18 columns (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA). The eluate was dried under the nitrogen stream and stored at -80°C until the assays were performed. The concentrations of isoprostanes (8-iP$F_{2\alpha}$-III) were determined using the enzyme-linked immunosorbent assay (ELISA) method with the use of the reagent set BIOXYTECH® 8-Isoprostane Assay Kit (Oxis Research, Portland, OR, USA) (Morrow et al. 1992, 1999, Morrow & Roberts 1997, Roberts & Morrow 2000, Wang et al. 1995)

Total 8-iP$F_{2\alpha}$-III isoprostane measurements in amniotic fluid, peripheral blood and cord blood plasma

Samples of 0.5 ml of amniotic fluid, peripheral blood and cord blood plasma were extracted using Folch mixture (chloroform:methanol, 1:2; v/v). The received extracts were then purified (0.043% liquid $MgCl_2$ solution) and centrifuged (3824*g*, 10 min, 4°C). When the supernatant had separated, the lower organic layer was transferred to another test tube and dried under the nitrogen stream. The dried extracts were then dissolved in methanol and hydrolysed using 15% KOH (37°C; 30 min). Next, the samples were purified and condensed in Bakerbond spe™ RP-18 columns. The eluate was dried under the nitrogen stream and stored at -80°C until the assays were performed. The concentrations of isoprostane (8-iP$F_{2\alpha}$- III) were determined using the ELISA method with the use of the reagent set BIOXYTECH® 8-Isoprostane Assay Kit (Morrow et al. 1992, 1999, Morrow & Roberts 1997, Roberts & Morrow 2000, Wang et al. 1995).

Esterified 8-iP$F_{2\alpha}$-III isoprostane measurements in amniotic fluid, peripheral blood and cord blood plasma

Esterified isoprostane concentrations in amniotic fluid, and maternal and cord blood plasma were calculated as the difference between the total and free isoprostane concentrations.

Statistical analyses

The assessment of normality of distribution of continuous variables (Shapiro–Wilk test) was conducted and showed non-normal distributions of parameters. Each parameter tested was characterized with the use of the arithmetic mean, median and standard deviation. To assess the differences between the parameters tested, the Mann–Whitney test for unpaired variables (to compare the values between groups) and Wilcoxon's test

for paired variables (to compare the values between compartments) were used. The strength of correlation between the parameters was measured with the use of the Spearman's rank correlation coefficient (*R*s). The results were processed statistically with the use of STATISTICA PL v. 7.1 (Statsoft, Kraków, Poland). The *p*-value was considered significant at a level lower than 0.05 ($p < 0.05$). Eighty per cent statistical power for significance minimal detectable differences between any two groups were: (1) 0.1 ng l^{-1} free or esterified 8-iPF$_{2\alpha}$- III, and 0.1 free/total (F/T) ratio value in amniotic fluid, (2) 0.2 ng l^{-1} free, 0.7 ng l^{-1} esterified 8-iPF$_{2\alpha}$- III and 0.1 F/T ratio value in maternal and umbilical cord plasma.

Results

The concentrations of free and esterified forms of 8-iPF$_{2\alpha}$- III, and mean F/T coefficient values measured in maternal plasma, amniotic fluid and cord blood plasma are presented in Tables 2–4 and Figures 1–3. The mean free isoprostane concentrations, examined in amniotic fluid and maternal plasma in PROM patients were significantly higher than in C2 individuals ($p < 0.01$). Moreover, the mean levels of free 8-iPF$_2\alpha$-III measured in blood plasma from C1 women, were significantly lower compared with those from patients in the pPROM, PROM at term and C2 groups ($p < 0.001$, $p < 0.00001$ and $p < 0.00001$, respectively).

The statistical analysis of mean concentrations of esterified isoprostanes in all types of examined fluids revealed no significant differences between the groups.

The mean values of the F/T coefficients found in maternal plasma from C2 patients were significantly lower than those from the PROM at term or pPROM groups ($p < 0.01$, $p < 0.05$, respectively). However, the mean F/T values found in maternal plasma from pPROM women were significantly lower than in C1 individuals ($p < 0.00001$). In PROM and C1 patients significantly higher F/T values were observed than in women from the pPROM and C2 groups ($p < 0.00001$).

Table 2. Maternal plasma 8-iPF$_2\alpha$-III concentrations in the study and control groups.

8-iPF$_{2\alpha}$-III (ng l^{-1})	pPROM	PROM at term	Control 1 (C1)	Control 2 (C2)
Free (F)				
Mean ± SD	0.12 ± 0.12*	0.16 ± 0.40†	0.01 ± 0.01‡	0.04 ± 0.02^^
Median (IQR)	0.07 (0.21)	0.07 (0.08)	0.01 (0.01)	0.03 (0.03)
Esterified (E)				
Mean ± SD	0.64 ± 0.73	0.91 ± 1.06	0.69 ± 0.51	0.57 ± 0.41
Median (IQR)	0.46 (0.66)	0.55 (0.89)	0.56 (0.54)	0.47 (0.52)
F/T ratio				
Mean ± SD	0.19 ± 0.16**¶	0.13 ± 0.12$^{\#\S}$	0.02 ± 0.01¶	0.07 ± 0.04***†
Median (IQR)	0.15 (0.19)	0.09 (0.12)	0.02 (0.02)	0.06 (0.05)

pPROM, preterm premature rupture of membranes; PROM, premature rupture of membranes; F/T ratio, free/free+esterified ; IQR, interquartile range.

*p <0.001; and **p <0.00001, level of significance for difference between mean pPROM compared with C1 and C2; ***p <0.05, level of significance for difference between mean pPROM compared with C2; †p <0.01, level of significance for difference between mean PROM at term compared with C2; ‡p <0.00001, level of significance for difference between mean PROM at term compared with C1; §p < 0.05, level of significance for difference between mean PROM at term compared with C2; ¶p <0.00001, level of significance for difference between mean C1 compared with C2 and mean pPROM compared with PROM at term.

Table 3. Amniotic fluid 8-iPF$_2\alpha$-III concentrations in the study and control groups.

8-iPF$_{2\alpha}$-III (ng $l^{-1)}$	pPROM	PROM at term	Control 1 (C1)	Control 2 (C2)
Free (F)				
Mean ± SD	0.11 ± 0.12	0.13 ± 0.15$^{\#\#}$	NA	0.05 ± 0.03
Median (IQR)	0.07 (013)	0.07 (0.06)	NA	0.04 (0.05)
Esterified (E)				
Mean ± SD	0.12 ± 0.13	0.11 ± 0.12	NA	0.10 ± 0.14
Median	0.09 (0.12)	0.06 (0.07)	NA	0.06 (0.08)
F/T ratio				
Mean ± SD	0.48 ± 0.11***	0.51 ± 0.11†	NA	0.40 ± 0.15
Median	0.50 (0.09)	0.49 (0.15)	NA	0.40 (0.29)

pPROM, preterm premature rupture of membranes; PROM, premature rupture of membranes; NA, not assayed; F/T ratio, free/free+esterified; IQR, interquartile range.

***p <0.05, level of significance for difference between mean pPROM compared with C2; $^{\dagger}p$ <0.01, level of significance for difference between mean PROM at term compared with C2.

Table 4. Umbilical cord plasma 8-iPF$_{2\alpha}$-III concentrations in the study and control groups.

8-iPF$_{2\alpha}$-III (ng l^{-1})	pPROM	PROM at term	Control 1 (C1)	Control 2 (C2)
Free (F)				
Mean ± SD	0.08 ± 0.07	0.20 ± 0.40	NA	0.06 ± 0.03
Median (IQR)	0.06 (0.05)	0.07 (0.06)	NA	0.06 (0.04)
Esterified (E)				
Mean ± SD	0.55 ± 0.80	0.71 ± 1.23	NA	0.39 ± 0.23
Median (IQR)	0.31 (0.50)	0.32 (0.30)	NA	0.36 (0.36)
F/T ratio				
Mean ± SD	0.20 ± 0.13***	0.21 ± 0.12†	NA	0.14 ± 0.09
Median (IR)	0.19 (0.20)	0.18 (0.13)	NA	0.13 (0.29)

pPROM, preterm premature rupture of membranes; PROM, premature rupture of membranes; NA, not assayed; F/T ratio, free/free+esterified; IQR, interquartile range.

***p <0.05, level of significance for difference between mean pPROM compared with C2; $^{\dagger}p < 0.05$, level of significance for difference between mean PROM at term compared with C2;

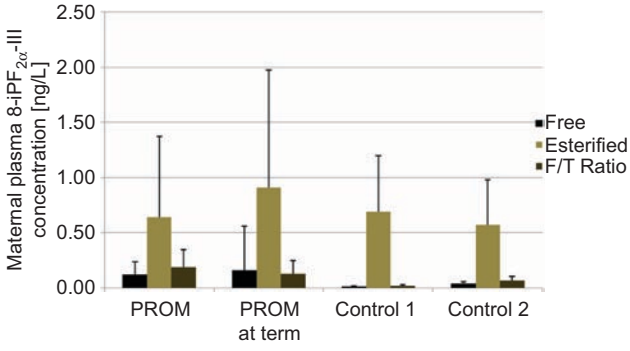

Figure 1. Maternal plasma 8-iPF$_{2\alpha}$-III concentrations in the study and control groups. PROM, premature rupture of membranes.

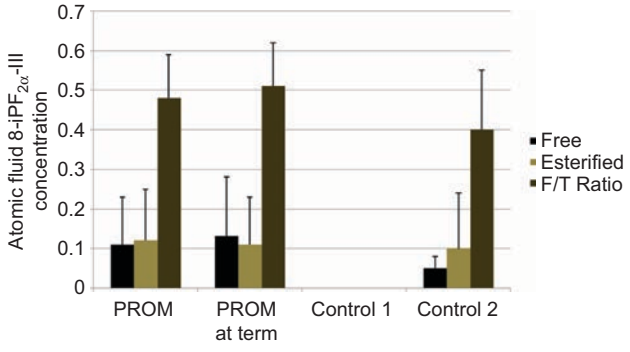

Figure 2. Amniotic fluid 8-iPF$_{2\alpha}$-III concentrations in the study and control groups. PROM, premature rupture of membranes.

The statistical differences between mean concentrations of examined parameters in various types of the biological samples are presented in Table 5. In the pPROM, PROM at term and C2 groups mean esterified isoprostane concentrations, as well as the mean F/T coefficient values found in the amniotic fluid, significantly differed from those measured in maternal and cord blood plasma. In the PROM at term and C2 groups the comparison of mean esterified isoprostane concentrations and F/T values measured in maternal plasma revealed significant differences in relation to the concentrations found in cord blood plasma.

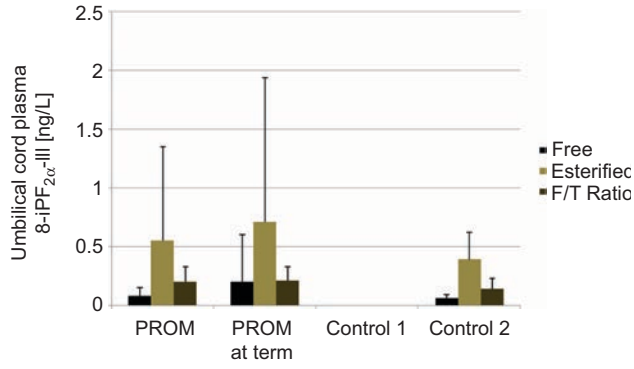

Figure 3. Umbilical cord plasma 8-iPF$_{2\alpha}$-III concentrations in the study and control groups. PROM, premature rupture of membranes.

In the pPROM group a significant negative correlation between mean maternal plasma F/T values and gestational age was found (r = -0.59; p = 0.001). Moreover, a significant positive correlation between plasma free isoprostane levels and gestational age in C2 women was observed (r = 0.40; p = 0.05).

Discussion

The fact that pregnancy is always accompanied by intensified oxidative stress is supported by various researchers, who diagnosed elevated levels of isoprostanes in pregnant women in relation to non-pregnant individuals (Basu 2004). In our research we did not observe any significant differences in esterified isoprostane concentrations in all types of the examined biological samples. However, we observed significant differences in free isoprostane concentrations, mainly in maternal plasma.

Esterified isoprostanes are a source of free isoprostanes that possess strong and varied biological activity. Significant differences in maternal and cord blood plasma esterified isoprostane concentrations were observed only in patients who carried the foetus to term, independently

Table 5. Comparison of 8-iPF$_{2\alpha}$-III [ng/L] concentrations between various samples from the study groups.

	pPROM	PROM at term	Control 2
Maternal plasma/ amniotic fluid			
Free 8-iPF$_{2\alpha}$-III	NS	NS	NS
Esterified 8-iPF$_{2\alpha}$-III	0.0001	0.000001	0.00001
F/T ratio	0.0004	0.000001	0.0002
Maternal plasma/umbilical plasma			
Free 8-iPF$_{2\alpha}$-III	NS	NS	NS
Esterified F$_{2\alpha}$-III	NS	0.03	0.03
F/T ratio	NS	0.01	0.001
Amniotic fluid/ umbilical plasma			
Free 8-iPF$_{2\alpha}$-III	NS	NS	NS
Esterified 8-iPF$_{2\alpha}$-III	0.0002	0.000001	0.0001
F/T ratio	0.00003	0.00001	0.0001

pPROM, preterm premature rupture of membranes; PROM, premature rupture of membranes; F/T ratio, free/free+esterified; NS, not significant.

of PROM development. Esterified isoprostanes are connected to cellular membranes and lipoproteins, and their local concentration depends on the local oxidative stress intensity, the amount of substrates for ROS (phospholipids), and on the eventual increase of the physiological barrier permeability. Large lipoproteins or cells normally do not penetrate through existing barriers, and this may explain the differences between isoprostane concentrations in various examined samples. Barden et al. (1996) examined isoprostane levels in women with pre-eclampsia. They found significant differences in free isoprostane concentrations between hypertensive and normotensive women; however, no significant differences were observed when a comparison of total isoprostane levels (which were a sum of free and esterified forms) was performed.

In all our groups we did not observe any significant differences between examined samples in relation to free isoprostane concentrations. Significantly elevated maternal plasma free isoprostane levels were observed in patients in whom ROM was diagnosed. Interestingly, no significant differences between pPROM and PROM at term patients in relation to maternal plasma free isoprostane concentrations were determined; however, such alterations were observed in non-PROM patients, when a comparison of pregnancy maturity was analysed. The levels of free isoprostanes found in amniotic fluid of pPROM and PROM at term patients were significantly higher than in non-PROM individuals. In our research no statistically significant differences between free isoprostane concentrations in cord blood plasma were found and these results confirm those obtained by Comporti et al. (2004).

Isoprostanes are 'released' from cellular membrane phospholipids and from lipoproteins mostly via phospholipase A_2 activity (Morrow et al. 1992). An increase in plasma PLA2 concentration is observed in both acute and chronic inflammation (Kudo & Murakami 2002). Factors that accelerate PLA2 activity and generation are interleukin-1β, tumour necrosis factor-α and amphoterin protein, which is synthesized in apoptotic cells (Hansen et al. 1999, Jaulmes et al. 2006, Brant & Caruso 2006). Many authors highlight the elevated percentage of apoptotic cells as a key factor that may explain the pathomechanism of PROM (Sagol et al. 2002, Kataoka et al. 2002, Menon et al. 2001).

Oxidative stress may directly cause an increase in PLA2 concentration (Staff et al. 2003), and this process may be molecularly modulated via nuclear factor-κB, the synthesis of which is induced by ROS (Blackwell & Christman 1997, Allen & Tresini 2000, Tak & Firestein 2001). An intensified PLA2 activity is responsible for a significant increase in both maternal plasma and amniotic fluid free isoprostane concentration in patients with PROM. Staff et al. analysed PLA2 activity and isoprostanes concentration in pre-eclamptic decidual tissue and demonstrated a significant correlation between phospholipase activity and isoprostane level (Staff et al. 2003).

In our research F/T coefficient values may indirectly indicate PLA2 activity. In amniotic fluid and in cord blood plasma, F/T values in pPROM and PROM patients were significantly higher than in the control groups. The mean F/T values in maternal plasma of the non-PROM preterm group were significantly higher than in pPROM patients; however, the comparison of the non-PROM and PROM term groups revealed significantly lower F/T values in non-PROM women.

In summary, free isoprostane and F/T coefficient values based on amniotic fluid and cord blood analysis may be considered as a laboratory marker of PROM development that is independent of gestational maturity. Moreover, the relatively strong negative correlation between gestational age and mean F/T coefficient values in pPROM patients suggests that plasma free isoprostane concentrations and F/T values estimated on the basis of maternal plasma examination indicate PROM-risk pregnancy.

In conclusion, measurement of free isoprostane concentration in maternal plasma may be considered as a laboratory marker of PROM-risk pregnancy.

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***Declaration of interest*:** There is no conflict of interest. The current study has not previously been published elsewhere.

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