Expression profile of C19MC microRNAs in placental tissue of patients with preterm prelabor rupture of membranes and spontaneous preterm birth

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Abstract. The aim of the study was to demonstrate that preterm birth (PTB) is associated with altered C19MC microRNA expression profile in placental tissues. Gene expression of 15 placental specific microRNAs (miR-512-5p, miR-515-5p, miR-516-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-519e-5p, miR-520a-5p, miR-520h, miR-524-5p, miR-525-5p, miR-526a and miR-526b-5p) was compared between groups: 34 spontaneous PTB, 108 preterm prelabor rupture of membranes (PPROM) and 20 term in labor pregnancies. Correlation between variables including relative microRNA quantification in placental tissues and the gestational age at delivery, white blood cell (WBC) count at admission and serum levels of C-reactive protein at admission in patients with PPROM and PTB was determined. Expression profile of microRNAs was different between PPROM and term in labor pregnancies, PTB and term in labor pregnancies, and between gestational age-matched PPROM and PTB groups. When compared with term in labor pregnancies, while C19MC microRNAs showed a downregulation in PPROM pregnancies (miR-525-5p), in PTB pregnancies C19MC microRNAs were upregulated (miR-515-5p, miR-516-5p, miR-518b, miR-518f-5p, miR-519a, miR-519e-5p, miR-520a-5p, miR-520h, and miR-526b-5p) or showed a trend to upregulation (miR-519d and miR-526a). In comparison to PTB pregnancies, the PPROM group demonstrated a significant portion of downregulated C19MC microRNAs (miR-516-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-519e-5p, miR-520a-5p, miR-520h, miR-525-5p, miR-526a and miR-526b-5p). In the group of PPROM pregnancies, a weak negative correlation between the gestational age at delivery and microRNA gene expression in placental tissue for all examined C19MC microRNAs was observed. PTB pregnancies showed a positive correlation (miR-512-5p, miR-515-5p, miR-519e-5p) or a trend to positive correlation (miR-516-5p, miR-518b, miR-520h) between particular C19MC microRNAs and maternal WBC count at admission. Our study demonstrates that upregulation of C19MC microRNAs is a characteristic phenomenon of PTB. PPROM pregnancies have a tendency to produce lower levels of miR-525-5p. All examined C19MC microRNAs displayed decreased expression with advancing gestational age in PPROM group.

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Introduction

Preterm delivery, defined as the delivery prior 37 complete weeks of gestation, occurs in 5-13% of all pregnancies (1-3). It is the most common cause of neonatal mortality and morbidity worldwide (2-5), accounts for approximately 70% of neonatal deaths (6,7). Preterm delivery results from three clinical conditions occurring with nearly similar rates: i) Medically indicated or elective preterm delivery (30-35%); ii) spontaneous preterm birth (PTB) with intact fetal membranes (40-45%); and iii) premature rupture of fetal membranes (PROM; 25-30%) (2,8,9).

Preterm delivery results usually in critical care emergencies, and is associated not only with short-term consequences to the health of the child including cerebral palsy, respiratory distress syndrome, neonatal infection/sepsis and intraventricular hemorrhage, but also with long-term adverse sequel (i.e., neurodevelopmental impairment, sensory defects, learning difficulties and behavioural problems) (5,10-15).

Since preterm delivery rates increase over the last two decades in many developed countries (16), early identification (weeks if not months before the clinical event) of patients with an increased risk for PTB and PROM, as a prerequisite for the effective use of interventions (use of steroids, transfer to appropriate hospital facilities), becomes crucial in reducing adverse perinatal outcomes and the costs of neonatal care (5,17-19).

Although etiology of preterm delivery is multifactorial and the exact causes remain unknown in most cases, intrauterine infection with activation of the innate immune system

and an exaggeration of inflammatory processes is found in 25-40% (9,20-23). Many other predisposing conditions have been proposed, including factors such as an incomplete cervix, uterine abnormalities, exposure to diethylstilbestrol (synthetic nonsteroidal estrogen; oral contraceptive) or environmental pollutants such as lead, pregnancy hypertension, intrauterine growth restriction, multiple pregnancy, maternal stress, heavy physical work, and smoking (24-27). Concerning intrauterine infection, development of histological chorioamnionitis is one of the main features and represents a pathological condition characterized by intraamniotic inflammation [AIA; inflammation of amniochorionic (fetal) membranes and placental chorion] in response to microbial invasion of the amniotic cavity (MIAC), which is predominated by Ureaplasma species, or to other pathological processes (28-30).

ROM is a natural physiological phenomenon that occurs before delivery. However, when the rupture of the amniotic membranes with release of the amniotic fluid occurs more than 1 h prior to the onset of labor, it is called PROM (31). PROM, that complicated 4.5-7.6% of deliveries (32,33), may be subdivided into term PROM (TPROM; i.e., PROM after 37 weeks of gestation) and preterm PROM (PPROM; i.e., spontaneous rupture of fetal membranes prior 37 weeks of gestation) (31). Most women with PPROM deliver within 48 h after rupture. The incidence of PPROM is 2-3.5% (31). The management of patients with PROM offered two general, but still controversial, options: i) Prompt delivery for women in labor, when infection or irreversible fetal distress occurs; and ii) complex decision concerning prolonging of gestation, reducing of complications of prematurity, timing of labor and choosing the route of delivery for women not in labor, especially in premature gestational ages (34).

Many possible mechanisms underlying spontaneous PPROM, including intra-amniotic infection and other factors mentioned above, have been identified: Reduction of membrane collagen content stretched membranes, vasculopathy in placentation, decidual hemorrhage, placental abruption, uterine overdistension, nutritional deficiences, and genetic factors (i.e., race and familiar clustering) (33,35-37). All these factors may play primary or secondary roles in the pathogenesis of PPROM.

Based on the known risk factors and pathways, diagnosis of preterm delivery comprises clinical evaluation and biological tests, which are useful in the case of clinically asymptomatic patients. These tests include vaginal pH determination (nitrazine, bromthymol blue test) and the measurement of prolactin, α-fetoprotein, inflammatory cytokine IL-6, diamine oxydase, insulin-like growth factor binding protein-1 (IGFBP-1), amniotic fluid intracellular adhesion molecule 1, human choriogonadotrophin levels, or fetal fibronectin in cervicovaginal fluid, respectively (11,38-44).

Several biomarkers in first trimester maternal blood samples have been tested to see if they can predict preterm delivery. Among those rise in C-reactive protein (CRP) and decrease in mean platelet volume (MPV), pregnancy-associated plasma protein-A, selenium and lead levels in maternal serum during early gestation demonstrate some ability to distinguish women at risk of experiencing PTB or PPROM, but the insufficient test characteristics (specificity and sensitivity) of such methods limit any application in current clinical practice (45-50).

In the last decade, the importance of microRNAs (miRNA) in both, health and disease was revealed which provide a new opportunity for biomarker discovery in the field of preterm delivery. MicroRNAs belong to small (18-25 nucleotides) highly conserved single-stranded RNA molecules that play a critical role in posttranscriptional gene regulation by degrading or blocking translation of target messenger RNA. Although microRNA analyses indicate that a variety of disease-affected tissues display microRNA expression profiles that are significantly different from normal tissues, there are only several existing studies that have explored their possible involvement in regulating the molecular mechanisms that contribute to the pathophysiology of preterm delivery. First of all, Montenegro *et al* (51) screened 157 miRNAs using quantitative PCR in the chorioamniotic membranes derived from term and preterm patients and revealed a tendency toward decreased expression for 13 miRNAs with advancing gestational age. Evaluation of preterm membranes with and without chorioamnionitis identified increased expression of miR-223 and miR-338 in the presence of chorioamnionitis. Additional microRNA microarray analysis of 455 miRNAs in the chorioamniotic membranes revealed 39 differentially expressed microRNAs between term and preterm groups, of which 31 were downregulated at term. Subsequent quantitative PCR analysis confirmed decreased expression of miR-338, miR-449, miR-136, and miR-199a* in chorioamniotic membranes at term (52).

Another study (53) based on microarray profiling of 820 microRNAs in placentas identified 141 miRNAs (113 upregulated and 23 downregulated) differentially expressed in spontaneous preterm delivery (≤35 weeks of gestation) compared to normal term pregnancies (elective caesarean section without labor). Validation analysis using quantitative PCR revealed that lower expression of miR-15b, miR-181, miR-210, miR-483-5p, and a trend toward higher expression of miR-496 were able to differentiate between preterm delivery, preeclampsia and term pregnancies.

Elovitz *et al* (54) analyzed miRNA expression levels in cervical cells obtained from the ectocervix by a cytobrush. Profiling of the 5,640 miRNAs in samples collected at 20 to 28 weeks of gestation showed 99 miRNAs expressed differentially in women who eventually had a preterm delivery compared with their term counterparts. Of these microRNAs, only three (miR-143, miR-145 and miR-199b-5p) were confirmed to be upregulated at 24 weeks to 28 weeks of gestation and just only one (miR-106b*) at 20 weeks to 24 weeks of gestation in women with a PTB.

Sanders *et al* (55), who studied microRNA expression levels in cervical cells obtained from swabs during pregnancy between 16 and 19 weeks of gestation, identified 6 miRNAs significantly associated with gestational age at the time of delivery. They found that the levels of certain microRNAs (miR-21, miR-30e, miR-142, miR-148b, miR-29b, and miR-223) in the human cervix during pregnancy were predictive of gestational age at delivery. Per each doubling in miR-21 or miR-30e, miR-142, miR-148b, miR-29b, and miR-223 expression, gestations were 0.9 or 1.0-1.6 days shorter, respectively.

Recent study by Elovitz *et al* (56) examined miRNA profile in maternal serum collected from women destined to have a preterm delivery compared with a term birth. Only

4 out of 5,640 miRNAs (miR-200a*, miR-4695-5p, miR-665, and miR-887) were significantly different between cases and control subjects, however the fold difference in expression did not exceed 2, which limits their potential for clinical utilization.

Numerous studies have shown that alterations in miRNA expression in the placenta are associated with various pregnancy complications, such as preeclampsia and fetal growth restriction (57-62). The objective of this study was to investigate expression profile of C19MC miRNAs (miR-512-5p, miR-515-5p, miR-516b-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-519e-5p, miR-520a-5p, miR-520h, miR-524-5p, miR-525-5p, miR-526a and miR-526b-5p) in placental tissues collected from women with PTB or PPROM. C19MC is the largest human miRNA gene cluster and consists of 46 genes encoding a total of 56 mature miRNAs (63). This cluster is only present in the primate and human genomes and expresses miRNAs almost exclusively in placenta (64), with expression detected in only a few other cell types such as embryonic stem cells and certain tumors (65-68). However, for testing we selected only those 15 C19MC microRNAs which, according to the miRNAMap database and the study presented by Liang *et al* (69), have been reported to be placenta specific (i.e., to be significantly expressed in the placenta while showing no or minimal expression in other tissues).

To our knowledge, no study on C19MC microRNA expression in PTB and PPROM has been carried out. Since placenta is a complex and vital organ that not only mediates the selective transfer of solutes and gasses between the mother and the fetus, but also produces hormones and other factors that support pregnancy, changes in miRNAs levels may lead to dysregulation of several proteins which can contribute to the mechanisms underlying pathogenesis of PTB or PPROM. We hypothesize that a distinct profile of C19MC miRNAs in placental tissues may differentiate between women with PTB, PPROM and term pregnancies in labor.

Materials and methods

Patients. The study was retrospective. Clinical samples were collected between 2013 and 2016 at the Institute for the Care of Mother and Child (Prague, Czech Republic). Samples processing and analyses were performed at the Department of Molecular Biology and Cell Pathology, Third Faculty of Medicine, Charles University (Prague, Czech Republic). The study protocol was approved by the appropriate Local Ethics Committees and all patients who participated in the study provided written informed consent.

Placental tissues were collected from pregnant women with singleton pregnancies only. The studied cohort consisted of 34 pregnancies with spontaneous PTB delivering within 25+1-36+5 (median 34+5) weeks of gestation, and 108 gestational age matched (range 24+0-36+6, median 34+2 weeks) pregnancies with PPROM. The control cohort consisted of 20 women at term in labor with normal course of gestation delivering healthy infants weighing >2,500 g at term (after 37 completed weeks of gestation). Gestational age was assessed using ultrasonography.

PTB was defined as the occurrence of regular uterine contractions at a minimum frequency of two contractions per 10 min, along with cervical changes, leading to delivery before the 37th week of gestation was completed.

PPROM was defined as amniotic fluid leakage preceding the onset of labor by at least 2 h. PPROM was diagnosed visually using a sterile speculum examination to confirm the pooling of amniotic fluid in the vagina and an alkaline pH of cervicovaginal discharge. When necessary, it was confirmed by a positive test for the presence of IGFBP-1 (ACTIM PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

The exclusion criteria included women with gestational hypertension, preeclampsia, diabetes mellitus, significant vaginal bleeding, foetuses with the presence of congenital or chromosomal fetal abnormalities, signs of fetal growth restriction (an estimated weight below the 10th percentile for appropriate gestational age) and fetal hypoxia.

Women with PPROM and PTB at less than 34 weeks of gestation with negative markers of inflammation [maternal white blood cell (WBC) count and serum CRP levels] were treated with corticosteroids to accelerate lung maturation (one or maximally two doses of betamethasone administered intramuscularly 24 h apart). Tocolysis was used only in those patients before 34 weeks of gestation with no sign of fetal infection, fetal distress, maternal infection and negative perineal and perianal culture for GBS to allow the first course of antenatal corticosteroids to be completed and/or to transfer the patient to a tertiary care center. Prophylactic antibiotics were given to the majority of patients with preterm delivery to prevent signs of prepartum, intrapartum and postpartum infection.

In case of PPROM, induction of labor was initiated or an elective Cesarean section was performed within 24 to 72 h after the rupture of the membranes, depending on the gestational age of the pregnancy, the fetal status, the maternal serum levels of CRP and cervicovaginal Streptococcus β colonization.

The clinical characteristics of normal and complicated pregnancies are presented in Table I.

Processing of samples. Samples of placenta were stored in RNAlater (Ambion, Austin, TX, USA) at -80˚C until further processing.

Total RNA was extracted from 25 mg of placental tissue followed by an enrichment procedure for small RNAs (siRNAs, microRNAs), according to manufacturer's instructions using mirVana microRNA Isolation kit (Ambion). To minimize DNA contamination, we treated the eluted RNA with 5 μl of DNase I (Fermentas international, Inc., Burlington, ON, Canada) for 30 min at 37˚C. Using this approach, a RNA fraction highly enriched in RNA species <200 nt was obtained, whose concentration and quality was assessed using a NanoDrop ND-1,000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A (260/280) absorbance ratio of isolated RNA was 1.8-2.0, demonstrating that the RNA fraction was pure and could be used for analysis. Additionally, the A (260/230) ratio was greater than 1.6, demonstrating negligible contamination by polysaccharides.

Reverse transcriptase reaction using a stem-loop primer. Each of the 15 microRNAs (miR-512-5p, miR-515-5p, miR-516-5p,

Table I. Maternal and neonatal characteristics of normal and complicated pregnancies.

Characteristic	Term in labor (n=20)	PTB (n=34)	PPROM (n=108)	P-value[a]	P-value[b]
Age (years)	31.00 (29.75-33.00)	30.00 (27.00-33.00)	31.00 (28.75-35.00)	0.430	0.248
Gestational age at delivery (weeks)	39.86 (38.71-40.57)	34.78 (31.00-35.92)	34.35 (31.14-36.00)	**<0.001**	0.956
Mode of delivery					0.445
Vaginal	16 (80%)	25 (73.5%)	70 (64.8%)	0.318	
Cesarean section	4 (20%)	9 (26.5%)	38 (33.2%)		
Fetal birth weight (g)	3450 (2954.5-3682.5)	2120 (1627.5.5-2742.5)	2310 (1540-2682.5)	**<0.001**	0.973
Fetal sex					0.216
Boy	12 (60%)	24 (70.6%)	61 (56.5%)	0.344	
Girl	8 (40%)	10 (29.4%)	47 (43.5%)		
BMI	26.64 (23.70-28.71)	25.00 (22.00-28.25)	24.97 (22.22-28.00)	0.199	0.929
Primiparity					0.342
Yes	-	13 (38.2%)	53 (49.1%)	NS	
No	-	21 (61.8%)	55 (50.9%)		
Administration of corticosteroids					0.492
Yes	-	20 (58.8%)	72 (66.7%)	NS	
No	-	14 (41.2%)	36 (33.3%)		
Administration of antibiotics					0.187
Yes	-	27 (79.4%)	102 (94.4%)	NS	
No	-	7 (20.6%)	6 (5.6%)		
Tocolytic therapy					0.130
Yes	-	15 (44.1%)	29 (26.9%)	NS	
No	-	19 (55.9%)	79 (73.1%)		
CRP levels (mg/l)	-	5.5 (2.9-20.0)	6.3 (3.0-10.2)	NS	0.587
WBC count ($x10^9$/l)	-	13.7 (11.0-16.3)	12.4 (10.4-15.8)	NS	0.344
Apgar score <7; 5 min	-	2 (5.9%)	5 (4.6%)	NS	0.305
Apgar score <7; 10 min	-	1 (2.9%)	2 (1.9%)	NS	0.235
Umbilical blood pH	-	7.33 (7.30-7.36)	7.31 (7.28-7.36)	NS	0.156

Data are presented as median (25-75 percentile) for continuous variables and as number (percent) for categorical variables. Statistically significant results are marked in bold. [a]P-value: The comparison among three groups. Continuous variables were compared using the Kruskal-Wallis test (exact two-tailed P-value). Categorical variables were compared using Fisher's exact test (exact two-tailed P-value). [b]P-value: The comparison among women with spontaneous PTB and PPROM. Continuous variables were compared using the Kruskal-Wallis test. Categorical variables were compared using a Chi-square test. PTB, preterm birth; PPROM, preterm prelabor rupture of membranes; BMI, Body mass index; CRP, C-reactive protein; WBC, white blood cells.

miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-519e-5p, miR-520a-5p, miR-520h, miR-524-5p, miR-525-5p, miR-526a and miR-526b-5p) was reverse transcribed into complementary DNA (cDNA) using a TaqMan MicroRNA Assay, containing microRNA-specific stem-loop RT primers, and a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Branchburg, NJ, USA) in a total reaction volume of 32 μl, according to manufacturer's instructions. Reverse transcriptase reactions were performed using a 7,500 Real-Time PCR system (Applied Biosystems) with the following thermal cycling parameters: 30 min at 16˚C; 30 min at 42˚C; 5 min at 85˚C; and then held at 4˚C. Finally, 20 ng of the RNA template was used for each RT reaction. The characteristics of studied C19MC microRNAs are outlined in Table II.

Relative quantification of microRNAs by quantitative PCR. 15 μl of cDNA, corresponding to each selected microRNA, were mixed with specific primers and the TaqMan MGB probe (TaqMan MicroRNA Assay; Applied Biosystems,), and the ingredients of the TaqMan Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of 35 μl. The analysis was performed using a 7,500 Real-Time PCR System. TaqMan PCR conditions were set as described in the TaqMan guidelines using 50 cycles of 95˚C for 15 sec and 60˚C for 1 min with 2-min preincubation at 50˚C required for optimal AmpErase UNG activity and 10-min preincubation at 95˚C required for activation of AmpliTaq Gold DNA polymerase. All PCRs were performed in duplicate. Multiple negative controls such as NTC (water instead of cDNA sample), NAC (non-transcribed RNA samples), and genomic DNA (isolated from equal biological samples) did not generate any signals during PCR reactions. Each sample was considered positive if the amplification signal occurred before the 40th quantification cycle (C_q <40).

The expression of particular microRNA was determined using the comparative C_q method (70) relative to normalization factor (geometric mean of three selected endogenous controls) (71). RNA isolated from a randomly selected placenta derived from a normal gestation was chosen as a reference for each comparison. RNA that was highly enriched with small RNA, isolated from the fetal part of the placenta (the part of the placenta derived from the chorionic sac that encloses the embryo, consisting of the chorionic plate and villi), was used as a reference sample for relative quantification throughout the study.

All 15 selected microRNAs were reliably detectable in the fetal part of the placenta, when a fixed concentration of RNA (5 ng/μl) was used in the analysis; however their expression differed significantly with respect to various C_q values, ranging from 17.4 to 35.2 (72).

The difference (ΔCq) between the C_q values of particular microRNA and the internal control (geometric mean of three selected endogenous controls: RNU6B, RNU38B and synthetic *C. elegans* microRNA cell-miR-39 was calculated for each sample). Synthetic *C. elegans* microRNA was used as an internal control for variations during the preparation of RNA, cDNA synthesis, and quantitative PCR. The comparative ΔΔCq calculation involved finding the difference between each sample's ΔCq and the reference's ΔCq. Finally, ΔΔCq

Table II. Characteristics of selected C19MC microRNAs.

Assay name	miRBase ID	NCBI location chromosome	microRNA sequence	Expression in placenta
hsa-miR-512-5p	hsa-miR-512-5p	Chr.19: 54169933 - 54170016 [+]	5'-CACUCAGCCUUGAGGGCACUUUC-3'	High expression
hsa-miR-515-5p	hsa-miR-515-5p	Chr.19: 54182257 - 54182339 [+]	5'-UUCUCCAAAAGAAAGCACUUUCUG-3'	High expression
hsa-miR-516-5p	hsa-miR-516b-5p	Chr.19: 58920508 - 58920592 [+]	5'-CAUCUGGAGGUAAGAAGCACUUU-3'	Exclusively expressed
hsa-miR-517*	hsa-miR-517-5p	Chr.19: 54215522 - 54215608 [+]	5'-CCUCUAGAUGGAAGCACUGUCU-3'	Exclusively expressed
hsa-miR-518b	hsa-miR-518b	Chr.19: 54205991 - 54206073 [+]	5'-CAAAGCGCUCCCCUUUAGAGGU-3'	Exclusively expressed
hsa-miR-518f*	hsa-miR-518f-5p	Chr.19: 54203269 - 54203355 [+]	5'-CUCUAGAGGGAAGCACUUUCUC-3'	High expression
hsa-miR-519a	hsa-miR-519a-3p	Chr.19: 54255651 - 54255735 [+]	5'-AAAGUGCAUCCUUUUAGAGUGU-3'	Exclusively expressed
hsa-miR-519d	hsa-miR-519d	Chr.19: 54216601 - 54216688 [+]	5'-CAAAGUGCCUCCCUUUAGAGUG-3'	Exclusively expressed
hsa-miR-519e*	hsa-miR-519e-5p	Chr.19: 54183194 - 54183277 [+]	5'-UUCUCCAAAAGGGAGCACUUUC-3'	High expression
hsa-miR-520a*	hsa-miR-520a-5p	Chr.19: 54194135 - 54194219 [+]	5'-CUCCAGAGGGAAGUACUUUCU-3'	High expression
hsa-miR-520h	hsa-miR-520h	Chr.19: 54245766 - 54245853 [+]	5'-ACAAAGUGCUUCCCUUUAGAGU-3'	High expression
hsa-miR-524-5p	hsa-miR-524-5p	Chr.19: 54214256 - 54214342 [+]	5'-CUACAAAGGGAAGCACUUUCUC-3'	High expression
hsa-miR-525	hsa-miR-525-5p	Chr.19: 54200787 - 54200871 [+]	5'-CUCCAGAGGGAUGCACUUUCU-3'	Exclusively expressed
hsa-miR-526a	hsa-miR-526a	Chr.19: 54209506 - 54209590 [+]	5'-CUCUAGAGGGAAGCACUUUCU-3'	High expression
hsa-miR-526b	hsa-miR-526b-5p	Chr.19: 54197647 - 54197729 [+]	5'-CUCUUGAGGGAAGCACUUUCUGU-3'	Exclusively expressed

values were transformed to absolute values using the formula $2^{-\Delta\Delta Cq}$. This distinctive approach allows long-term, large-scale analysis composed of multiple analyses performed at different periods.

Statistical analysis. Data normality was assessed using the Shapiro-Wilk test, which showed that our data did not follow a normal distribution. Therefore, microRNA levels were compared between groups using non-parametric tests (the Mann-Whitney U test for the comparison between two groups and the Kruskal-Wallis test for the comparison among multiple groups). P<0.05 was considered to indicate a statistically significant difference.

Data analysis was performed and box plots were generated using Statistica software (version 9.0; StatSoft, Inc., Tulsa, OK, USA). Each box encompasses the median (dark horizontal line) of log-normalized gene expression values for microRNAs of interest in cohorts; the upper and lower limits of the boxes represent the 75 and 25th percentiles, respectively. The upper and lower whiskers represent the maximum and minimum values that are no more than 1.5 times the span of the interquartile range (range of the values between the 25th and the 75th percentiles). Outliers are indicated by circles and extremes by asterisks.

Correlation between variables including relative microRNA quantification in placental tissues and the gestational age at delivery, maternal WBC count at admission ($x10^9$/l), maternal serum levels of CRP at admission (mg/l) in patients with PPROM and PTB was calculated using the Spearman's rank correlation coefficient (rho). If the correlation coefficient value is -1 or 1, there is a perfect negative or positive correlation. If it ranges within <-1; 0.5> or <0.5; 1>, there is a strong negative or positive correlation. If it varies from -0.5 to 0 and from 0 to 0.5, there is a weak negative or positive correlation. The significance level was established at a P-value of P<0.05.

Results

Initially, gene expression of C19MC microRNAs was compared between the groups of women at term in labor, spontaneous PTB and PPROM. Consecutively, an effect of gestational age on C19MC microRNA gene expression was evaluated in the group of PPROM and PTB patients.

Moreover, the association between C19MC microRNA gene expression in placental tissue and maternal WBC count and maternal serum CRP levels in patients with PPROM and PTB was determined.

Downregulation of C19MC microRNAs in PPROM pregnancies and upregulation of C19MC microRNAs in PTB pregnancies. Overall, the expression of C19MC microRNAs in placental tissue samples differed significantly or was on the border of statistical significance between the control group (term in labor pregnancies) and pregnancies affected with PPROM or PTB. While decreased expression of 1/15 C19MC microRNAs was observed in women with PPROM (miR-525-5p, P=0.025), the upregulation of 9/15 C19MC microRNAs was found in PTB pregnancies (miR-515-5p, P=0.040; miR-516b-5p, P=0.032; miR-518b, P=0.039; miR-518f-5p, P=0.036; miR-519a, P=0.032; miR-519e-5p, P=0.006; miR-520a-5p, P=0.014; miR-520h, P=0.039; and miR-526b-5p, P=0.022). The difference on the border of statistical significance was identified between the groups of PTB patients and term in labor pregnancies for miR-519d (P=0.067) and miR-526a (P=0.067) (Fig. 1, Table III).

Differentiation between gestational age-matched pregnancies with PTB and PPROM based on placental expression profile of C19MC microRNAs. Overall, the expression of miR-516b-5p (P=0.009), miR-517-5p (P=0.021), miR-518b (P=0.009, miR-518f-5p (P=0.033), miR-519a (P=0.022), miR-519d (P=0.003), miR-519e-5p (P=0.016), miR-520a-5p (P=0.018), miR-520h (P=0.005), miR-525-5p (P=0.015), miR-526a (P=0.006) and miR-526b-5p (P=0.007) differed significantly between the PTB group and pregnancies affected with PPROM. Lower expression rates were detected in patients with PPROM. A trend towards statistical significance for downregulation of miR-524-5p (P=0.064) was observed for PPROM pregnancies (Fig. 1, Table III).

The effect of the gestational age on C19MC microRNA expression in placental tissue within the groups of PPROM and PTB pregnancies. A weak negative correlation between the gestational age at delivery and microRNA gene expression in placental tissue within the group of PPROM patients was observed (miR-512-5p, ρ=-0.344, P=0.002; miR-515-5p, ρ=-0.342, P=0.002; miR-516b-5p, ρ=-0.293, P=0.010; miR-517-5p, ρ=-0.258, P=0.023; miR-518b, ρ=-0.375, P<0.001; miR-518f-5p, ρ=-0.316, P=0.005; miR-519a, ρ=-0.293, P=0.010; miR-519d, ρ=-0.333, P=0.003; miR-519e-5p, ρ=-0.278, P=0.014; miR-520a-5p, ρ=-0.257, P=0.024; miR-520h, ρ=-0.334, P=0.003; miR-524-5p, ρ=-0.351, P=0.001; miR-525-5p, ρ=-0.284, P=0.012; miR-526a, ρ=-0.308, P=0.006; and miR-526b-5p, ρ=-0.453, P<0.001), which means that the expression of all 15 examined C19MC microRNAs decreased with advancing gestational age at delivery (Fig. 2). On the other hand, no association between microRNA gene expression in placental tissue and the gestational age at delivery was found in the group of PTB patients.

The association between C19MC microRNA gene expression in placental tissue and maternal serum CRP levels within the groups of PPROM and PTB pregnancies. No association between maternal serum CRP levels at admission and C19MC microRNA gene expression levels in placental tissues was found in the group of PPROM pregnancies (miR-512-5p: ρ=-0.117, P=0.333; miR-515-5p, ρ=-0.131, P=0.276; miR-516b-5p, ρ=-0.105, P=0.384; miR-517-5p, ρ=-0.167, P=0.164; miR-518b, ρ=-0.112, P=0.354; miR-518f-5p, ρ=-0.154, P=0.202; miR-519a, ρ=-0.149, P=0.215; miR-519d, ρ=-0.160, P=0.183; miR-519e-5p, ρ=-0.167, P=0.166; miR-520a-5p, ρ=-0.142, P=0.238; miR-520h, ρ=-0.134, P=0.268; miR-524-5p, ρ=-0.188, P=0.117; miR-525-5p, ρ=-0.145, P=0.229; miR-526a, ρ=-0.143, P=0.235; and miR-526b-5p, ρ=-0.027, P=0.823) and PTB pregnancies (miR-512-5p, ρ=0.144, P=0.594; miR-515-5p, ρ=0.114, P=0.672; miR-516b-5p, ρ=-0.058, P=0.828; miR-517-5p, ρ=0.036, P=0.892; miR-518b, ρ=-0.151, P=0.575; miR-518f-5p, ρ=-0.091, P=0.736; miR-519a: ρ=-0.139, P=0.605; miR-519d, ρ=0.035, P=0.896; miR-519e-5p, ρ=-0.061, P=0.820; miR-520a-5p, ρ=-0.005, P=0.982; miR-520h, ρ=0.064, P=0.811; miR-524-5p,

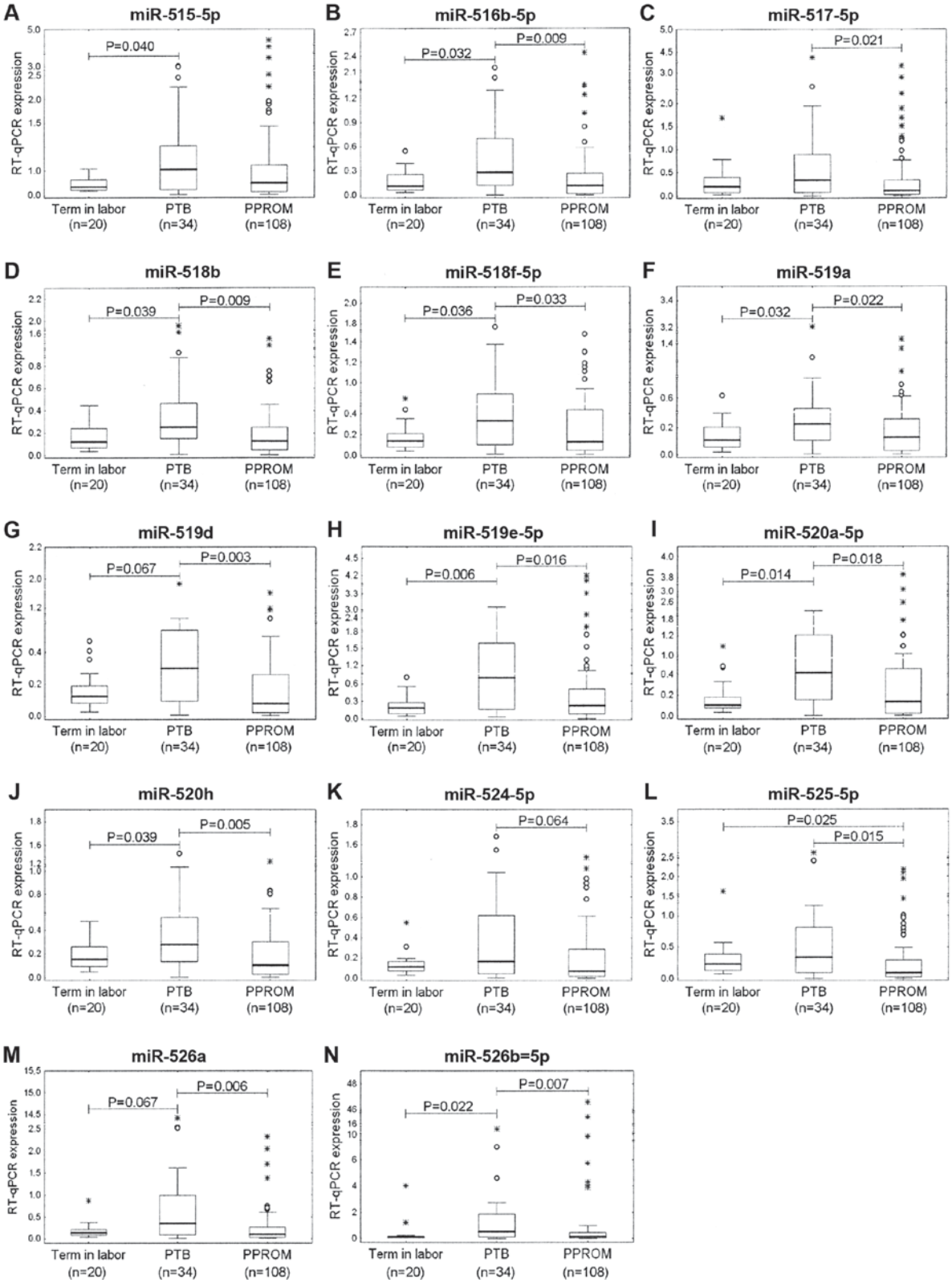

Figure 1. Downregulation of C19MC microRNAs in PPROM pregnancies and upregulation of C19MC microRNAs in PTB pregnancies. Differentiation between gestational age matched PTB and PPROM pregnancies. Gene expression of C19MC microRNAs was compared between the groups of women at term in labor (20), spontaneous preterm birth (34 PTB) and preterm prelabor rupture of membranes (108 PPROM) using the Kruskal Wallis test for the comparison among multiple groups. The significance level was established at $P<0.05$. While decreased expression of (L) miR-525-5p was observed in women with PPROM, the upregulation of (A) miR-515-5p, (B) miR-516b-5p, (D) miR-518b, (E) miR-518f-5p, (F) miR-519a, (H) miR-519e-5p, (I) miR-520a-5p, (J) miR-520h and (N) miR-526b-5p was found in PTB pregnancies. The expression of (B) miR-516b-5p, (C) miR-517-5p, (D) miR-518b, (E) miR-518f-5p, (F) miR-519a, (G) miR-519d, (H) miR-519e-5p, (I) miR-520a-5p, (J) miR-520h, (L) miR-525-5p, (M) miR-526a and (N) miR-526b-5p differed significantly between the PTB group and pregnancies affected with PPROM. Data analysis was performed and box plots were generated using Statistica software (version 9.0; StatSoft, Inc., Tulsa, OK, USA). Each box encompasses the median (dark horizontal line) of log normalized gene expression values for microRNAs of interest in cohorts; the upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers represent the maximum and minimum values that are no more than 1.5 times the span of the interquartile range (range of the values between the 25 and the 75th percentiles). Outliers are indicated by circles and extremes by asterisks.

Table III. C19MC microRNA gene expression differentiate between particular groups: Term in labor pregnancies, spontaneous PTB and PPROM pregnancies.

Gene expression	Term in labor (n=20)	PTB (n=24)	PPROM (n=75)	P-value[a]	P-value[b]	P-value[c]	P-value[d]
miR-512-5p [median (range)]	0.223 (0.054-1.382)	0.521 (0.002-3.013)	0.261 (0.011-4.758)	0.254	0.147	0.106	0.880
miR-515-5p [median (range)]	0.173 (0.084-0.543)	0.521 (0.014-3.013)	0.255 (0.015-4.758)	0.181	0.149	**0.040**	0.625
miR-516b-5p [median (range)]	0.112 (0.038-0.543)	0.275 (0.002-2.256)	0.119 (0.002-2.444)	**0.022**	**0.009**	**0.032**	0.543
miR-517-5p [median (range)]	0.194 (0.022-1.677)	0.329 (0.002-4.376)	0.106 (0.001-3.153)	**0.039**	**0.021**	0.243	0.161
miR-518b [median (range)]	0.117 (0.035-0.447)	0.249 (0.009-1.957)	0.127 (0.005-1.439)	**0.026**	**0.009**	**0.039**	0.858
miR-518f-5p [median (range)]	0.141 (0.044-0.712)	0.330 (0.012-1.768)	0.129 (0.010-1.473)	0.068	**0.033**	**0.036**	0.901
miR-519a [median (range)]	0.107 (0.019-0.618)	0.223 (0.006-3.204)	0.127 (0.004-1.545)	**0.048**	**0.022**	**0.032**	0.967
miR-519d [median (range)]	0.123 (0.026-0.469)	0.297 (0.006-1.968)	0.078 (0.004-1.299)	**0.007**	**0.003**	0.067	0.166
miR-519e-5p [median (range)]	0.184 (0.049-0.718)	0.711 (0.035-3.056)	0.226 (0.003-4.189)	**0.017**	**0.016**	**0.006**	0.451
miR-520a-5p [median (range)]	0.101 (0.034-1.091)	0.413 (0.003-2.318)	0.133 (0.003-3.821)	**0.033**	**0.018**	**0.014**	0.823
miR-520h [median (range)]	0.151 (0.053-0.475)	0.277 (0.007-1.529)	0.105 (0.004-1.221)	**0.010**	**0.005**	**0.039**	0.226
miR-524-5p [median (range)]	0.114 (0.033-0.549)	0.162 (0.005-1.683)	0.071 (0.004-1.475)	0.098	0.064	0.273	0.178
miR-525-5p [median (range)]	0.226 (0.073-1.757)	0.328 (0.003-2.999)	0.091 (0.002-2.122)	**0.011**	**0.015**	0.595	**0.025**
miR-526a [median (range)]	0.135 (0.034-0.865)	0.348 (0.004-14.414)	0.097 (0.003-2.327)	**0.014**	**0.006**	0.067	0.209
miR-526b-5p [median (range)]	0.093 (0.052-4.020)	0.505 (0.009-11.458)	0.120 (0.010-46.575)	**0.018**	**0.007**	**0.022**	0.945

PTB, preterm birth; PPROM, preterm prelabor rupture of membranes. Continuous variables were compared using a non-parametric Kruskal-Wallis or Mann-Whitney test. Statistically significant results are marked in bold. Continuous variables are presented as median (range). [a]P-value: A comparison among all three groups. [b]P-value: A comparison between group of women with spontaneous preterm birth and group of woman with preterm prelabor rupture of membranes. [c]P-value: A comparison between group of women at term in labor and group of woman with spontaneous preterm birth. [d]P-value: A comparison between group of women at term in labor and group of woman with preterm prelabor rupture of membranes.

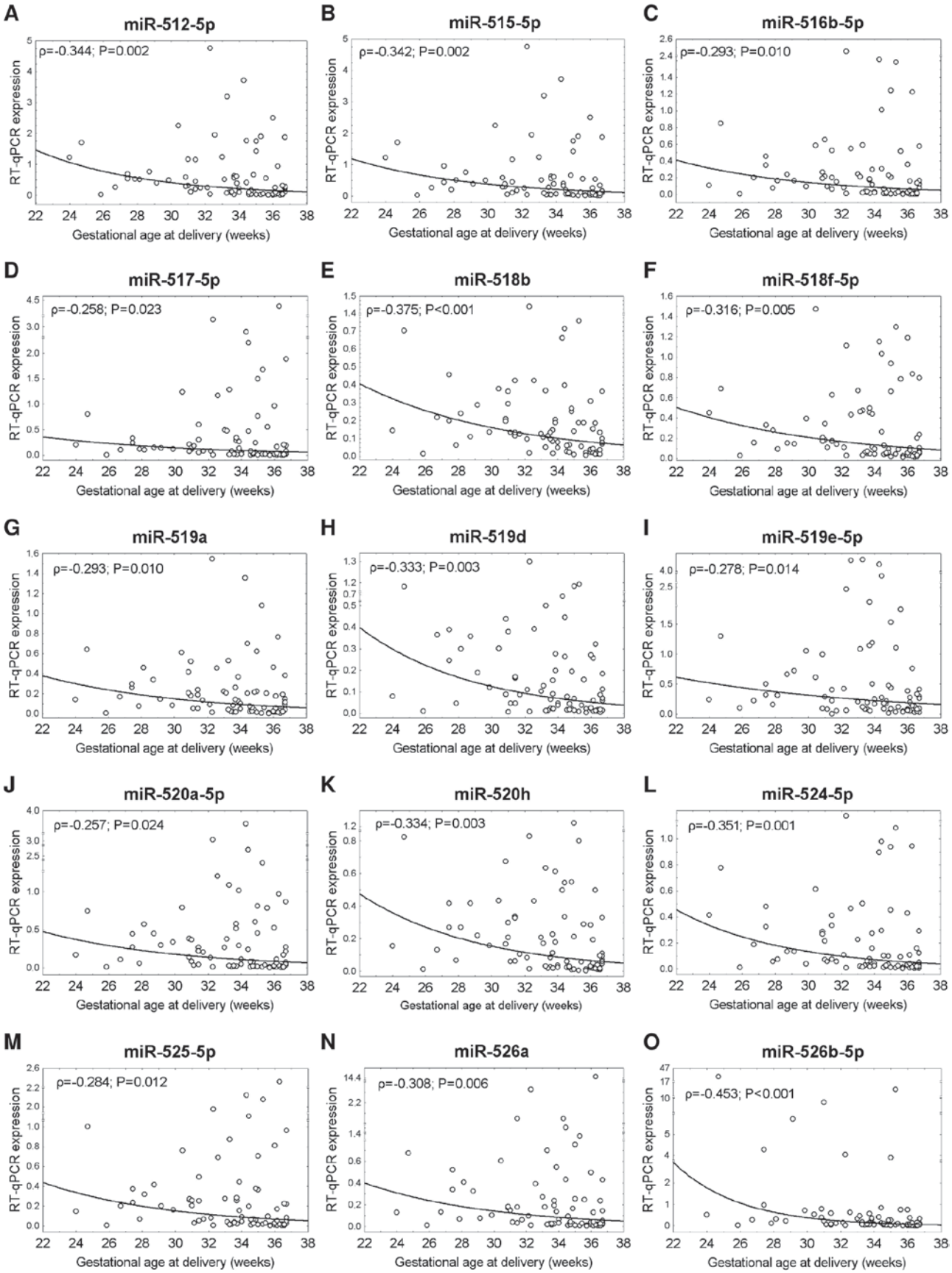

Figure 2. Decline of C19MC microRNA expression in placental tissues with advancing gestational age in the group of preterm prelabor rupture of membranes (PPROM) pregnancies. Correlation between variables including relative microRNA quantification in placental tissues and the gestational age at delivery in patients with PPROM was calculated using the Spearman's rank correlation coefficient (rho). If the correlation coefficient value varies from 0.5 to 0, there is a weak negative correlation. The significance level was established at $P<0.05$. A weak negative correlation between the gestational age at delivery and gene expression of (A) miR-512-5p, (B) miR-515-5p, (C) miR-516b-5p, (D) miR-517-5p, (E) miR-518b, (F) miR-518f-5p, (G) miR-519a, (H) miR-519d, (I) miR-519e-5p, (J) miR-520a-5p, (K) miR-520h, (L) miR-524-5p, (M) miR-525-5p, (N) miR-526a and (O) miR-526b-5p in placental tissue within the group of PPROM patients was observed.

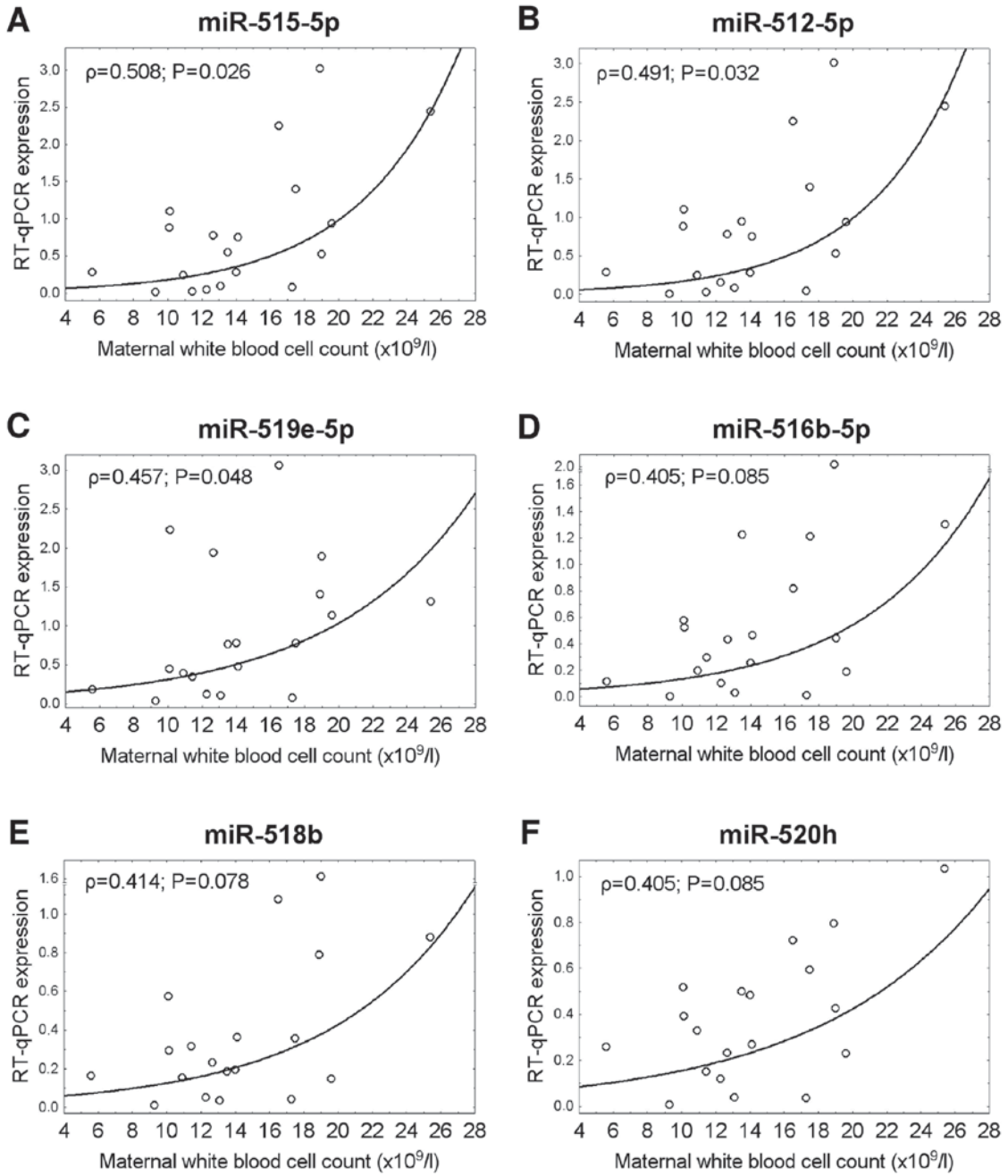

Figure 3. Increase of C19MC microRNA expression in placental tissues with rising maternal WBC count in the group of PTB pregnancies. Correlation between variables including relative microRNA quantification in placental tissues and maternal WBC count at admission (x10^9/l) in patients with PTB was calculated using the Spearman's rank correlation coefficient (rho). If the correlation coefficient value ranges within <0.5; 1>, there is a strong positive correlation. If it varies from 0 to 0.5, there is a weak positive correlation. The significance level was established at P<0.05. PTB pregnancies showed a strong positive correlation between maternal WBC count at admission and gene expression of (A) miR-515-5p in placental tissues. A positive correlation between the levels of (B) miR-512-5p and (C) miR-519e-5p in placental tissues of PTB patients and maternal WBC count at admission was observed. A trend to positive correlation between the levels of (D) miR-516-5p, (E) miR-518b and (F) miR-520h in placental tissues of PTB patients and maternal WBC count at admission was observed. WBC, white blood cell; PTB, preterm birth.

ρ=-0.132, P=0.624; miR-525-5p, ρ=0.069, P=0.799; miR-526a, ρ=-0.035, P=0.896; and miR-526b-5p, ρ=-0.117, P=0.948).

The association between C19MC microRNA gene expression in placental tissue and maternal WBC count within the groups of PPROM and PTB pregnancies. No association between maternal WBC count at admission and C19MC microRNA gene expression levels in placental tissues was found in the group of PPROM pregnancies.

Nevertheless, in the group of PTB pregnancies a strong positive correlation (miR-515-5p: ρ=0.508, P=0.026), a positive correlation (miR-512-5p: ρ=0.491, P=0.032; and miR-519e-5p: ρ=0.457, P=0.048), or a trend to positive correlation (miR-516-5p: ρ=0.405, P=0.085; miR-518b: ρ=0.414, P=0.078; and miR-520h: ρ=0.405, P=0.085) between maternal WBC count at admission and some C19MC microRNA gene expression levels in placental tissues was observed (Fig. 3).

Discussion

Different C19MC microRNA expression profiles in different cell types within villous tissue and in different areas of placental tissues were documented. The expression of C19MC microRNAs has been observed at least in first-trimester and full-term placental tissues (73,74), human first and third trimester trophoblast cell lines, ACH-3P and AC1-M59 (75), and placenta-derived stromal cells (67). In our initial study, we have observed the presence of all 15 tested C19MC microRNAs (miR-512-5p, miR-515-5p, miR-516-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-519e-5p, miR-520a-5p, miR-520h, miR-524-5p, miR-525-5p, miR-526a and miR-526b-5p) on the fetal side of the placenta (72). In addition, the set of microRNAs (miR-517c, miR-518a, miR-519d, and miR-520h) forming a cluster on chromosome 19q13 was observed to be expressed in umbilical cord blood $CD34^+$ cells (76).

In addition, our recent study demonstrated that pregnancy-related complications such as gestational hypertension, preeclampsia and fetal growth restriction were associated with downregulation of those C19MC microRNAs that were previously demonstrated to be highly or exclusively expressed in placental tissues (77). The downregulation of 4 of 15 (miR-517-5p, miR-519d, miR-520a-5p and miR-525-5p), 6 of 15 (miR-517-5p, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p and miR-525-5p) and 11 of 15 (miR-515-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p, miR-520h, miR-524-5p, miR-525-5p and miR-526a) microRNAs was associated with gestational hypertension, fetal growth restriction, and preeclampsia, respectively. The aim of the current study was to demonstrate if spontaneous PTB and PPROM are also associated with alterations in placental microRNA expression. Overall, the expression profile of studied C19MC microRNAs was different between spontaneous PTB, PPROM, and term in labor pregnancies.

Most of examined C19MC microRNAs were dysregulated in PTB pregnancies. The analysis revealed the upregulation of 9 C19MC microRNAs (miR-515-5p, miR-516-5p, miR-518b, miR-518f-5p, miR-519a, miR-519e-5p, miR-520a-5p, miR-520h and miR-526b-5p) and a trend toward upregulation for other 2 C19MC microRNAs (miR-519d, and miR-526a) in PTB pregnancies. Therefore, it seems that spontaneous PTB has a dissimilar course as term in labor pregnancies.

With regard to PPROM, the analysis indicated that the levels of only 1 out of 15 C19MC microRNAs were significantly decreased in placental tissues samples (miR-525-5p), which indicates that the course of labor in PPROM pregnancies did not differ as much from term in labor pregnancies as the course of labor in PTB pregnancies. Nevertheless, in some ways similar findings to other pregnancy-related complications may be observed in PPROM pregnancies. Downregulation of miR-525-5p was also found in placental tissues derived from patients with gestational hypertension, preeclampsia, and fetal growth restriction (77).

Moreover, clear evidence was brought, that the pathogenesis of spontaneous PTB and PPROM is different. The analysis demonstrated the difference in expression in almost all examined C19MC microRNAs (12 out of 15 C19MC microRNAs reached statistical significance and 1 out of 15 C19MC microRNAs was on the border of statistical significance). C19MC microRNAs (miR-516b-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-519e-5p, miR-520a-5p, miR-520h, miR-525-5p, miR-526a and miR-526b-5p) were found to be downregulated in placental tissues derived from PPROM pregnancies when compared with gestational age matched PTB pregnancies. A trend toward statistical significance for downregulation of miR-524-5p was observed in PPROM pregnancies.

As the cause of labor still remains elusive, the exact cause of PTB is also unsolved. In fact, the cause of 50% of PTBs is never determined. Labor is a complex process involving many factors. Four different pathways have been identified that can result in PTB and have considerable evidence: precocious fetal endocrine activation, uterine overdistension (placental abruption), decidual bleeding, and intrauterine inflammation/infection (78). Activation of one or more of these pathways may happen gradually over weeks, even months. From a practical point a number of factors have been identified that are associated with PTB, however, an association does not establish causality. Nevertheless, altered miRNA networks may be a consequence of abnormal physiology leading to PTB. In addition, microRNA alterations can disrupt protein homeostasis and may be at the root of PTB.

While the full repertoire of the biological action of C19MC microRNAs remains to be established, data from various expression studies of C19MC microRNAs imply a role for them in cell proliferation, self-renewal, angiogenesis, and particularly in pro-/anti-cancer activity (79-81). In fact, there is not much research data about the function of C19MC microRNAs in the literature.

Available prediction algorithms usually predict hundreds of potential target genes for a single microRNA, but often generate false-positive candidates (82). Although methods to comprehensively identify miRNAs that regulate individual genes of interest are currently available, pathways involving miRNAs are often complex regulatory networks, whose regulation is difficult to understand. Additionally, it makes the direct interpretation of experimental data complicated. Many genes are targeted for repression by a high number of miRNAs, which seem to regulate those genes cooperatively (83).

The decreased levels of C19MC microRNAs in placental tissues of patients with PPROM may lead to upregulation of relevant proteins involved in the direction of key biological pathways such as premature aging of the fetal membranes where senescence, apoptosis and proteolysis play an important role (84-86). The cause of PPROM is multifactorial and next to intraamniotic infection, reduction in membrane collagen content, stretched membranes, vasculopathy in placentation and decidual haemorrhage are considered to be possible mechanisms underlying PPROM (33,35). We have previously published an extensive list of predicted target genes of all downregulated C19MC microRNAs in patients with gestational hypertension, preeclampsia and fetal growth restriction involved in the regulation of the immune system and the inflammatory response (77).

Among these predicted target genes a lot of those involved in apoptosis were identified (TP53, tumor protein p53; CASP2, Caspase-2, apoptosis-related cysteine peptidase; CASP3, Caspase-3, apoptosis-related cysteine peptidase; CASP10,

Caspase-10, apoptosis-related cysteine peptidase; BCL2, B-cell CLL/lymphoma 2; BCL10, B-cell CLL/lymphoma 10; TNF, Tumor necrosis factor; TRAF6, TNF receptor associated factor 6; E3, Ubiquitin protein ligase) that have been previously shown to be upregulated in placental tissues derived from patients with preeclampsia and IUGR (77,87-99). Therefore the downregulation of C19MC microRNAs in placental tissues of patients with PPROM may result in increased levels of these proteins, whose levels are exaggerated in pregnancies with other pregnancy-related complications such as preeclampsia and IUGR.

No association between C19MC microRNA gene expression in placental tissues and maternal serum CRP at admission in groups of PPROM and PTB patients was observed.

Likewise, no effect of maternal WBC count on C19MC microRNA gene expression levels of any of the microRNAs in the group of PPROM patients was demonstrated. Both CRP and leukocyte levels were shown to be increased in serum of patients preceding PPROM (46,100). Nevertheless, maternal WBC count and CRP levels are not specific to intrauterine infections and may be influenced by other factors (101). However, in the group of patients with PTB a positive correlation between 3 out of 15 studied C19MC microRNAs (miR-512-5p, miR-515-5p and miR-519e-5p) and maternal WBC count was identified. A trend towards upregulation was observed for other 3 C19MC microRNAs (miR-516-5p, miR-518b and miR-520h) in the group of PTB patients with increased WBC levels.

Nevertheless, in PPROM group all examined C19MC microRNAs displayed decreased expression with advancing gestational age, which suggests a functional involvement of microRNAs in the translational inhibition of multiple mRNA targets. Parallel, Montenegro *et al* (51) brought similar finding for other 13 microRNAs (miR-199b, miR-373, miR-218, miR-154, miR-338, miR-198, miR-214, miR-370, miR-213, miR-107, miR-199a, miR-222, and miR-330), whose levels were also decreased with advancing gestational age, but this study was performed on chorioamniotic membranes in patients with preterm labor without histologic chorioamnionitis.

In conclusion, this study demonstrated for the first time that PPROM and PTB were associated with altered C19MC microRNA expression profile. The expression profile of placental specific microRNAs was the most distinct between PTB group and women at term in labor, and between gestational age-matched PPROM and PTB groups.

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