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Expression Profile of C19MC microRNAs in Placental Tissue in Pregnancy-Related Complications

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To demonstrate that pregnancy-related complications are associated with alterations in placental microRNA expression. Gene expression of 15 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, miR-526a, and miR-526b) was assessed in placental tissues, compared between groups (21 gestational hypertension [GH], 63 preeclampsia, 36 fetal growth restriction [FGR], and 42 normal pregnancies), and correlated with the severity of the disease with respect to clinical signs, delivery date, and Doppler ultrasound parameters. The expression profile of microRNAs was different between pregnancy-related complications and controls. The downregulation of 4 of 15 (miR-517-5p, miR-519d, miR-520a-5p, and miR-525), 6 of 15 (miR-517-5p, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p, and miR-525), 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, and miR-526a) microRNAs was associated with GH, FGR, and preeclampsia, respectively. Sudden onset of severe preeclampsia requiring immediate termination of gestation and mild forms of preeclampsia (persisting for several weeks) were associated with similar microRNA expression profile (downregulation of miR-517-5p, miR-520a-5p, miR-524-5p, and miR-525). In addition, miR-519a was found to be associated with severe preeclampsia. The longer the pregnancy-related disorder lasted, the more extensive was the downregulation of microRNAs (miR-515-5p, miR-518b, miR-518f-5p, miR-519d, and miR-520h). The downregulation of some C19MC microRNAs is a common phenomenon shared between GH, preeclampsia, and FGR. On the other hand, some of the C19MC microRNAs are only downregulated just in preeclampsia.

Introduction

Preeclampsia (PE) and fetal growth restriction (FGR) are major complications affecting 2–10% of pregnancies responsible for maternal and perinatal morbidity and mortality (WHO, 1988; Bamfo and Odibo, 2011). Preeclampsia usually develops after 20 weeks of gestation and is characterized by chronic or gestational hypertension (GH) combined with proteinuria (ACOG practice bulletin, 2002), which results from defective placentation, eliciting inadequate uteroplacental blood perfusion and ischemia (Khong *et al.*, 1986; Khan *et al.*, 2006). The causes of preeclampsia and FGR remain unknown; however, preeclampsia is thought to be an implantation disorder (Miko *et al.*, 2013). The actual hypothesis regarding the etiology of preeclampsia is based on the presumption that inadequate trophoblast invasion and placentation is linked to maladaptation of the local maternal immune response to paternal antigens expressed by extravillous cytotrophoblast at the fetal–maternal interface (Huppertz, 2008; Roberts and Hubel, 2009).

Recent evidence suggests that preeclampsia can be further subdivided into early PE (before 34 weeks of gestation), intermediate PE (between 34 and 37 weeks of gestation), and late PE (after 37 weeks of gestation) (Poon *et al.*, 2010; Akolekar *et al.*, 2011). The concepts of early and late PE are recent; it is widely accepted that these two entities have different etiologies and should be regarded as different forms of the disease, whereas early-onset PE and intrauterine growth restriction (IUGR) are considered placenta-mediated diseases (von Dadelszen *et al.*, 2003; Huppertz, 2008; Valensise *et al.*, 2008).

MicroRNAs belong to the family of small noncoding RNAs (18–25 nucleotides) that regulate gene expression at the post-transcriptional level by degrading or blocking translation of target messenger RNA (Lai, 2002; Bartel, 2004). MicroRNA analyses indicate that a variety of tissues display microRNA expression profiles that are significantly different from normal tissues (Calin and Croce, 2006), which may be useful for a wide range of applications in clinical diagnostics (Rosenfeld *et al.*, 2008). Recent studies

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have shown that preeclampsia and FGR are associated with alterations in microRNA expression in the placenta (Pineles *et al.*, 2007; Hu *et al.*, 2009; Zhu *et al.*, 2009; Enquobahrie *et al.*, 2011; Maccani *et al.*, 2011; Mayor-Lynn *et al.*, 2011; Noack *et al.*, 2011; Ishibashi *et al.*, 2012; Higashijima *et al.*, 2013). For example, miR-210, upregulated in placental tissues derived from patients with preeclampsia (Pineles *et al.*, 2007; Zhu *et al.*, 2009; Enquobahrie *et al.*, 2011; Ishibashi *et al.*, 2012), was shown to play a role in endothelial cell response to hypoxia, formation of capillary-like structures, vascular endothelial growth factor-driven cell migration, cell differentiation, and survival (Chan *et al.*, 2012). A link between miR-155, also overexpressed in preeclamptic placentas (Pineles *et al.*, 2007; Zhang *et al.*, 2010), and the innate immune response has been suggested. Tili *et al.* (2007) reported that lipopolysaccharide (LPS) stimulation of macrophages resulted in the upregulation of miR-155 and TNF-α, which is one of the main cytokines involved in the response to LPS. Simultaneously, miR-155 was shown to function as an oncogene and as a common target of a broad range of inflammatory mediators, suggesting that miR-155-inducing signals use the c-Jun N-terminal kinase (JNK) pathway (O'Connell *et al.*, 2007).

Patients with established preeclampsia also had increased levels of miR-181a in placental tissues (Hu *et al.*, 2009; Zhu *et al.*, 2009). T-cell sensitivity to antigen is intrinsically regulated during maturation to ensure proper development of immunity and tolerance. Li *et al.* (2007) showed that increasing miR-181a expression in mature T cells augmented the sensitivity to peptide antigens, while inhibiting miR-181a expression in immature T cells reduced sensitivity and impaired both positive and negative selection.

Most pregnancy-associated microRNAs have been shown to be encoded by genes located inside chromosome 19 miRNA clusters (C19MC and miR-371-3 cluster) or the chromosome 14 miRNA cluster (C14MC) (Seitz *et al.*, 2004; Bentwich *et al.*, 2005; Liang *et al.*, 2007; Lin *et al.*, 2010; Morales-Prieto *et al.*, 2013).

In this study, we compared gene expression of C19MC microRNAs in normal and complicated pregnancies. Chromosome 19 microRNA cluster involves 46 microRNA genes altogether (Bentwich *et al.*, 2005; Bortolin-Cavaillé *et al.*, 2009; Lin *et al.*, 2010; Morales-Prieto *et al.*, 2013). The study preferentially used those microRNAs that were previously demonstrated to be exclusively expressed in placental tissues (miR-516-5p, miR-517-5p, miR-518b, miR-519a, miR-519d, miR-525, and miR-526b) and those microRNAs that were reported to be highly expressed in placental tissues (miR-512-5p, miR-515-5p, miR-518f-5p, miR-519e-5p, miR-520a-5p, miR-520h, miR-524-5p, and miR-526a) (Hsu *et al.*, 2008; Kotlabova *et al.*, 2011; Hromadnikova *et al.*, 2012).

To our knowledge, no study on C19MC microRNA expression in GH has been carried out. Our study also describes, for the first time, the expression of most members of C19MC microRNAs in preeclampsia (miR-512-5p, miR-515-5p, miR-516-5p, miR-517-5p, miR-519a, miR-519d, miR-520a-5p, miR-520h, miR-524-5p, and miR-526a) or FGR (miR-512-5p, miR-516-5p, miR-517-5p, miR-518f-5p, miR-519a, miR-519e-5p, miR-524-5p, and miR-526a).

The relationship between C19MC microRNA gene expression and identified risk factors for poorer perinatal outcome was analyzed. C19MC microRNA gene expression was analyzed in relation to the severity of the disease with respect to the degree of clinical signs (mild and severe preeclampsia), delivery date (before or after 34 weeks of gestation), and Doppler ultrasonography parameters (the pulsatility index [PI] in the umbilical artery [UA], the PI in the middle cerebral artery [MCA], and the cerebroplacental ratio [CPR]).

Doppler velocimetry of the placental and fetal circulations is currently the main tool used in clinical practice to identify patients at risk for or those with established ischemic placental disease. Doppler velocimetry is considered abnormal when the PIs of the UA and the MCA are >95th and <5th percentiles for a given gestational age. The CPR is calculated by simple division of the MCA by the UA PIs (Cruz-Martinez, 2009). Indeed, animal models have demonstrated that this ratio is better correlated with hypoxia than its individual components (Arbeille *et al.*, 1995).

Consecutively, the comprehensive online databases for miRNA target prediction and functional annotations were used to collect predicted targets of aberrantly expressed C19MC microRNAs in patients with established pregnancy-related complications; the main subjects of interest were predicted targets that were closely related to regulation of the immune system and the inflammatory response.

Materials and Methods

Patients

The study was retrospective. The studied cohort consisted of 162 consecutive Caucasian pregnant women involving 63 pregnancies with clinically established preeclampsia (PE), 36 pregnancies complicated by FGR, 21 patients with GH, and 42 normal pregnancies. Of the 63 patients with preeclampsia, 30 had symptoms of mild preeclampsia and 33 were diagnosed with severe preeclampsia. Twenty-two preeclamptic patients required delivery before 34 weeks of gestation, and 41 patients delivered after 34 weeks of gestation. Preeclampsia both occurred in previously normotensive patients (44 cases) and was superimposed on preexisting hypertension (19 cases). Eight growth-retarded fetuses were delivered before 34 weeks of gestation, and 28 were delivered after 34 weeks of gestation. Oligohydramnios or anhydramnios were present in 12 growth-restricted fetuses.

An examination of blood flow (Doppler ultrasonography) showed an abnormal PI in the UA (6 preeclampsia and 17 FGR) and/or in the MCA (1 preeclampsia and 8 FGR). The CPR, expressed as a ratio between the MCA and the UA PIs, was below the fifth percentile in nine cases (nine FGR). Absent or reversed end-diastolic velocity waveforms in the UA occurred in four cases (one preeclampsia and three FGR). The clinical characteristics of the normal and complicated pregnancies are presented in Table 1.

Women with normal pregnancies were defined as those without medical, obstetrical, or surgical complications at the time of the study and who subsequently delivered full-term, singleton healthy infants weighing >2500 g after 37 completed weeks of gestation. Preeclampsia was defined as blood pressure $>140/90$ mmHg in two determinations 4 h apart that was associated with proteinuria >300 mg/24 h after 20 weeks of gestation (ACOG practice bulletin, 2002). Severe preeclampsia was diagnosed by the presence of one or more of the following findings: (1) a systolic blood pressure >160 mmHg or a diastolic blood pressure

Table 1. Clinical Characteristics of the Normal and Complicated Pregnancies

	Healthy pregnant women (n = 42)	*Preeclamptic patients (n = 63)*	*FGR patients (n = 36)*	*GH patients (n = 21)*
Age (years)	30.6 (4.4)	31.7 (5.0)	30.4 (5.5)	29.4 (2.9)
Blood pressure (mmHg)				
Systolic	121.6 (14.1)	152.6 (14.2)	123.1 (16.4)	149.8 (9.2)
Diastolic	75.1 (8.2)	96.8 (8.9)	79.0 (11.6)	95.2 (9.8)
Proteinuria (g/24 h)	None	2.7 (6.1)	None	None
Gestational age at delivery (weeks)	39.0 (1.9)	34.3 (4.3)	35.9 (3.7)	38.5 (1.6)
Pregnancy body mass index	26.9 (3.2)	29.6 (4.8)	26.3 (3.8)	31.5 (6.0)
Fetal birth weight (g)	3440.7 (427.1)	2030.6 (889.3)	2111.3 (690.0)	3173.7 (477.9)
Mode of delivery				
Vaginal	36 (85.7%)	9 (14.3%)	8 (22.2%)	15 (71.4%)
Cesarean section	6 (14.3%)	54 (85.7%)	28 (77.8%)	6 (28.6%)
Fetal sex				
Boy	26 (61.9%)	23 (36.5%)	20 (55.6%)	7 (33.3%)
Girl	16 (38.1%)	40 (63.5%)	16 (44.4%)	14 (66.7%)
Glucose status				
Normal	38 (90.5%)	60 (95.2%)	36 (100%)	21 (100%)
GDM/DM	4 (9.5%)	3 (4.8%)	0 (0%)	0 (0%)

Data are presented as mean and (standard deviation) for continuous variables and as number (percent) for categorical variables.
FGR, fetal growth restriction; GDM/DM, gestational diabetes mellitus/diabetes mellitus; GH, gestational hypertension.

> 110 mmHg, (2) proteinuria greater than 5 g of protein in a 24-h sample, (3) very low urine output (less than 500 mL in 24 h), (4) signs of respiratory problems (pulmonary edema or cyanosis), (5) impairment of liver function, (6) signs of central nervous system problems (severe headache, visual disturbances), (7) pain in the epigastric area or right upper quadrant, (8) thrombocytopenia, and (9) the presence of severe FGR (ACOG practice bulletin, 2002).

FGR was diagnosed when the estimated fetal weight, calculated using the Hadlock formula (Astraia Software GmbH), was below the tenth percentile for the evaluated gestational age; adjustments were made for the appropriate population standards of the Czech Republic.

Patients with a complicated gestation demonstrating premature rupture of membranes, *in utero* infections, fetal anomalies or chromosomal abnormalities, and fetal demise *in utero* or stillbirth were excluded from the study.

All patients who participated in this study provided written informed consent. The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University in Prague.

Processing of samples, preparation of microRNAs

Samples of placenta were collected at the Institute for the Care of Mother and Child and stored at −80°C until further processing.

Total RNA was extracted from 25 mg of placental tissue preserved in RNAlater (Ambion) followed by an enrichment procedure for small RNAs (siRNAs, microRNAs), according to the manufacturer's instructions using a mirVana microRNA Isolation kit (Ambion). To minimize DNA contamination, we treated the eluted RNA with 5 μL of DNase I (Fermentas International) for 30 min at 37°C. Using this novel approach, an RNA fraction highly enriched in RNA species < 200 nt was obtained, whose concentration and quality was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). 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. In addition, 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, 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, miR-526a, and miR-526b) was reverse transcribed into complementary DNA (cDNA) using a TaqMan MicroRNA Assay, containing microRNA-specific stem-loop reverse transcription (RT) primers (Table 2), and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in a total reaction volume of 32 μL, according to the manufacturer's instructions. Reverse transcriptase reactions were performed using a 7500 Real-Time PCR system (Applied Biosystems) with the following thermal cycling parameters: 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C, and then held at 4°C. Finally, 20 ng of the RNA template was used for each RT reaction.

Relative quantification of microRNAs by real-time polymerase chain reaction

Fifteen microliters 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. TaqMan polymerase chain reaction (PCR) conditions were set as described in the TaqMan guidelines.

Table 2. 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

miRNAMap 2.0 designers themselves performed the quantitative polymerase chain reaction (Q-PCR) experiments for monitoring the expression profiles of 224 human miRNAs in 18 major normal tissues in humans, including placental tissues. The miRNAMap release 2.0 is now available at http://miRNAMap.mbc.nctu.edu.tw/ (Hsu *et al.,* 2008).

The analysis was performed using a 7500 Real-Time PCR System. All polymerase chain reactions (PCRs) were performed in duplicate. Multiple negative controls such as no template control (NTC) (water instead of cDNA sample), no amplification control (NAC) (nontranscribed 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 threshold cycle ($Ct < 40$).

The expression of particular microRNA was determined using the comparative Ct method (Livak and Schmittgen, 2001) relative to synthetic Caenorhabditis *elegans* microRNA (cel-miR-39; Qiagen), which was used as an internal control for variations during the preparation of RNA, cDNA synthesis, and real-time PCR. Due to a lack of generally accepted standards, all experimental real-time quantitative real-time polymerase chain reaction (qRT-PCR) data were normalized to cel-miR-39, as it shows no sequence homology to any human microRNA. One microliter of 100 nM cel-miR-39 was spiked in after incubation with miRNA Homogenate Additive (a component of mirVana microRNA Isolation kit; Ambion) to the human placental samples.

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. The difference (ΔCt) between the Ct values of particular microRNA and the internal control (cel-miR-39) was calculated for each sample. The comparative $\Delta\Delta$Ct calculation involved finding the difference between each sample's ΔCt and the reference's ΔCt. Finally, $\Delta\Delta$Ct values were transformed to absolute values using the formula $2^{-\Delta\Delta Ct}$. 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 followed a normal distribution. Therefore, microRNA levels were compared between groups using parametric tests. To test for a significant difference among multiple groups, the one-way analysis of variance and the *post hoc* Bonferroni's test was applied. The Benjamini–Hochberg (BH) correction was applied to the normalized expression data to adjust for multiple comparisons in order to limit a false discovery rate (FDR). The significance level was established at an adjusted *p*-value ($q^* = 0.022$ for group comparison; $q^* = 0.022$ for comparison of preeclampsia severity with respect to clinical signs; $q^* = 0.025$ for comparison of preeclampsia severity with respect to delivery date; q^* is the corrected significance level after Benjamini and Hochberg for multiple testing). Similarly, the two-tailed Student's t-test was used for the comparisons between two groups. Again, if necessary, the FDR approach was used to correct for multiple comparisons ($q^* = 0.0045$ for comparison of disease severity with respect to Doppler ultrasonography of UA flow).

Data analysis was performed, and box plots were generated using Statistica software (version 9.0; StatSoft, Inc.). Each box encompasses the mean (dark horizontal line) of

log-normalized gene expression values for microRNAs of interest in cohorts, one standard error above and below the mean in the box, and the 95% confidence interval in the bars (standard deviation). Outliers are indicated by circles, and extremes are indicated by asterisks.

C19MC microRNA target prediction

The predicted targets of studied C19MC microRNAs were collected from the miRDB database (http://mirdb.org/miRDB/) and the miRWalk database (www.umm.uni-heidelberg.de/apps/zmf/mirwalk). miRDB is an online database for miRNA target prediction and functional annotations. All the targets were predicted using MirTarget2 bioinformatics tool, which was developed by analyzing thousands of genes impacted by miRNAs with an SVM learning machine (Wang and El Naqa, 2008). miRDB hosts predicted miRNA targets in five species: human, mouse, rat, dog, and chicken.

The miRDB database is connected to the NCBI database (www.ncbi.nlm.nih.gov/gene), where the description of proteins encoded by predicted genes is provided. Comprehensive and systematic search for each predicted target of a particular C19MC microRNA, which have been shown to be downregulated in patients with the onset of GH, preeclampsia, and FGR in relation to the regulation of the immune system and inflammatory response, was made using the PubMed database (www.ncbi.nlm.nih.gov/pubmed).

Further, miRWalk database (www.umm.uni-heidelberg.de/apps/zmf/mirwalk) and the Validated Targets module were used to provide information on an experimentally verified interaction between appropriate microRNA and specific genes (Dweep *et al.*, 2011). miRWalk is a comprehensive database that provides information on miRNA from human, mouse, and rat on their predicted as well as validated binding sites on their target genes.

Results

After correction for FDR ($q^* = 0.043$), overall, significantly decreased expression of *miR-515-5p* ($F = 3.893$, df 3,160, $p = 0.01$), *miR-516-5p* ($F = 3.135$, df 3,160, $p = 0.027$), *miR-517-5p* ($F = 10.532$, df 3,160, $p < 0.001$), *miR-518b* ($F = 3.950$, df 3,160, $p = 0.009$), *miR-518f-5p* ($F = 4.989$, df 3,160, $p = 0.002$), *miR-519a* ($F = 5.449$, df 3,160, $p = 0.001$), *miR-519d* ($F = 5.179$, df 3,160, $p = 0.002$), *miR-519e-5p* ($F = 3.316$, df 3,160, $p = 0.021$), *miR-520a-5p* ($F = 8.162$, df 3,160, $p < 0.001$), *miR-520h* ($F = 5.122$, df 3,160, $p = 0.002$), *miR-524-5p* ($F = 4.645$, df 3,160, $p = 0.004$), *miR-525* ($F = 6.810$, df 3,160, $p < 0.001$), and *miR-526a* ($F = 4.377$, df 3,160, $p = 0.006$) was observed in women with pregnancy-related complications (GH, preeclampsia, and FGR) compared with all together with normal pregnancies. Bonferonni correction for multiple comparisons ($q^* = 0.0033$) confirmed downregulation of the following microRNA biomarkers (*miR-517-5p*, *miR-518f-5p, miR-519a*, *miR-519d*, *miR-520a-5p*, *miR-520h*, and *miR-525*).

Downregulation of miR-517-5p, miR-519d, miR-520a-5p, *and* miR-525 *is a common feature of GH, preeclampsia, and FGR*

After an FDR adjustment for multiple testing ($q^* = 0.022$), it was found that the expression of *miR-517-5p, miR-519d, miR-520a-5p,* and *miR-525* differed significantly between the control group and pregnancies affected with pregnancy complications. Lower expression rates were detected in patients with GH (*miR-517-5p*: 0.589 ± 0.666 vs. 3.821 ± 4.938, $p < 0.001$; *miR-519d*: 0.671 ± 0.772 vs. 2.423 ± 3.454, $p = 0.019$; *miR-520a-5p*: 1.066 ± 1.609 vs. 4.320 ± 6.586, $p = 0.006$; *miR-525*: 0.901 ± 1.872 vs. 4.089 ± 6.931, $p = 0.017$), preeclampsia (*miR-517-5p*: 0.873 ± 1.452 vs. 3.821 ± 4.938, $p < 0.001$; *miR-519d*: 0.934 ± 1.823 vs. 2.423 ± 3.454, $p = 0.004$; *miR-520a-5p*: 1.093 ± 1.867 vs. 4.320 ± 6.586, $p < 0.001$; *miR-525*: 0.905 ± 2.236 vs. 4.089 ± 6.931, $p < 0.001$), and FGR (*miR-517-5p*: 1.239 ± 2.242 vs. 3.821 ± 4.938, $p < 0.001$; *miR-519d*: 0.959 ± 0.974 vs. 2.423 ± 3.454, $p = 0.021$; *miR-520a-5p*: 1.061 ± 1.075 vs. 4.320 ± 6.586, $p < 0.001$; *miR-525*: 0.904 ± 1.352 vs. 4.089 ± 6.931, $p = 0.002$) (Fig. 1). Using the more stringent Bonferroni-corrected significance level for multiple comparison ($q^* = 0.001$), a significant decrease in *miR-517-5p* expression was confirmed for all three diagnoses.

Downregulation of miR-518f-5p *and* miR-519a *represents a common feature of preeclampsia and FGR*

After FDR analysis ($q^* = 0.022$), a significant difference in *miR-518f-5p* and *miR-519a* expression was found between the control group and pregnancies affected with preeclampsia (0.798 ± 1.643 vs. 2.262 ± 3.475, $p = 0.004$; 1.258 ± 2.954 vs. 9.992 ± 21.452, $p = 0.001$) and FGR (0.793 ± 0.872 vs. 2.262 ± 3.475, $p = 0.018$; 2.508 ± 4.829 vs. 9.992 ± 21.452, $p = 0.022$) (Fig. 2).

Downregulation of miR-515-5p, miR-518b, miR-520h, miR-524-5p, and miR-526a represents a unique feature of preeclampsia

After FDR correction ($q^* = 0.022$), statistical analysis revealed that placental expression of *miR-515-5p* (0.923 ± 2.317 vs. 2.581 ± 3.981, $p = 0.013$), *miR-518b* (1.096 ± 3.087 vs. 3.235 ± 5.757, $p = 0.021$), *miR-520h* (0.798 ± 1.449 vs. 1.943 ± 2.366, $p = 0.003$), *miR-524-5p* (0.672 ± 1.055 vs. 2.434 ± 3.784, $p = 0.002$), and *miR-526a* (0.832 ± 2.122 vs. 5.939 ± 14.978, $p = 0.007$) differed significantly between preeclamptic and normal pregnancy groups (Fig. 3).

The association study of microRNA expression and the severity of preeclampsia with respect to clinical signs and delivery date

Gene expression of those C19MC microRNAs that were found to be downregulated in preeclampsia were analyzed in relation to the severity of the disease with respect to the degree of clinical signs (mild vs. severe preeclampsia) and delivery dates (before or after 34 weeks of gestation). When compared with normal pregnancies, using FDR analysis ($q^* = 0.022$), significant downregulation of four microRNAs was observed in both mild (*miR-517-5p*: 0.636 ± 1.495 vs. 3.821 ± 4.938, $p < 0.001$; *miR-520a-5p*: 0.963 ± 2.421 vs. 4.320 ± 6.586, $p = 0.006$, *miR-524-5p*: 0.572 ± 1.343 vs. 2.434 ± 3.784, $p = 0.008$ and *miR-525*: 0.876 ± 3.071 vs. 4.089 ± 6.931, $p = 0.017$) and severe preeclampsia (*miR-517-5p*: 1.076 ± 1.383 vs. 3.821 ± 4.938, $p = 0.001$; *miR-520a-5p*: 1.205 ± 1.192 vs. 4.320 ± 6.586, $p = 0.008$, *miR-524-5p*: 0.757 ± 0.712 vs. 2.434 ± 3.784, $p = 0.014$ and *miR-525*:

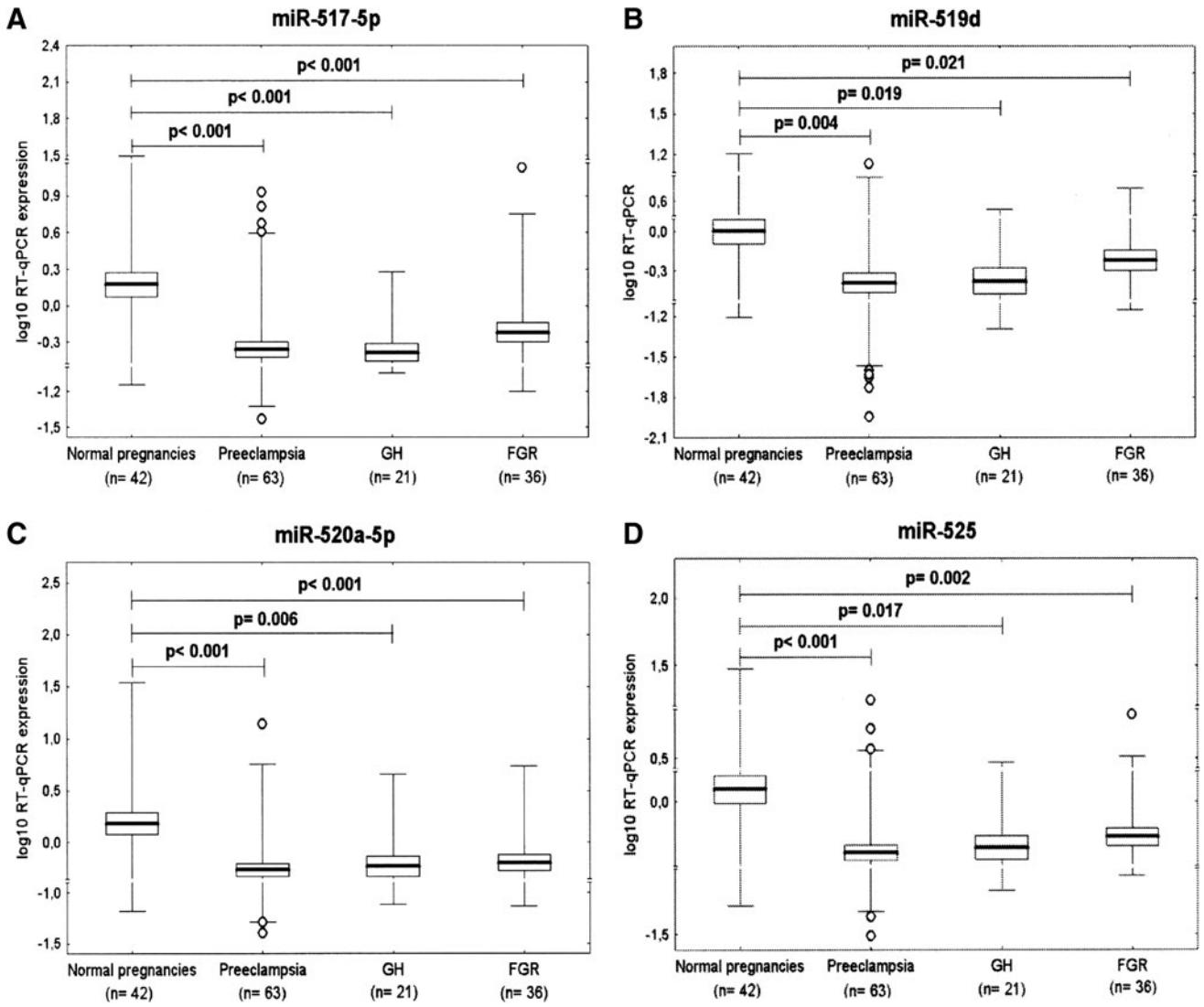

FIG. 1. Downregulation of **(A)** *miR-517-5p*, **(B)** *miR-519d*, **(C)** *miR-520a-5p*, and **(D)** *miR-525* is a common feature of gestational hypertension, preeclampsia, and fetal growth restriction (FGR). GH, gestational hypertension.

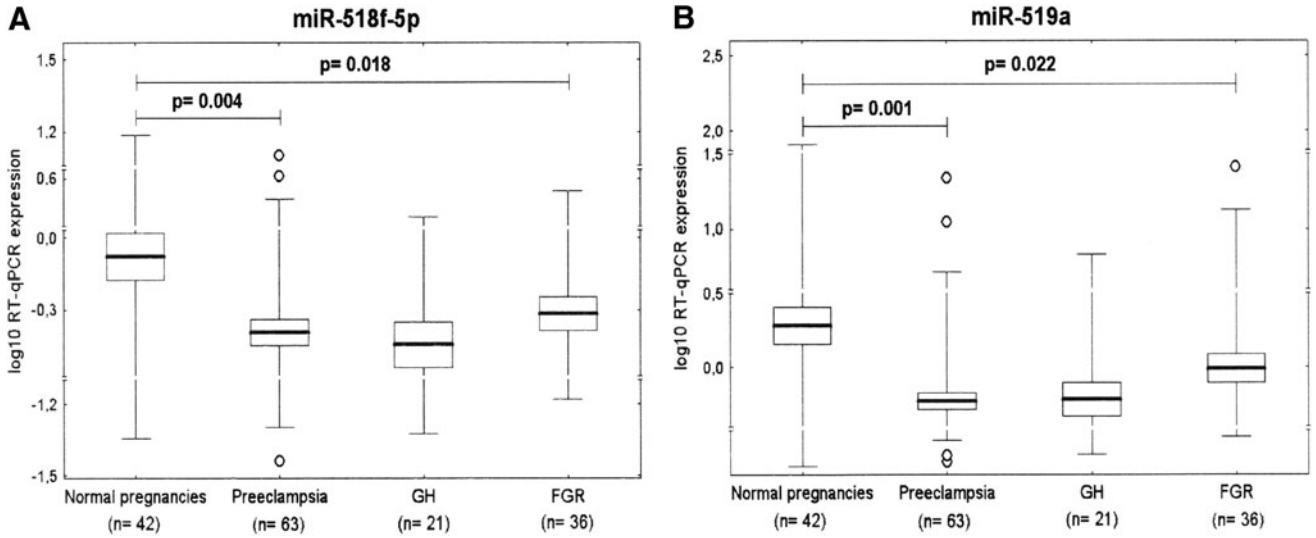

FIG. 2. Downregulation of **(A)** *miR-518f-5p* and **(B)** *miR-519a* represents a common feature of preeclampsia and FGR.

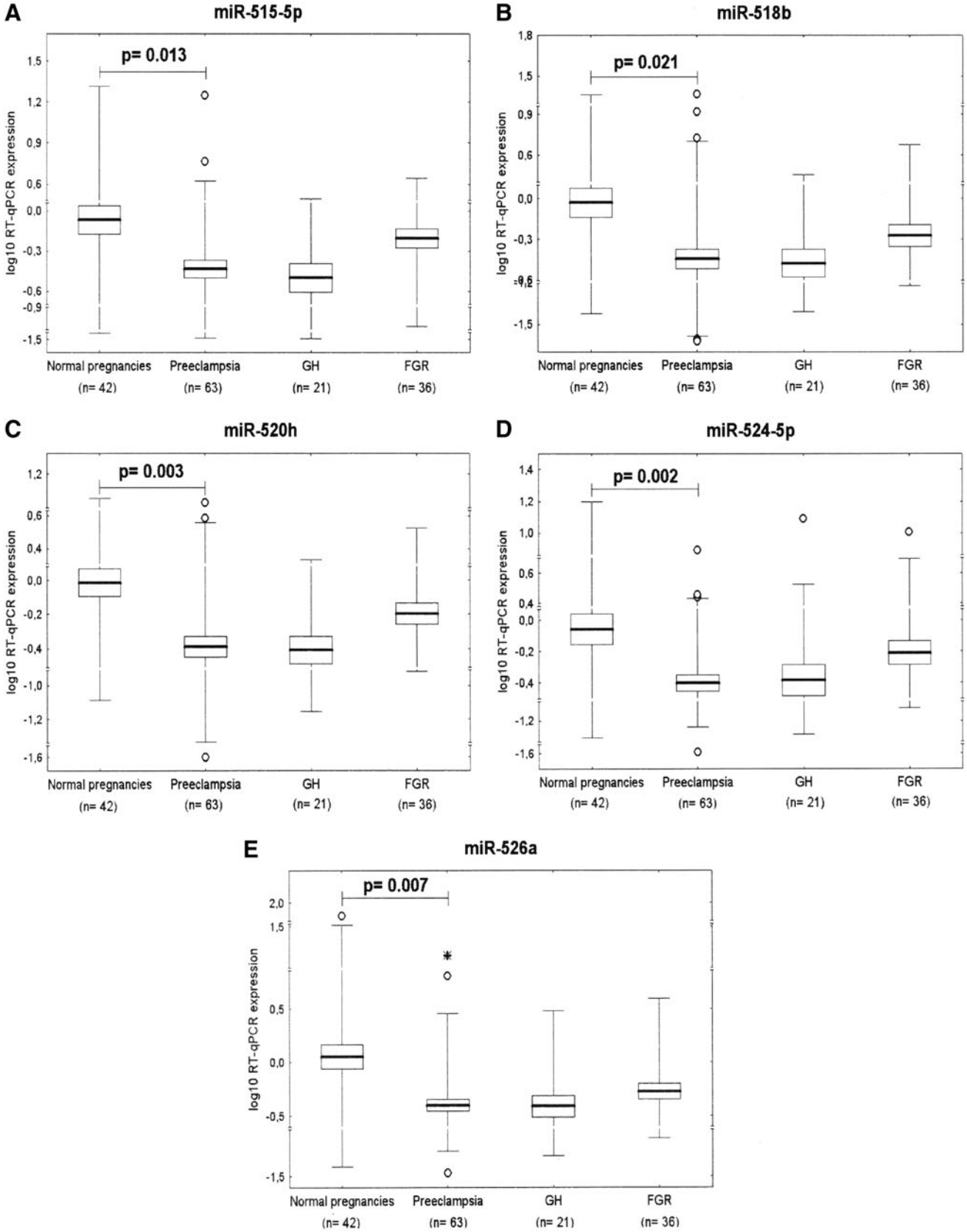

FIG. 3. Downregulation of **(A)** *miR-515-5p*, **(B)** *miR-518b*, **(C)** *miR-520h*, **(D)** *miR-524-5p*, and **(E)** *miR-526a* represents a unique feature of preeclampsia.

0.929 ± 1.094 vs. 4.089 ± 6.931, $p = 0.013$). Expression of *miR-519a* was significantly lower (1.266 ± 1.866 vs. 9.992 ± 21.452, $p = 0.021$), but only in patients with severe preeclampsia. Similarly, decreased expression of *miR-520h* (0.690 ± 1.887 vs. 1.943 ± 2.366, $p = 0.020$) was observed exclusively in patients with mild preeclampsia (Fig. 4).

After FDR analysis ($q^* = 0.025$), while gene expression of C19MC microRNAs differed significantly between preeclamptic pregnancies delivering after 34 weeks of gestation and normal pregnancies (*miR-515-5p*: 1.613 ± 3.483 vs. 2.581 ± 3.981, $p = 0.009$; *miR-517-5p*: 0.694 ± 0.935 vs. 3.821 ± 4.938, $p < 0.001$; *miR-518b*: 0.622 ± 1.373 vs. 3.235 ± 5.757, $p = 0.023$; *miR-518f-5p*: 0.547 ± 0.710 vs. 2.262 ± 3.475, $p = 0.008$; *miR-519a*: 0.902 ± 1.702 vs. 9.992 ± 21.452, $p = 0.010$; *miR-519d*: 0.637 ± 0.941 vs. 2.423 ± 3.454, $p = 0.007$; *miR-520a-5p*: 0.856 ± 0.995 vs. 4.320 ± 6.586, $p = 0.002$; *miR-520h*: 0.540 ± 0.728 vs. 1.943 ± 2.366, $p = 0.003$; *miR-524-5p*: 0.488 ± 0.503 vs. 2.434 ± 3.784, $p = 0.002$ and *miR-525*: 0.571 ± 0.884 vs. 4.089 ± 6.931, $p = 0.003$), the difference between preeclamptic pregnancies requiring delivery before 34 weeks of gestation and controls was only observed for *miR-517-5p* (1.179 ± 2.017 vs. 3.821 ± 4.938, $p = 0.007$) (Fig. 5).

However, microRNA gene expression levels did not differ between 17 pregnancies with severe preeclampsia that required delivery before 34 weeks of gestation and 23 mild preeclampsia patients delivering after 34 weeks of gestation (*miR-515-5p*: $p = 1.0$; *miR-517-5p*: $p = 0.885$; *miR-518b*: $p = 1.0$; *miR-518f-5p:* $p = 1.0$; *miR-519a:* $p = 1.0$; *miR-519d*: $p = 1.0$; *miR-520a-5p*: $p = 1.0$; *miR-520h:* $p = 0.955$; *miR-524-5p*: $p = 0.828$ and *miR-525:* $p = 1.0$). Similarly, no differences in microRNA expression were observed in 18 patients suffering from severe preeclampsia who delivered after 34 weeks of gestation compared with 23 patients with mild preeclampsia who delivered after 34 weeks of gestation (*miR-517-5p*: $p = 0.797$; *miR-518b*: $p = 1.0$; *miR-518f-5p:* $p = 1.0$; *miR-519a:* $p = 1.0$; *miR-519d*: $p = 1.0$; *miR-520a-5p*: $p = 1.0$; *miR-520h:* $p = 1.0$; *miR-524-5p*: $p = 1.0$ and *miR-525:* $p = 1.0$). Since few patients with mild preeclampsia delivered before 34 weeks of gestation due to other reasons such as acute respiratory failure, they were excluded from mutual comparison.

In addition, the association between C19MC microRNA gene expression and the occurrence of previous hypertension in the group of patients with preeclampsia was determined. The statistical analyses revealed no difference between the group of preeclampsia superposed on chronic hypertension and/or GH and the group of patients with unexpected onset of preeclampsia (*miR-515-5p*: $p = 0.392$, *miR-517-5p*: $p = 0.792$, *miR-518b*: $p = 0.423$, *miR-518f-5p:* $p = 0.348$, *miR-519a:* $p = 0.336$, *miR-519d*: $p = 0.370$, *miR-520a-5p*: $p = 0.298$, *miR-520h:* $p = 0.465$, *miR-524-5p*: $p = 0.206$, *miR-525:* $p = 0.437$, and *miR-526a:* $p = 0.352$).

The association study of microRNA expression and the severity of the disease with respect to Doppler ultrasonography monitoring

The association between gene expression of particular C19MC microRNAs (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, and miR-526a) and Doppler ultrasonography parameters (the PI in the UA, the PI in the MCA, and the CPR) was analyzed in the cohort of pregnancies complicated with preeclampsia or FGR. The FDR approach ($q^* = 0.0045$) used to correct for multiple comparisons revealed the difference in 1 out of the 11 microRNAs tested (*miR-524-5p*, 0.472 ± 0.394 vs. 1.216 ± 1.676, $p = 0.0041$) within the group of complicated pregnancies with normal and abnormal values of flow rate in the UA. Pregnancy-related complications with normal blood flow velocity waveforms showed significantly decreased expression of *miR-524-5p* compared with patients with abnormal values of flow rate in the UA (Fig. 6). Nevertheless, statistical analysis showed no effect of the PI in the MCA and the CPR on microRNA gene expression levels of any of the microRNAs identified to be downregulated in placental samples derived from patients with preeclampsia and/or FGR (*miR-515-5p*: $p = 0.661$, $p = 0.724$; *miR-517-5p*: $p = 0.721$, $p = 0.380$; *miR-518b:* $p = 0.820$, $p = 0.522$; *miR-518f-5p:* $p = 0.797$, $p = 0.729$; *miR-519a*: $p = 0.705$, $p = 0.252$; *miR-519d:* $p = 0.953$, $p = 0.333$; *miR-520a-5p*: $p = 0.836$, $p = 0.491$; *miR-520h*: $p = 0.982$, $p = 0.841$; *miR-524-5p:* $p = 0.485$, $p = 0.653$; *miR-525*: $p = 0.803$, $p = 0.477$; and *miR-526a*: $p = 0.926$, $p = 0.290$).

C19MC microRNA target prediction

The extensive file of predicted targets of all downregulated C19MC microRNAs in patients with established GH, preeclampsia, or FGR indicates that a large group of genes is potentially involved in the regulation of the immune system and the inflammatory response (Tables 3 and 4).

Discussion

Gene expression of C19MC microRNAs was compared between normal and complicated pregnancies. We focused on those miRNAs that were exclusively expressed in placental tissues (miR-516-5p, miR-517-5p, miR-518b, miR-519a, miR-519d, miR-525, and miR-526b) and those with high expression levels in placental tissues (miR-512-5p, miR-515-5p, miR-518f-5p, miR-519e-5p, miR-520a-5p, miR-520h, miR-524-5p, and miR-526a). These choices were based on the miRNAmap database (Hsu *et al.*, 2008) and on the results of our previous research (Kotlabova *et al.*, 2011; Hromadnikova *et al.*, 2012).

Overall, the expression profile of 13 of the 15 C19MC microRNAs was different between complicated pregnancies and controls. With regard to individual pregnancy-related disorder subtypes, downregulation of C19MC microRNAs was most common in patients with preeclampsia and less common in GH.

While downregulation of only 4 out of 15 studied C19MC microRNAs was found in placental tissues derived from patients with GH (miR-517-5p, miR-519d, miR-520a-5p, and miR-525), placental tissues from pregnancies with clinically established preeclampsia showed decreased expression in 11 out of 15 C19MC microRNAs (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, and miR-526a). In addition, 6 out of 15 placental-specific microRNAs were also identified to be associated with FGR pregnancies (miR-517-5p, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p, and miR-525).

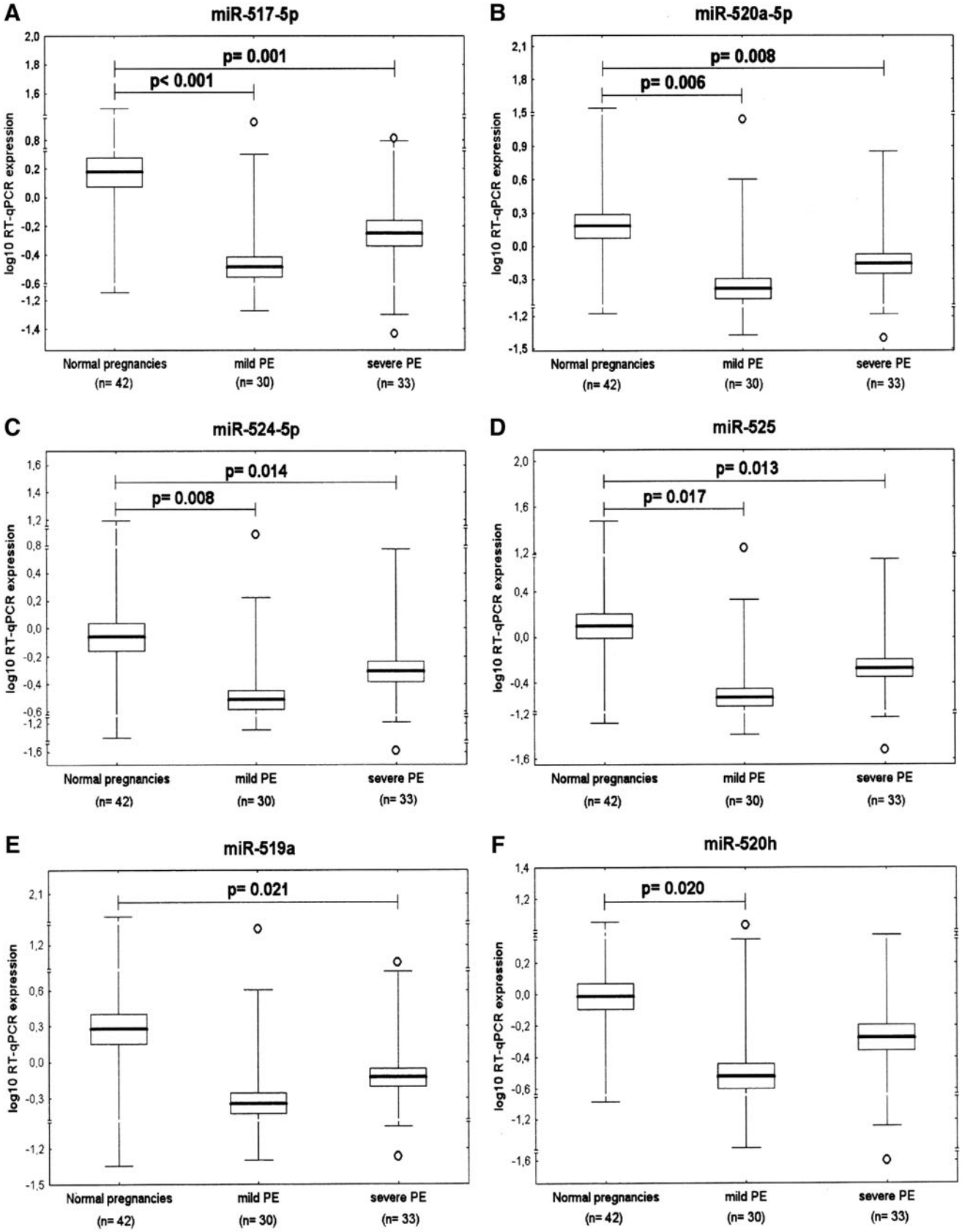

FIG. 4. The association study of microRNA expression and the severity of preeclampsia with respect to clinical signs shows downregulation of **(A)** *miR-517-5p*, **(B)** *miR-520a-5p*, **(C)** *miR-524-5p*, and **(D)** *miR-525* in both, mild and severe PE, and significantly lower expression of **(E)** *miR-519a* in mild and **(F)** *miR-520h* in severe PE. PE, preeclampsia.

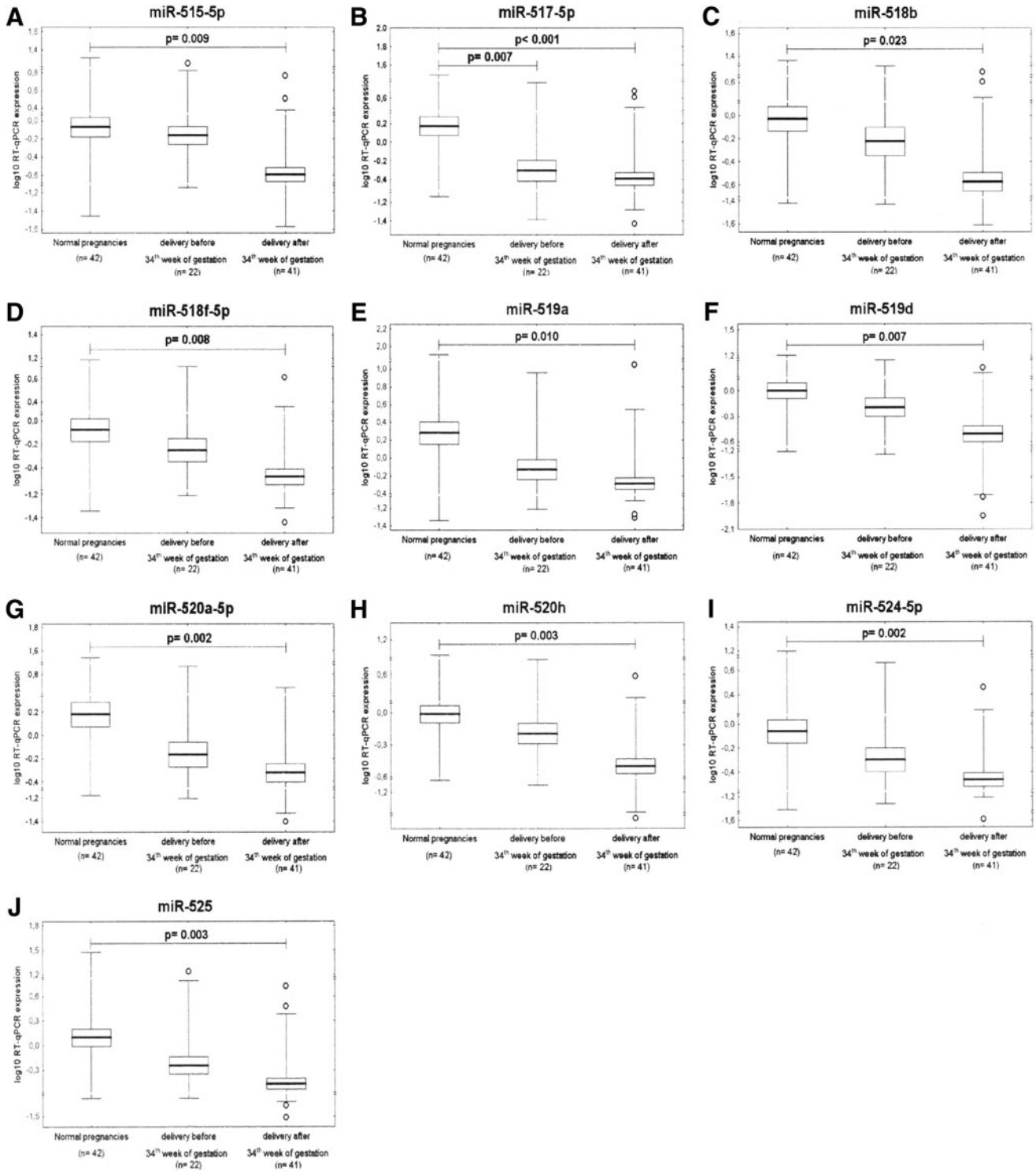

FIG. 5. The association study of microRNA expression and the severity of preeclampsia with respect to delivery date reveals downregulation of **(A)** *miR-515-5p*, **(B)** *miR-517-5p*, **(C)** *miR-518b*, **(D)** *miR-518f-5p*, **(E)** *miR-519a*, **(F)** *miR-519d*, **(G)** *miR-520a-5p*, **(H)** *miR-520h*, **(I)** *miR-524-5p* and **(J)** *miR-525* in PE cases delivering after 34th week of gestation. Lower expression in PE cases requiring delivery before 34th week of gestation is evident only in **(B)** *miR-517-5p*.

Our finding that miR-518b expression was decreased in preeclampsia is consistent with the observation of Guo *et al.* (2011), who also independently demonstrated significantly decreased miR-518b expression levels in preeclampsia compared with controls. A study by Mayor-Lynn *et al.* (2011) showed a 0.74-fold change, but without a significant difference between preeclampsia and normal pregnancies. On the other hand, studies by Zhu *et al.* (2009) and Xu *et al.* (2014) demonstrated upregulation of miR-518b in severe preeclampsia compared with pregnancies with normal

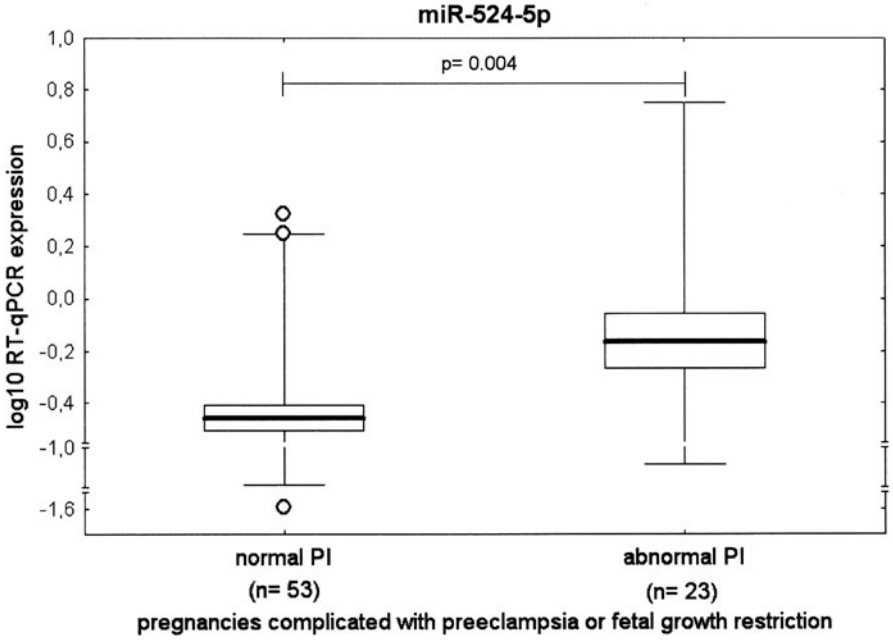

FIG. 6. The association study of microRNA expression and the severity of the disease with respect to Doppler ultrasonography monitoring (the pulsatility index in the umbilical artery).

gestation. The difference in the expression levels of placenta specific miR-518b might be explained by different experimental approaches. Zhu *et al.* (2009) performed the study mainly using tissue from the decidual side of the placenta (a collection of pooled tissue fragments derived from 10 randomly selected sites on the placenta) using microarray and real-time RT-PCR, with the experimental data normalized to U6snRNA. Nevertheless, Xu *et al.* (2014) observed upregulated expression of miR-518b in basal plates of severe preeclampsia placentas using qRT-PCR. However, our group focused on the analysis of microRNA gene expression in the area of the central cotyledon and experimental real-time qRT-PCR data were normalized to synthetic *C. elegans* microRNA (cel-miR-39). The placenta is divided into cotyledons, each supplied by a major branch of the UA, and drained by a major tributary of the umbilical vein. These vessels enter stem villi, which branch and re-branch, similar to a tree, to form microscopic terminal villi suspended within the intervillous space. Each cotyledon has several anchoring villi that extend into the decidua basalis and are anchored to it by syncytial cells and fibrin.

With regard to miR-525, our study produced similar findings to Guo *et al.* (2011), in which they reported significantly lower levels in preeclamptic placentas. Nevertheless, our data are inconsistent with the study of Ishibashi *et al.* (2012), who found miR-525 to be significantly upregulated in preeclamptic placentas. While Ishibashi *et al.* (2012) also revealed upregulation of miR-518f-5p, miR-526b, and miR-519e-5p in preeclamptic placentas, our study found downregulation of miR-518f-5p and no statistically significant change in miR-519e-5p and miR-526b gene expression levels. Ishibashi *et al.* combined large-scale profiling of miRNA expression using high-throughput sequencing with comprehensive quantitative analysis of miRNA expression with real-time PCR-based array analysis. However, they did not provide details regarding the location of placental tissue sampling.

Our data confirmed the preliminary data of Mayor-Lynn *et al.* (2011), who also found no difference in miR-526b expression between patients with preeclampsia and normal pregnancies. Mayor-Lynn *et al.* (2011) used microarray miRNA profiling followed by validation analysis using real-time PCR with normalization to RNU6B and 18SrRNA.

A unique study by Higashijima *et al.* (2013) identified significantly decreased expression of miR-515-5p, miR-518b, miR-519d, miR-520h, and miR-526b in idiopathic FGR pregnancies with an undefined cause of FGR. In conformity with Higashijima *et al.* (2013), our independent study also showed reduced expression of miR-519d in placentas affected with FGR; however, this downregulation was also present in preeclampsia and GH. Nevertheless, in our setting, miR-515-5p, miR-518b, and miR-520h were significantly lower, but just in placentas from preeclamptic pregnancies. Higashijima *et al.* (2013) also observed no change in gene expression of miR-520a-5p and miR-525 in placentas derived from fetal growth-restricted fetuses; however, we observed significantly decreased expression levels of miR-520a-5p and miR-525 in placentas affected by all of the currently studied pregnancy-related complications.

Higashijima *et al.* (2013) carried out the study on a larger group of patients by identifying aberrantly expressed microRNAs using next-generation sequencing analysis with subsequent confirmation from quantitative real-time RT-PCR. However, they performed only the quantitative microRNA measurement, and similar to most investigators, they did not specify the location of placental tissue sampling.

Based on the results of our study, we further studied the association between microRNA expression in placental tissues and the severity of the disease with respect to the degree of clinical signs, delivery date (before or after 34 weeks of gestation), and Doppler ultrasound examination. Placental-specific microRNA gene expression appeared to be linked to the sudden onset of severe clinical symptoms requiring urgent termination of pregnancy by Caesarean section, to avoid potentially serious maternal and perinatal outcomes. Our results showed that five C19MC microRNAs (*miR-517-5p*, *miR-520a-5p*, *miR-524-5p*, *miR-525*, and *miR-*

Table 3. The List of Predicted Target Genes of Differentially Expressed C19MC microRNAs in Gestational Hypertension, Preeclampsia, and Fetal Growth Restriction in Relation to the Immune System and the Inflammatory Response

microRNA	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	miR-526a
Number of predicted target genes	571	239	30	215	685	844	486	462	916	482	215
Unique target genes	API5	FAS	CD5	LILRA1	BAG5	BCL10	ELF4	DIDO1	API5	TOX	BCAP29
	CIAPIN1	IL9R	PRDX6	EGLN3	CD69	CASP2	CCL7	IFNAR1	BCL10	OLR1	CD302
	HSPA12A	IRAK3	TOLLIP	FLT3	CXCL12	CD69	SOCS3	PIAS2	CD276	IGF1R	DNAJC21
	HSPH1	LILRA2		TLR6	DNAJB9	CD84	OLR1	RFX4	CD47	TLR7	HSP90AA1
	IL16	MTDH		TNFRSF19	FZD6	DNAJB6	IGF1R	TGFB2	CD8A	ACVR2B	IGFBP1
	IL6R	PAPPA		CXCL11	HIF1A	HSPA8	TLR7	THAP2	HSPA1B	ATRN	TLR2
	TNFAIP8L1	BCLAF1[a] (Yoshitomi *et al.,* 2011)		TNFSF4	HSPA13	IL17RD	ACVR2B	TLR5	IL1A	CD2	TNFRSF19
	XIAP	IFNA1		ADAM19	HSPA8	IL8	ATRN	CD38	IL1RL1	CD300LB	TNFSF15
	CASP10	IL6		TLR2	IER3IP1	MCL1	CD2	SMAD6	SMAD4	CD46	TRAF6
	CD46	NFKB1		ADAM9	NFAT5	PDCD1LG2	CD300LB	TGFBR2	SMURF2	CD93	
	ATRN	CASP10			PDCD1LG2	SMAD5	CD46	VEGFA[a] (Ye *et al.,* 2008)	SOCS4	HSF5	
	IL10RA	BCL2			TNF	TGFBR2	CD93	HIF1A	TNFAIP1	IL10RA	
	TGFBR2				TNFRSF10D	TNFAIP1	HSF5	PAFAH1B2	CD46	MMD2	
					BBC3	TNFSF11	IL10RA	BNIP3L	CD1A	PPARA	
					BCL2L2[a] (Huang *et al.,* 2011)	BID	MMD2	IRAK4	IRAK2	AHSA2	
					CASP3[a] (Huang *et al.,* 2011)	CPAMD8	PPARA	SMAD2	IRAK4		
					CISH	PPARA[a] (Martinelli *et al.,* 2010)	AHSA2	SMAD3	SMAD2		
					FLT1	TNFRSF1A		SMAD7	SMAD3		
					BCL2[a] (Huang *et al.,* 2011)	TP53[a] (Fornari *et al.*, 2012)					
					BNIP3L[a] (Ye *et al.,* 2008)	F3					
					IRAK2	HIF1A					
					SMAD7	PAFAH1B2					
					TGFBR2	BNIP3L					
					TNFRSF21	IRAK4					
					TOLLIP	SMAD7					
						TNFRSF21					

[a]Experimentally verified targets according to miRWalk database.

Table 4. The Role of Predicted Target Genes of Differentially Expressed C19MC microRNAs in the Pathogenesis of Preeclampsia, Fetal Growth Restriction, and Gestational Hypertension

Gene official symbol	*Gene full name*	*The role in the pathogenesis of pregnancy-related complications*
VEGFA	Vascular endothelial growth factor A	VEGFA, a major factor in angiogenesis, binds to two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), and regulates endothelial cell proliferation, migration, vascular permeability, secretion, and other endothelial functions. Immunohistochemical studies have shown high expression of VEGFA in the placental villous structures of patients with preeclampsia (Dubova *et al.*, 2013).
FLT1	fms-related tyrosine kinase-1 (Flt-1), Vascular endothelial growth factor receptor 1 (VEGFR-1)	Placental overexpression of soluble fms-related tyrosine kinase-1 (sFLT1), an alternatively spliced form of VEGFR-1, specifically in the fetal-derived trophoblast cells, has been implicated as the underlying cause of preeclampsia (Maynard *et al.*, 2003; Fan *et al.*, 2014). Immunohistochemical studies show high expression of VEGFR-1 in the placental villous structures of patients with preeclampsia (Dubova *et al.,* 2013).
PPARA	Peroxisome proliferator-activated receptor alpha	PPARs are involved in trophoblast invasion, placental development, parturition, and pregnancy-specific diseases. In clinical pregnancy-specific disorders, including preeclampsia, gestational diabetes, and intrauterine growth restriction, aberrant regulation of components of the PPAR system parallels dysregulation of metabolism, inflammation, and angiogenesis. The reduction of transcriptional activity observed in the sera of preeclamptic women has been seen with PPARα. The reduction in potential circulating PPAR activators has been observed weeks and sometimes months before the onset of maternal symptoms and a clinical diagnosis of PE. Partially characterized inflammatory, angiogenic, and metabolic disturbances in pregnancy-related diseases suggest that synthetic PPAR agonists may be of potential use in these conditions (Wieser *et al.*, 2008).
HIF1A	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	HIF-1 functions as a master regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes, including those involved in energy metabolism, angiogenesis, and apoptosis. Levels of HIF-1α in the placentas of preeclamptic patients have been found to be higher than the levels in placentas of healthy pregnant women (Tal, 2012; Sezer *et al.*, 2013).
F3	Coagulation factor III (thromboplastin, tissue factor)	Higher expression and/or release of plasma tissue factor (F3) from the placenta may contribute toward a pathological hypercoagulable state in preeclampsia patients (Teng *et al.*, 2010). The F3 level in the preeclampsia group was significantly higher than that in the control group.
PAFAH1B2	Platelet-activating factor acetylhydrolase IB, regulatory subunit 1 (45 kDa)	The PAFAH1B2 gene encodes the beta subunit of PAF-AH that inactivates PAF. Increased PAF-AH activity in trophoblasts from preeclamptic placenta has been detected (Gu *et al.*, 2006).
OLR1	Oxidized low-density lipoprotein (lectin-like) receptor 1	The expression levels of oxidized low-density lipoprotein (lectin-like) receptor 1 (LOX-1 protein encoded by OLR1 gene) were significantly higher in the placental tissues of the early-onset preeclampsia than in normal pregnancies (Meng *et al.*, 2008).
TP53	Tumor protein p53 (p53)	Preeclampsia is characterized by exaggerated apoptosis of villous trophoblast of placental villi. p53, encoded by TP53 gene, and FAS play a central role in regulation of programmed cell death. Protein expression of p53 was significantly increased in pregnancies complicated by PE (Sharp *et al.*, 2014).
TNFRSF6	FAS receptor (FasR), also known as apoptosis antigen 1 (APO-1 or APT), cluster of differentiation 95 (CD95), or tumor necrosis factor receptor superfamily member 6	Preeclampsia is characterized by exaggerated apoptosis of villous trophoblast of placental villi. Fas-mediated apoptosis may play a role in conditions related to abnormal placentation, such as preeclampsia (Neale and Mor, 2005).
CASP10	Caspase-10, apoptosis-related cysteine peptidase	Caspase-10 is involved in apoptosis in the preeclamptic placenta.The expression of caspase-10 has been found to be significantly increased in full-term preeclamptic placentas (Han *et al.*, 2006).

(*continued*)

Table 4. (Continued)

Gene official symbol	*Gene full name*	*The role in the pathogenesis of pregnancy-related complications*
CASP3	Caspase-3, apoptosis-related cysteine peptidase	Apoptotic marker caspase-3 is significantly increased in villous trophoblasts of patients with preeclampsia, HELLP syndrome, and IUGR, indicating increased placental apoptosis (Cali *et al.*, 2013). Hypoxia induces apoptosis in trophoblastic cells accompanied by increased expression of Caspase-3 (Ishioka *et al.*, 2006).
BCL2	B-cell CLL/lymphoma 2	This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells. Severe preterm FGR, with or without PE, are associated with increased expression of BCL2 in both the placenta and maternal blood. In preterm PE, but not FGR, there is increased placental expression of BCL-XL and BCL2, but only BCL2 is significantly increased in the maternal blood. Increased expression of genes of the intrinsic apoptosis pathway reflects the severity of FGR as determined by deteriorations in umbilical artery Doppler velocimetry. In severe early-onset FGR, there is increased expression of genes regulating intrinsic apoptosis in both the placenta and maternal blood (Whitehead *et al.*, 2013).
BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	A member of the BCL2/adenovirus E1B 19 kd-interacting protein (BNIP) family protects cells from virally induced cell death and interacts with E1B 19 kDa-like sequences of BCL2, another apoptotic protector. The pro-apoptotic proteins BNip3 and Nix are expressed in the human placenta. Pregnancies with placental dysfunction and hypertensive pregnancy disorders with different clinical manifestations are characterized by a significantly decreased placental expression of BNip3 and Nix proteins (Stepan *et al.*, 2005).
AHSA2	AHA1, activator of heat shock 90 kDa protein ATPase homolog 2	The upregulation of Hsp90, found in placental tissues affected with mild preeclampsia (Hromadnikova *et al.*, 2015), may be associated with downregulation of miR-520a* and miR-525. Both microRNAs have been predicted to regulate the expression of the AHSA2 gene, which is an activator of Hsp90 protein ATPase homolog 2.
HSF5	Heat shock transcription factory family member 5	The upregulation of Hsp27, Hsp90, and HspBP1, found in placental tissues affected with mild preeclampsia (Hromadnikova *et al.*, 2015), may be associated with downregulation of miR-520a* and miR-525. Both microRNAs have been predicted to regulate the expression of HSF5 gene, a transcriptional activator of heat shock genes.
HSP90AA1	Heat shock protein 90 kDa alpha (cytosolic), class A member 1 (Hsp90)	The upregulation of Hsp90, found in placental tissues affected with mild preeclampsia (Hromadnikova *et al.*, 2015), may be associated with downregulation of miR-526a, which has been predicted to regulate expression of the HSP90AA1 gene encoding heat shock protein 90 (Hsp90), a molecular chaperon essential for activating many signaling proteins in the eukaryotic cells.
IGFBP1	Insulin-like growth factor-binding protein 1 (IBP-1), also known as placental protein 12 (PP12)	The expression of IGFBP-1 in hypertensive disorder complicating pregnancy group was higher than that in the control group. Its expression in mild and severe preeclampsia patients was higher than that in control group (Shang *et al.*, 2005).
TLR2	Toll-like receptor 2	TLRs may modulate the innate immune response of the maternal syndrome of preeclampsia and might trigger the differential inflammatory response existing between early-onset preeclampsia and normotensive IUGR. Women with preclampsia had increased TLR2 mRNA and protein expressions in peripheral blood compared with other groups (Xie *et al.*, 2010). Another study demonstrated the associations between the presence of periodontal pathogens and the increased expression of TLR-2 in the placental tissue of patients with hypertensive disorders compared with the placentas of healthy normotensive patients (Chaparro *et al.*, 2013). TLR2 engagement by the synthetic lipoprotein FSL-1 enhances pro-inflammatory and pro-labor mediators in human fetal membranes and myometrium via MyD88/TRAF6/NF-κB (Lim *et al.*, 2014).
TLR5	Toll-like receptor 5	TLR5 engagement by bacterial flagellin enhances pro-inflammatory and pro-labor mediators in human fetal membranes and myometrium via MyD88/TRAF6/NF-κB (Lim *et al.*, 2014).

(*continued*)

Table 4. (Continued)

Gene official symbol	*Gene full name*	*The role in the pathogenesis of pregnancy-related complications*
TLR7	Toll-like receptor 7	RNA-mediated activation of TLR7/8 plays a key role in the development of PE. TLR7/8 activation by single-stranded RNA expressed by viruses and/or released from necrotic cells initiates a pro-inflammatory immune response. Placental immunoreactivity and mRNA levels of TLR7 and TLR8 were significantly increased in women with PE compared with normotensive women (Chatterjee *et al.*, 2012).
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	Inflammatory processes are present during preeclampsia and in normal pregnancies. Maternal inflammatory reactions may change toward the end of the term. The study evaluating genome signaling in peripheral blood during preeclampsia and toward the end of the term using microarrays revealed upregulation of TNFSF15 (Dahlstrøm *et al.*, 2010).
IL16	Interleukin 16	The upregulation of the IL-16 profile in both maternal and placental systems in PE was demonstrated, suggesting that IL-16 could be an important cytokine engaged in the altered immune system and exaggerated inflammatory response in PE syndrome (Gu *et al.*, 2008).
CXCL12	Chemokine (C-X-C motif) ligand 12	CXCL12 expression is significantly greater in the placenta of preeclamptic women compared with normal women. This may represent part of a compensatory mechanism associated with preeclampsia (Hwang *et al.*, 2012).
TNF	Tumor necrosis factor	The ischemic placenta releases cytokines such as TNF-α, which causes maternal endothelial dysfunction (Lamarca, 2012). Expression of TNF-α in placental tissues and maternal sera was significantly increased in preeclamptic patients, as well as in those with IUGR. TNF-α is mainly expressed in trophoblasts, vascular endothelial cells, decidual cells, and the stroma of the stem villi. The findings suggest that an increased concentration of TNF-alpha in the maternal and umbilical serum plays a significant role in the pathogenesis of preeclampsia (Tosun *et al.*, 2010; Zhou *et al.*, 2012).
IL6	Interleukin 6	Decidual cells from preeclamptic patients exhibit high IL6 production (Lockwood *et al.*, 2008). Interestingly, a study showing increased expression of *IL6* mRNA together with reduced frequency of regulatory macrophages in decidual tissue of women who later progressed to develop pregnancy-induced hypertension suggests that this disturbance originates during the first trimester, before overt symptoms appear (Prins *et al.*, 2012). One function of IL6 in influencing the progression of preeclampsia may be exerted at the level of vascular activation. IL6 production in endothelial cells is elevated after endothelial cells phagocytose necrotic trophoblast debris, and it is implicated in spreading the vascular activation response (Chen *et al.*, 2009). Elevated circulating IL6 in preeclamptic women may exacerbate and amplify this response, by acting on trophoblasts to promote the excessive shedding of necrotic debris considered to underpin this disease (Chen *et al.*, 2010). Other findings also suggest that increased concentrations of IL-6 in maternal and umbilical serum play a significant role in pathogenesis of preeclampsia (Tosun *et al.*, 2010).
IL6R	Interleukin 6 receptor	Placentas of preeclamptic patients produce less sIL6R, but more gp130 (IL-6 receptor subunit beta) than placentas of normal pregnant patients (Wilczyński *et al.*, 2002).
IL8	Interleukin 8	The findings suggest that an increased concentration of IL-8 in the maternal and umbilical serum plays a significant role in pathogenesis of preeclampsia (Tosun *et al.*, 2010). IL-8 gene expression is also altered in first-trimester trophoblasts derived from women developing PE later (Farina *et al.*, 2011).

(*continued*)

Table 4. (Continued)

Gene official symbol	*Gene full name*	*The role in the pathogenesis of pregnancy-related complications*
CD46	CD46 molecule, complement regulatory protein	CD46 has cofactor activity for inactivation of complement components C3b and C4b by serum factor I. The activation of neutrophils and monocytes takes place during the uteroplacental passage in preeclamptic pregnancies. Such a local inflammatory response involving enhanced leukocyte/endothelial interaction may contribute to the pathogenesis of this disorder. Increased expression of adhesion molecules and complement-related markers (CD46 and CD59) on neutrophils and monocytes has been detected (Mellembakken *et al.*, 2002).
SMAD2	SMAD family member 2	Smad2 expression, the molecule involved in TGF-β1 signaling in the normal villous tree, is enhanced in placental tissues derived from severe fetal growth restriction—preeclampsia cases characterized by early onset and absent end diastolic velocities in the umbilical arteries (Todros *et al.*, 2007).
BID	BH3-interacting domain death agonist	Hypoxia induces apoptosis in trophoblastic cells accompanied by decreased expression of Bid (Ishioka *et al.*, 2006).
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase	TLR2 engagement by the synthetic lipoprotein FSL-1 enhances pro-inflammatory and pro-labor mediators in human fetal membranes and myometrium via MyD88/TRAF6/NF-κB (Lim *et al.*, 2014).
IGF1R	Insulin-like growth factor 1 receptor	A significant association between newborn birth weight and placental IGF-1R number may indicate that IGF-1R is associated with fetal growth. Indeed, the reported decrease of IGF-1R mRNA and protein in intrauterine growth retardation in rats supports this affirmation (Reid *et al.*, 2002). On the other hand, the absence of a significant correlation between blood pressure and the IGF-1R characteristics suggests that hypertension is a pathological condition that does not significantly affect the affinity or number of placental IGF-1 receptors (Díaz *et al.*, 2005).
PRDX6	Peroxiredoxin 6	Downregulation of Prdx6 proteins in human and rat IUGR group placentas may have led to the formation of oxidative stress that may have impaired proliferation and invasion of cytotrophoblasts (Acar *et al.*, 2014).
HSPA8	Heat shock 70 kDa protein 8, also known as heat shock cognate 71 kDa protein or Hsc70 or Hsp73	Downregulation of Hsc70 in placentas might play an important role in the pathogenesis of PE (Gharesi-Fard *et al.*, 2010).
MCL1	Myeloid cell leukaemia 1	MiR-29b induces apoptosis and inhibits invasion and angiogenesis of trophoblast cells. Additional studies have confirmed that miR-29b regulates the expression of MCL1 (Li *et al.*, 2013).

PAF-AH, platelet-activating factor acetylhydrolase; PE, preeclampsia; PPARs, peroxisome proliferator-activated receptors.

519a) were dysregulated in severe preeclamptic pregnancies, and one C19MC microRNA (*miR-517-5p*) was altered in preeclampsia requiring termination before 34 weeks of gestation. These data suggest the involvement of these microRNAs in the pathogenesis of preeclampsia. Equally, mild forms of preeclampsia persisting for several weeks (carefully monitored from the onset of the disease until delivery) were observed to be associated with similar placental-specific microRNA expression profile (*miR-517-5p*, *miR-520a-5p*, *miR-524-5p*, *miR-525*, and *miR-520h*). Nevertheless, the longer the pregnancy-related disorder lasted, the more extensive the downregulation of placental-specific microRNAs appeared. This suggests that the dysregulation of some C19MC microRNAs (*miR-515-5p*, *miR-518b, miR-518f-5p*, and *miR-519d*) does not drive the pathological process itself, but could be reflective of a compensatory mechanism.

In addition, the analysis suggested that there was no relationship between microRNA expression and previous occurrence of hypertension in patients with established preeclampsia. Similarly, no substantial changes in microRNA gene expression in placental tissues, relative to Doppler ultrasonography parameters associated with poorer outcome in preeclampsia and/or FGR, were observed.

The decreased levels of C19MC microRNAs in placental tissues may lead to upregulation of relevant proteins involved in the direction of key biological pathways such as angiogenesis, stress response, apoptosis, and hemocoagulation. The occurrence of defective placental angiogenesis, inadequate uteroplacental blood perfusion, hypoxia, and ischemia can induce local stress responses, abnormal apoptosis of placental trophoblasts, and dysfunction of the blood coagulation-fibrinolysis system, and, finally, it can induce development of a generalized maternal systemic inflammatory response (Khong *et al.*, 1986; Redman *et al.*, 1999). Some predicted targets of C19MC microRNAs, which were a subject of interest in this study, have been previously shown to be upregulated in placental tissue samples derived from patients with pregnancy-related complications (e.g., sFLT-1, VEGFA, HIF-1α, PAF-AH, F3, LOX-1, CASP3, CASP10, p53, TLR2, TLR7, CD46, Hsp90, AHA1, IGFBP-1, TRAF6, IL-16, CXCL12, TNF-α, IL-6, IL-6R, IL-8, BCL-2, and SMAD2) (Tables 3 and 4). The role of predicted target genes of currently identified differentially expressed C19MC microRNAs in the pathogenesis of preeclampsia, FGR, and GH is discussed in detail in Table 4.

Nevertheless, the decreased levels of some proteins (e.g., Prdx6, Hsc70, MCL1, BID, IGF-1R, and BNip3) observed in placental tissues of patients with pregnancy-related complications, predicted to also be targeted by currently examined C19MC microRNAs, are contrary to the observed microRNA downregulation (Tables 3 and 4).

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. In addition, 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 (Schmitz *et al.*, 2013).

We have previously identified C19MC microRNAs (miR-516-5p, miR-517-5p, miR-518b, miR-520a-5p, miR-520h, miR-525, and miR-526a) present in maternal plasma that can differentiate between normal pregnancies and nonpregnant individuals (Kotlabova *et al.*, 2011). Our recent study also showed that the upregulation of circulating miR-516-5p, miR-517-5p, miR-520a-5p, miR-525, and miR-526a is a characteristic phenomenon of established preeclampsia (Hromadnikova *et al.*, 2013). While plasma levels of microRNAs between the control cohort and the cohorts of patients with FGR and GH did not differ, increased levels were detected in the group of patients with established preeclampsia.

It seems that while the placental tissues are able to respond to various pregnancy-related disorders, including pregnancy-induced hypertension, FGR, and/or preeclampsia via downregulation of C19MC microRNAs (miR-517-5p, miR-520a-5p, miR-525, and miR-526a), upregulation of these particular microRNAs appears in maternal circulation, but only in cases of preeclampsia. This inconsistent finding may be explained in several ways. First, the expression of various biomarkers may vary in different placental zones as has been previously shown. For example, the expression of various biomarkers in the inner region of placenta can differ compared with the outer part of the tissue (Abdulsid *et al.*, 2013). Furthermore, the expression may be different between a thrombus and an infarction (Wataba *et al.*, 2004). In this study, we focused on the examination of microRNA levels in a specific area, that is, in the central cotyledon zone, where the umbilical cord is inserted into the chorionic plate. However, the placenta is being continuously remodeled during normal placental development, and extracellular nucleic acids of both fetal and placental origin, packed into either trophoblast-derived apoptotic bodies or shedding syncytiotrophoblast micro-particles, may be detected in maternal circulation during the course of normal gestation (Nelson, 1996; Oudejans *et al.*, 2003; Huppertz and Kingdom, 2004; Orozco *et al.*, 2006; Hromadnikova, 2012). It is obvious that the levels of circulating nucleic acids, mainly the levels of circulating placental-specific C19MC microRNAs, present in maternal circulation during gestation reflect the overall status of the placenta.

Conclusion

This study demonstrated that pregnancy-related complications were associated with alterations in placental microRNA expression. The study revealed that downregulation of some C19MC microRNAs is a common phenomenon shared between GH, preeclampsia, and FGR. On the other hand, the study pointed to the fact that some C19MC microRNAs are exclusively downregulated, but only in association with preeclampsia.

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Disclosure Statement

No competing financial interests exist.

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