

## Interleukin 18 messenger RNA and proIL-18 protein expression in chorioamniotic membranes from pregnant women with preterm prelabor rupture of membranes

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### ABSTRACT

**Objective:** To quantify the expression of IL-18 mRNA and protein in the chorioamniotic membranes of pregnant women with PPRM and correlate expression with histological chorioamnionitis.

**Study design:** A case control study that included 42 pregnant women not in labor in the following groups: PPRM ( $n = 28$ ) and controls with intact membranes submitted to selective cesarean section at term ( $n = 14$ ). Expression of IL-18 mRNA in chorioamniotic membranes was determined by real-time polymerase chain reaction, and IL-18 protein expression was measured by western blot. Histopathological analyses and immunolocalization of IL-18 by immunohistochemistry were also performed. Analyses were performed using the Mann–Whitney or Fisher's exact tests and the group effect was considered significant if the adjusted  $p$ -values were  $<0.05$  and the magnitude of change was greater than 2-fold for mRNA expression.

**Results:** IL-18 mRNA was present in 100% of samples and no difference in expression was observed between term vs. PPRM membranes (fold-change 0.12;  $p = 0.88$ ). In the PPRM group, no difference was observed in IL-18 mRNA regarding gestational age (fold-change 0.11;  $p = 0.42$ ) or the presence of histological chorioamnionitis (fold-change 0.26;  $p = 0.15$ ). ProIL-18 was present in all samples. IL-18 was immunolocalized to amnion, chorion and decidua cells, with intense immunohistochemical staining at the choriodecidual junction.

**Conclusion:** Chorioamniotic membranes are sources of IL-18 mRNA and proIL-18, and their expression is unrelated to PPRM or histological chorioamnionitis.

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### 1. Introduction

Rupture of membranes (ROM) is a physiological process occurring during labor. When it occurs spontaneously before 37 weeks of gestation in the absence of labor, it is referred to as preterm prelabor rupture of membranes (PPROM) [1], and is one of the major causes of prematurity [2]. Prelabor rupture can result from various mechanisms, including bacterial invasion of the amniotic cavity and histological chorioamnionitis [3], physiological membrane weakening, diminished repair potential and in utero stretch forces with altered physical properties, which induce transcription and activation of proteins that promote apoptosis

and remodeling of the chorioamniotic membranes [4]. Recent studies have demonstrated that fetal membranes from women with PPRM presented a higher apoptotic index [5] and that an increase in apoptosis occurs in the presence of histological chorioamnionitis compared with its absence in PPRM [6] and term [7] membranes.

The two major pathways that trigger apoptosis in fetal membranes during PPRM are p53 and Fas-FasL/TNF-TNFR p55 (TNFR1) mediated pathways [8–10]. These activators can incite inflammatory changes, with fetal membrane degradation and weakening [11]. Moreover, apoptosis can also promote matrix degrading enzyme activation, promoting membrane degradation and rupture [8]. Both mechanisms result in the activation of nuclear factor (NF)- $\kappa$ B, a transcription factor that is involved in the synthesis and regulation of proinflammatory cytokines and prostaglandins [12–14].

IL-18 is a novel multifunctional cytokine that paradoxically participates in the regulation of both Type 1 and Type 2 helper T cell (Th1 and Th2) responses and increases both innate and

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acquired immunity [15,16]. In driving the Th1 response, IL-18 appears to act in association with IL-12 in inducing IFN- $\gamma$  [17,18]. Moreover, IL-18 has the ability to induce the synthesis of TNF- $\alpha$ , IL-1 $\beta$ , IL-8, and other cytokines, as well as to activate effector cells and molecules, like NF- $\kappa$ B [19]. IL-18 shares structural similarities with IL-1 $\beta$  and is produced as a 24-kD inactive precursor lacking a signal peptide (proIL-18) [20,21]. This form of IL-18 requires cleavage to an active cytokine by the IL-1 $\beta$ -converting enzyme (caspase-1), an intracellular cysteine protease [22,23].

IL-18 is expressed by human gestational tissues [24,25], but studies of IL-18 in relation to its role in pregnancy have been inconclusive. Some investigations showed an increase in serum and amniotic fluid (AF) IL-18 levels throughout pregnancy and during labor [26,27]. Ekelund et al. [28] reported that serum from women delivering before 34 weeks' gestation showed significantly lower levels of IL-18 compared with women delivering after 34 weeks. Conversely, Daskalakis et al. [29] demonstrated recently that AF from preterm deliveries contained significantly higher concentrations of IL-18 compared with term pregnancies. In noninfected women with PPRM, higher AF levels of IL-18 have been detected compared with women who underwent preterm labor with intact membranes or term delivery [25].

Considering that proinflammatory cytokine-induced collagen remodeling and apoptosis correlate directly with fetal membrane physical weakness [11] and that IL-18 has proinflammatory properties, the objective of the present study was to investigate the expression of IL-18 mRNA and protein in chorioamniotic membranes in relation to PPRM and chorioamnionitis.

## 2. Materials and methods

This was a case control study designed to evaluate the expression of IL-18 in chorioamniotic membranes from pregnant women with PPRM. All the patients studied were admitted to the Obstetrics Unit of the Hospital of Botucatu Medical School, located in the central region of the State of São Paulo, Brazil, in 2008. From a total of 1560 deliveries, 96 (6.2%) presented PPRM. Incomplete labor and delivery information, difficulties in sample collection and exclusion criteria resulted in the inclusion of 28 women with PPRM without labor and 14 control pregnant women at >37 weeks of gestation. All these women were scheduled for an elective cesarean section for the following indications: previous cesarean sections or breech presentation. None of the women at term were in labor or presented with rupture of membranes prior to surgery. Women with multiple pregnancies, preeclampsia, diabetes, fetal anomalies and placenta previa were excluded. Gestational age was calculated from the first day of the last menstruation and/or from the first trimester ultrasound.

PROM was diagnosed by sterile speculum examination confirming pooling of amniotic fluid in the vagina, positive nitrazine paper test, positive fern test and fetal cells in cervicovaginal secretion, and was defined according to the Brazilian Ministry of Health guidelines, available at: <http://portal.saude.gov.br/portal/arquivos/pdf/Manual%20Puerperio%202006.pdf>. The Human Research Ethics Committee of the institution approved the study and written informed consent was obtained from all participants.

Chorioamniotic membrane samples were immediately obtained after placental delivery and stored in RNA later<sup>®</sup> solution at  $-80^{\circ}\text{C}$  until RNA extraction and IL-18 expression analysis. Additional samples were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological evaluation and IL-18 and caspase-1 immunolocalization by immunohistochemistry. Other chorioamniotic samples were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until protein expression analysis.

Total RNA was extracted from tissue samples stored in RNA later using a commercial RNA isolation kit (Illustra RNAspin Mini Isolation Kit (GE Healthcare)). A total of 1.0  $\mu\text{g}$  of RNA from each sample were reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), in accordance with the manufacturer's recommendations. The qPCR reactions were assembled based on the TaqMan Universal PCR Master Mix protocol (Applied Biosystems), using GAPDH for quantification of the housekeeping gene and the TaqMan gene expression assay (Hs 00155517\_m1) for IL-18 mRNA. Data were collected by the ABI Prism 7300 Sequence Detection System (Applied Biosystems). The results are expressed as the ratio of IL-18 to GAPDH expression and the threshold cycle number (CT) was used to determine relative IL-18 mRNA expression, using the ddCT method [30]. A mix from some studied samples was used as a calibrator on all plates to account for eventual plate effects. Relative mRNA expression for IL-18 is given as relative folds, increased or decreased, between the studied groups.

For immunohistochemical analysis, eight micron-thick sections from formalin-fixed, paraffin-embedded tissue blocks were mounted on slides, dried at  $37^{\circ}\text{C}$  for 24 h and subjected to deparaffinization followed by antigen-retrieval by boiling in Trilogy<sup>™</sup> solution (Cell Marque, Hot Springs, AR) for 30 min. Sections were then immediately transferred to fresh hot Trilogy<sup>™</sup> solution in a second staining dish for 5 min and washed in PBS buffer (Biocare Medical, Concord, CA, USA). Before the primary antibodies for IL-18 [mouse monoclonal anti-human IL-18 (1:12,000) (MBL, Naka-ku, Nagoya, Japan)] or caspase-1 [mouse monoclonal anti-human caspase-1 (1:1200) (ab54932, Abcam Inc., Cambridge, MA, USA)] were applied, endogenous peroxidase activity was quenched with Peroxidized 1 (Biocare Medical) for 10 min at room temperature and sections were washed again in PBS buffer. The primary antibodies were applied for 3 h at room temperature. Staining was performed using the MACH 4 universal detection system (Biocare Medical), in accordance with the manufacturer's recommendations, and was visualized with DAB (3,3'-diaminobenzidine tetrahydrochloride) (Biocare Medical). Sections were counterstained with Harris hematoxylin. Negative controls were performed for each specimen, in which sections were incubated without the primary monoclonal antibodies. Sections from normal human prostate and pancreatic tissue were obtained from the Department of Pathology Archives at the Botucatu Medical School and applied as positive and negative controls for caspase-1 expression [31,32], respectively.

Snap frozen chorioamniotic membranes samples were lysed in a lysis buffer [PBS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and protease inhibitor (10  $\mu\text{L}/\text{mL}$ , Amersham<sup>®</sup>)]. The insoluble material was then removed by centrifugation at  $1500 \times g$  for 20 min at  $4^{\circ}\text{C}$ . The concentration of protein in each cell lysate was determined using a BCA protein assay kit (Bio-Rad Laboratories, Inc.) with bovine serum albumin (BSA) as the standard. An identical amount of protein (30  $\mu\text{g}$ ) from each sample was loaded onto a 10% SDS-PAGE gel and electrophoresed at 200 V. The resolved proteins were transferred to an Hybond-P membrane (Amersham Biosciences, GE Healthcare Ltd., Chalfont St. Giles, Bucks., UK) using a wet transfer apparatus (Bio-Rad Laboratories<sup>®</sup>). The membranes were blocked in Tris-Buffered Saline (TBS) containing 0.1% Tween 20 (TBS-T) and 5% skim milk for 1 h at room temperature. Blots were incubated with anti-IL-18 [mouse monoclonal anti-human IL-18 (1/1000 dilution) (MBL, Naka-ku, Nagoya, Japan)] and anti- $\beta$ -actin specific primary antibody [(1:6000) mouse monoclonal anti-human beta-actin (1/6000 dilution) (ab6276, Abcam Inc., Cambridge, MA, USA)] at  $4^{\circ}\text{C}$  and shaken overnight. Blots were washed three times with TBS-T and then incubated with peroxidase-conjugated goat anti-mouse IgG secondary antibody [(1/5000 dilution) SAB – Signalway

**Table 1**  
Demographic and obstetrics characteristics of study subjects.

	Term (n=14)	PPROM (n=28)	p
Maternal age (years) <sup>a</sup>	30 (27–37)	23.5 (19–29.5)	0.007
Ethnicity <sup>b</sup>			
White	11 (78.6)	26 (92.8)	0.31
Non-white	3 (21.4)	2 (7.2)	
Smoking habits <sup>b</sup>			
Yes	2 (14.3)	2 (7.2)	0.59
No	12 (85.7)	26 (92.8)	
Gestational age at delivery (weeks, days) <sup>a</sup>	38w4d (38w to 38w6d)	34w3d (33w to 35w4d)	<0.0001
Gravida <sup>b</sup>			
1	1 (7.1)	17 (60.7)	<0.001
>1	13 (92.9)	11 (39.3)	
Histological chorioamnionitis			
Yes	–	12 (42.8)	0.003
No	14 (100.0)	16 (57.2)	
Prior adverse gestational outcomes <sup>b</sup>			
Yes	7/13 (53.8)	5/11 (45.5)	1.000
No	6/13 (46.2)	6/11 (54.5)	

<sup>a</sup> Median (interquartile range); Mann–Whitney test.

<sup>b</sup> n(%); Fisher's exact test.

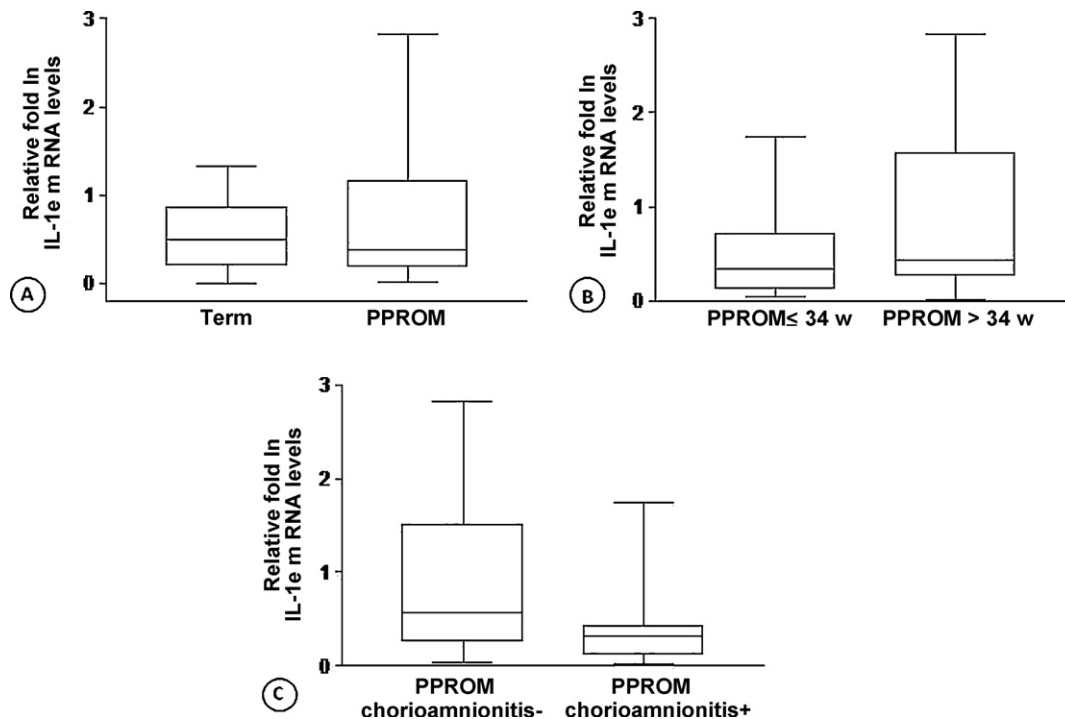
Antibody] for 1 h at room temperature. All blots were developed using chemiluminescence reagents ECL Plus Western Blotting Detection System (Amersham™), in accordance with the manufacturer's recommendations. The signals were captured by the Alpha Innotech FluorChem® FC2 Imager.

Statistical analyses were performed with SigmaStat Software version 3.1. After checking for data normality, maternal age, gestational age at delivery and IL-18 mRNA concentrations were compared between the groups studied using the Mann Whitney test. Ethnicity, smoking habits, parity, previous pregnancy complications and histological chorioamnionitis were compared using Fisher's exact test. A *p* value <0.05 was considered to be statistically significant.

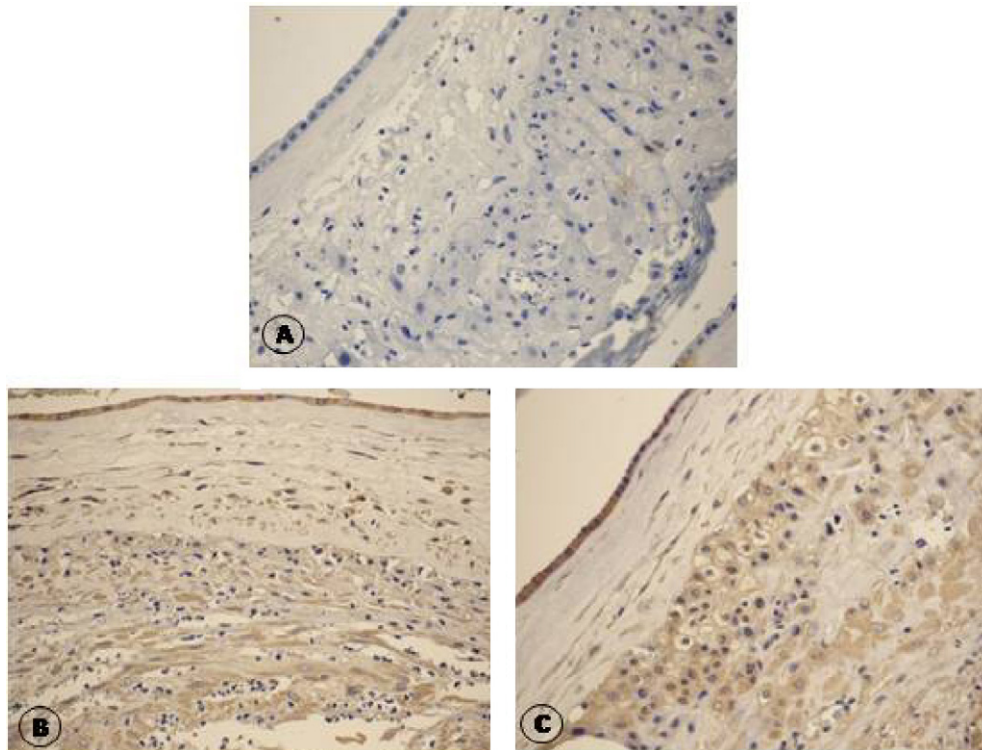
### 3. Results

The sociodemographic and clinical characteristics of the study population are shown in Table 1. Maternal age was lower in the PPRM group compared with term women. Gravida and the presence of histological chorioamnionitis were statistically different between the groups studied, while ethnicity, smoking habits and previous pregnancy complications were similar.

IL-18 mRNA was present in 100% of the samples, demonstrating constitutive gene expression in chorioamniotic membranes. No statistical difference in the relative fold expression of IL-18 was observed in the PPRM group vs. the term group (Fig. 1A). A similar finding was observed regarding gestational age in the PPRM



**Fig. 1.** Box plot representing IL-18 mRNA expression in chorioamniotic membranes, analyzed by real time PCR. (A) Comparison between term pregnancies vs. PPRM group (fold-change 0.12; *p* = 0.88). (B) IL-18 mRNA in relation to gestational age (<34 weeks of gestation and >34 weeks of gestation) in the PPRM group (fold-change 0.11; *p* = 0.42). (C) IL-18 mRNA in relation to histological chorioamnionitis in the PPRM group (fold-change 0.26; *p* = 0.15). Mann–Whitney test.



**Fig. 2.** Immunohistochemical detection of interleukin (IL)-18 in chorioamniotic membranes from the PPROM group. Negative control, sections were incubated without monoclonal anti-human IL-18 (A). Sections incubated with anti-human IL-18 in the presence (B) or absence (C) of histological chorioamnionitis (original magnification of all images, 400×).

group, stratified into two subgroups: less than 34 weeks' gestation and between 34 and 36 weeks' gestation (Fig. 1B); and regarding the presence or absence of histological chorioamnionitis (Fig. 1C).

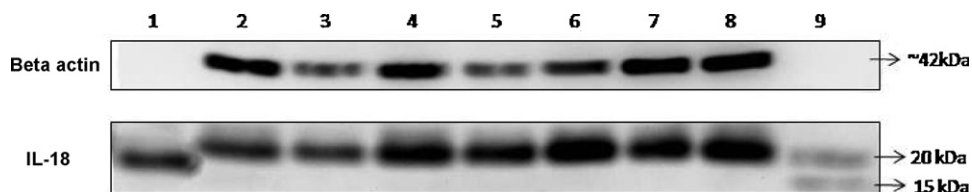
Samples subjected to immunohistochemistry showed the presence of IL-18 localized in amnion, chorion and decidua cells (Fig. 2). The staining was most intense at the area of the choriodecidual junction.

In order to quantify IL-18 levels in chorioamniotic membranes, western blot was performed. Only the 24 kDa immunoreactive band corresponding to the immature form of IL-18 (proIL-18) was detected. The 18 kDa mature bioactive form was not observed (Fig. 3), perhaps due to the fact that predominantly intracellular material was being analyzed. However, due to the absence of mature IL-18, the samples were also evaluated for the presence of the enzyme caspase-1, which creates mature IL-18 from its precursor. Discrete immunoperoxidase staining of caspase-1 was observed in amniotic, chorionic and decidual cells (Fig. 4).

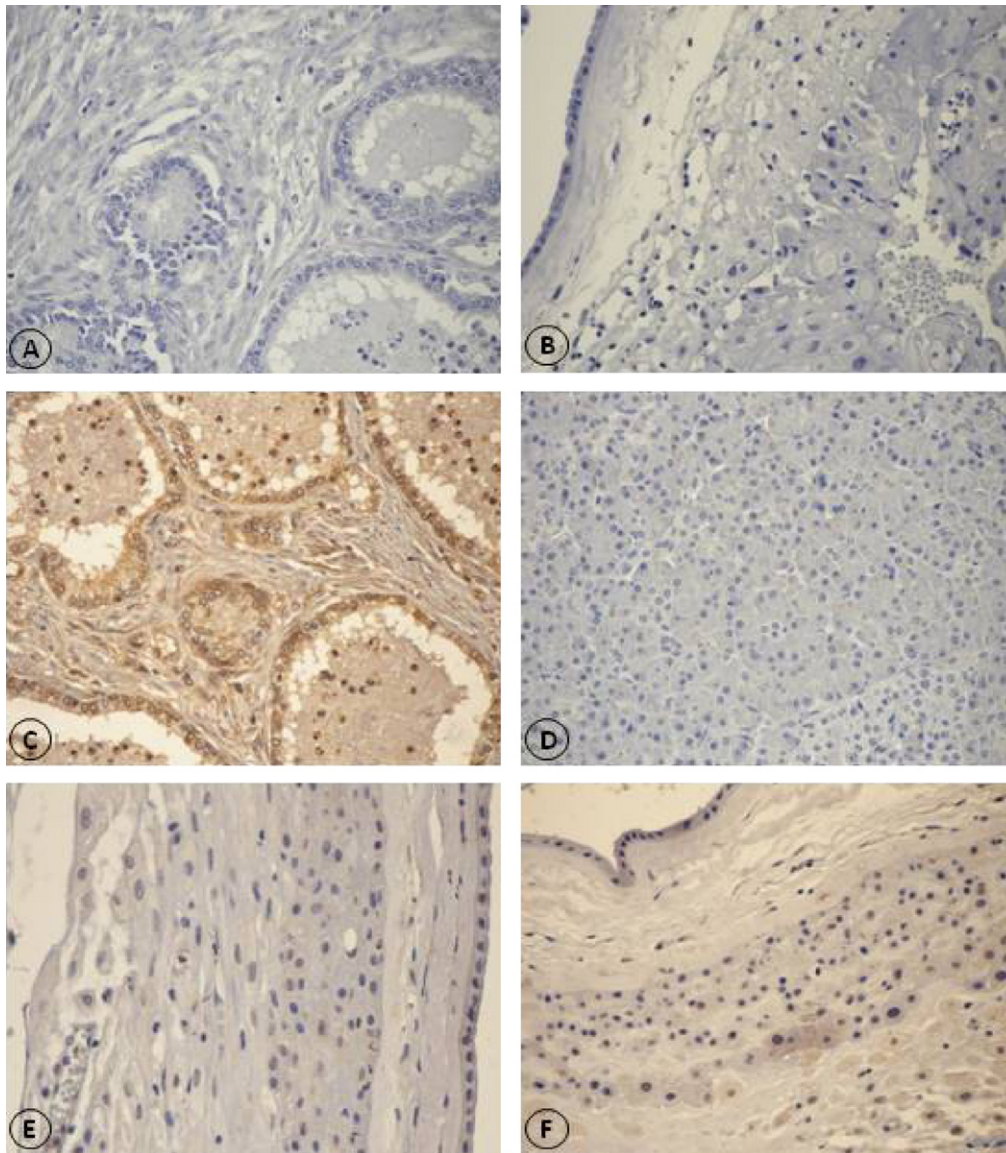
**4. Comment**

The present study demonstrates that chorioamniotic membranes from pregnant women not in labor are constitutive producers of

IL-18 mRNA and that the extent of this expression is unrelated to the presence or absence of PPROM and/or histological chorioamnionitis. Furthermore, the precursor proIL-18 protein was detected within the chorioamniotic membranes together with caspase-1. Presumably mature IL-18 was identified at the choriodecidual junction. We cannot exclude the possibility that small amounts of bioactive IL-18, below the detection limit of Western blot analysis, may have been present within the chorioamniotic membrane preparations. The antibody used to measure IL-18 in this study recognized both the mature and unprocessed forms. A prior publication by Gotsch et al. [33] concluded that caspase-1 levels in amniotic fluid were absent at mid-trimester and were highest in samples obtained from women in labor at term. Thus, production of bioactive IL-18 may only occur to a limited extent prior to parturition. Serum IL-18 concentrations were lower in women in preterm labor compared with women in term labor [28]. Similarly, reduced amniotic fluid IL-18 levels were identified in women who delivered preterm [34]. Although caspase-1 cleavage of proIL-18 is not exclusive and recent reports suggest other proteases, such as proteinase 3 [35] and granzyme B [36], also generate biological activity from precursor IL-18, little is known regarding the expression and regulation of these proteins in gestational tissues.



**Fig. 3.** Western blot analysis of interleukin (IL)-18 in chorioamniotic membranes using monoclonal anti-human IL-18. Lane 1: recombinant IL-18 protein (positive control, 10 ng, 18 kDa). Lanes 2–8: chorioamniotic membranes samples from the PPROM group in the absence (2–4) and presence (5–6) of chorioamnionitis and from the control group (7–8), showing the characteristics bands of proIL-18 (24 kDa). Lane 9: prestained protein marker (20 and 15 kDa).



**Fig. 4.** Immunohistochemical detection of caspase-1 in chorioamniotic membranes. Negative controls from prostate tissue and chorioamniotic membranes, sections were incubated without the monoclonal antibody anti-human caspase-1 (A and B, respectively). Sections from prostate and pancreatic tissues incubated with antibody anti-human IL-18 (C and D, respectively). Chorioamniotic membranes from the PPROM group incubated with anti-human caspase-1 (E and F) (original magnification of all images, 400 $\times$ ).

Our findings indicate that IL-18 is not specifically involved in the induction of prelabor rupture of the chorioamniotic membranes nor is it a factor in chorioamnionitis-related membrane disruption, rather that IL-18 is activated and functional during term labor and participates in the parturition process. Previous studies demonstrated no relationship between the amniotic fluid concentrations of IL-18 and PPRM [26,27]. In addition, Amash et al. [24] showed the levels of IL-18 in the amnion, chorion and placenta from uncomplicated term pregnancies were unchanged in the presence of LPS. A possible function for IL-18 prior to parturition can be implied from experiments in mice. Deletion of the gene coding for IL-18 was shown to result in a markedly elevated rate of fetal loss compared to intact control mice [37].

Further investigations are required to clarify the molecular mechanisms involved in IL-18 activation at the maternal–fetal interface, to evaluate protease and inflammasome activity in chorioamniotic membranes from women not in labor and to define the biological roles of IL-18 following term labor initiation.

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