Circulating C19MC MicroRNAs in Preeclampsia, Gestational Hypertension, and Fetal Growth Restriction

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The objective of the study was to identify the profile of circulating C19MC microRNAs (miR-516-5p, miR-517∗, miR-518b, miR-520a∗, miR-520h, miR-525, and miR-526a) in patients with established preeclampsia (n = 63), fetal growth restriction (n = 27), and gestational hypertension (n = 23). We examined the correlation between plasmatic concentrations and expression levels of microRNAs and the severity of the disease with respect to clinical signs, requirements for the delivery, and Doppler ultrasound parameters. Using absolute and relative quantification approaches, increased extracellular C19MC microRNA levels (miR-516-5p, P = 0.037, P = 0.009; miR-517∗, P = 0.033, P = 0.043; miR-520a∗, P = 0.001, P = 0.009; miR-525, P = 0.026, P = 0.01; miR-526a, P = 0.03, P = 0.035) were detected in patients with preeclampsia. The association analysis pointed to no relationship between C19MC microRNA plasmatic concentrations and expression profile and identified risk factors for a poorer perinatal outcome. However, the dependence between the levels of plasmatic C19MC microRNAs and the pulsatility index in the middle cerebral artery and the values of cerebroplacental ratio was demonstrated. The study brought the interesting finding that the upregulation of miR-516-5p, miR-517∗, miR-520a∗, miR-525, and miR-526a is a characteristic phenomenon of established preeclampsia.

1. Introduction

Normal pregnancy is associated with a systemic inflammatory response. Many of the physiologic changes of normal pregnancy are part of an acute-phase reaction, which is generated by an inflammatory response. The placenta is the proximal cause of these problems [1]. Since the placenta is being continuously remodelled during normal placental development, extracellular nucleic acids of both fetal and placental origin, packed into either trophoblast-derived apoptotic bodies or shedding syncytiotrophoblast microparticles, may be detected in maternal circulation during the course of normal gestation [2–6].

Circulating syncytiotrophoblast debris has been hypothesized to contribute to maternal inflammation and some of the features of the maternal syndrome [7]. Signs of maternal inflammation which appear to be present in normal pregnancies at term are exaggerated in preeclampsia (PE) and fetal growth restriction (FGR) and can account for their clinical features [1, 8].

Preeclampsia and fetal growth restriction (FGR) are major complications affecting about 2–10% of pregnancies responsible for maternal and perinatal morbidity and mortality [9, 10]. Preeclampsia usually develops after 20 weeks of gestation and is characterized by chronic or gestational hypertension combined with proteinuria, which results from defective placentation eliciting inadequate uteroplacental blood perfusion and ischemia [8, 11, 12]. The causes of preeclampsia and FGR remain unknown. Trophoblastic debris and the microparticles shed during normal pregnancy are proinflammatory and this process is amplified in preeclampsia [13]. A hypoxic environment induces excessive
trophoblast cell death and increased shedding of placenta debris into the maternal circulation; as a result, placental insufficiency related pregnancy complications are associated with abnormal levels of extracellular fetal DNA and mRNA transcripts [5, 14].

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) [15, 16]. The concept of early and late PE is modern, and it is widely accepted that these two entities have different etiologies and should be regarded as different forms of the disease, where early onsets of PE and IUGR are considered to be placenta-mediated diseases [17–19].

There has been a trend over the last 10 years to develop noninvasive methods utilizing quantification of cell-free nucleic acids inclusive of microRNAs in maternal circulation [6, 20–39]. The diagnostic potential of particular molecular biomarkers and their implementation in the current predictive and diagnostic algorithms for pregnancy related complications is subject of interest [6].

MicroRNAs belong to a family of small noncoding RNAs that regulate gene expression at the posttranscriptional level by degrading or blocking translation of messenger RNA (mRNA) targets [40, 41].

Recent studies have shown that preeclampsia is associated with alterations in extracellular microRNA expression. Using real-time PCR analysis, Gunel et al. [42] demonstrated the upregulation of miR-210 and downregulation of miR-152 in patients with preeclampsia. The application of next generation sequencing technology revealed a broader profile of dysregulated circulating microRNAs in preeclampsia. Compared to controls, 15 microRNAs were found to be upregulated (miR-521, miR-520h, miR-517c, miR-519d, miR-520g, miR-517b, miR-542-3p, miR-136, let-7f-1*, miR-518e, let-7a*, miR-125b, miR-125a-5p, miR-519a, and miR-29a) and 7 microRNAs were found to be downregulated (let-7f, miR-223, miR-1260, let-7d, miR-320c, miR-185, and miR-1272) in four examined preeclamptic serum samples [43].

Later, using microarray analysis Wu et al. [44] reported the upregulation of 13 microRNAs (miR-574-5p, miR-26a, miR-151-3p, miR-130a, miR-181a, miR-130b, miR-30d, miR-145, miR-103, miR-425, miR-221, miR-342-3p, and miR-24) and down-regulation of 2 microRNAs (miR-144, miR-16) in patients with severe preeclampsia. Seven of these 13 microRNAs (miR-574-5p, miR-26a, miR-130b, miR-181a, miR-342-3p, miR-103, and miR-24) were validated by real-time PCR analysis to be elevated in plasma from severe preeclamptic pregnancies.

In a small-scale analysis, Mouillet et al. [45] did not observe any differentiation between pregnancies with normal and fetal growth restricted fetuses when compared circulating microRNA expression levels (miR-27a, miR-30d, miR-141, miR-200c, miR-205, miR-424, miR-451, miR-491, miR-517a, miR-518b, miR-518e, and miR-524).

However, most of investigators focused on the study of those microRNAs, whose genes are located outside chromosome 19 miRNA clusters (C19MC and miR-371-3 cluster) or the chromosome 14 miRNA cluster (C14MC) that encode pregnancy-associated microRNAs [46–50].

We have previously identified C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) present in maternal plasma differentiating between normal pregnancies and nonpregnant individuals [51]. We selected from the chromosome 19 microRNA cluster, which involves 46 microRNA genes altogether, [48–50, 52] preferentially those microRNAs that were previously demonstrated to be exclusively expressed in placental tissues (miR-20a*, miR-516-5p, miR-517*, miR-518b, miR-519a, miR-524-5p, miR-525, miR-526a, miR-526b, and miR-520h) and those microRNAs that were reported to be highly expressed in placental tissues (miR-512-5p, miR-515-5p, miR-518f*, miR-519d, and miR-519e*) [51, 53, 54].

Later, we demonstrated significant increases in extracellular C19MC microRNAs levels (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) over time in normally progressing pregnancies [51, 54].

The results of our pilot study indicated no differentiation between normal and complicated pregnancies, but could not come to definitive conclusions due to the low number of studied subjects involved [51, 54]. The current study is a followup of our previous studies [51, 54] and describes comprehensively for the first time the expression profile of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) in the entirely new sample set of patients with clinically established preeclampsia and/or fetal growth restriction. To our knowledge, no study describing the profile of circulating C19MC microRNAs in gestational hypertension has been carried out.

2. Materials and Methods

2.1. Patients. The studied cohort consisted of Caucasian women involving 63 preeclampsia (PE) or w/o fetal growth restriction (FGR), 27 FGR, 23 gestational hypertension (GH), and 55 controls. Twenty-four women had signs of mild preeclampsia, 39 women were diagnosed with severe preeclampsia, 24 preeclamptic patients required the delivery before 34 weeks of gestation and 39 patients delivered after 34 weeks of gestation. In 18 cases, preeclampsia superposed on previous hypertension; otherwise, it occurred in normotensive patients (45 cases). Eight growth-retarded foetuses were delivered before 34 weeks of gestation and 19 those after 34 weeks of gestation. Oligohydramnios or anhydramnios were present in 7 growth restricted foetuses.

Doppler studies showed an abnormal pulsatility index (PI) in the umbilical artery (14 preeclampsia ± FGR and 14 FGR) and/or in the middle cerebral artery (10 preeclampsia ± FGR and 11 FGR). Cerebroplacental ratio (CPR), expressed as a ratio between umbilical artery and middle cerebral artery pulsatility index, was below the fifth percentile in 21 cases (9 preeclampsia ± FGR and 12 FGR). Absent or reversed enddiastolic velocity waveforms in the umbilical artery occurred in 8 cases (2 preeclampsia + FGR and 6 FGR). Normal pregnancies were defined as those without complications who delivered full term, singleton, healthy infants.
weighting >2500 g after 37 completed weeks of gestation. Preeclampsia was defined as blood pressure >140/90 mmHg in two determinations 4 hours apart that was associated with proteinuria >300 mg/24 h after 20 weeks of gestation. Severe preeclampsia was diagnosed by the presence of one or more of the findings according to the guidelines of ACOG Committee [11].

Fetal growth restriction was diagnosed when the estimated fetal weight (EFW), calculated using the Hadlock formula (Astraia Software GmbH), was below the tenth percentile for the evaluated gestational age.

All patients provided written informed consent. The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University in Prague. The samples for the study were chosen on the basis of equal times in storage and gestation age. Gestational age was assessed using ultrasonography.

2.2. Processing of Samples. Nine millilitres of peripheral blood were collected into EDTA tubes and centrifuged twice at 1200 g for 10 min at room temperature. Plasma samples were stored at −80°C until subsequent processing.

Total RNA was extracted from 1 mL of plasma and 25 mg of normal placental tissue preserved in RNAlater (Ambion, Austin, USA) followed by an enrichment procedure for small RNAs using a mirVana microRNA Isolation kit (Ambion, Austin, USA). Trizol LS reagent was used in plasma samples for total RNA extraction from biological fluids (Invitrogen, Carlsbad, USA) and preceded the small RNAs enrichment procedure. To minimize DNA contamination, we treated the eluted RNA with 5 μL of DNase I (Fermentas International, ON, Canada) for 30 min at 37°C.

2.3. Reverse Transcriptase Reaction. Each microRNA was reverse-transcribed into complementary DNA using TaqMan MicroRNA Assay, containing microRNA-specific stem-loop RT primers (Table I), and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Branchburg, USA) in a total reaction volume of 50 μL on a 7500 real-time PCR system (Applied Biosystems, Branchburg, USA) with the following thermal cycling parameters: 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and then held at 4°C.

2.4. Quantification of MicroRNAs. 15 μL of cDNA corresponding to each microRNA was mixed with components of TaqMan MicroRNA Assay and the ingredients of the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, USA) in a total reaction volume of 35 μL. TaqMan PCR conditions were set as described in the TaqMan guidelines. The analysis was performed using a 7500 real-time PCR system. All PCRs were performed in duplicates. A sample was considered positive if the amplification signal occurred before the 40th threshold cycle. Concentrations of individual microRNAs were expressed as pg of total RNA enriched for small RNAs per millilitre of plasma.

The expression of particular microRNA in maternal plasma was determined using the comparative Ct method [55] relative to the expression of the same microRNA in the reference sample, randomly selected placenta derived from gestation with normal course.

RNA fraction highly enriched for 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 to build-up the standard curves and as a reference sample for relative quantification throughout the study.

Synthetic C. elegans microRNA (cel-miR-39, Qiagen, Hilden, Germany) 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 qRT-PCR data were normalized to cel-miR-39, as it shows no sequence homology to any human microRNA. 1 μL of 0.1 mM cel-miR-39 was spiked in after incubation with Trizol LS reagent to the human plasma samples.
2.5. Statistical Analysis. MicroRNA levels were compared between groups by nonparametric tests (the Mann-Whitney U test for the comparison between two groups and the Kruskal-Wallis test for the comparison between three or more groups) using Statistica software (StatSoft Inc., USA). Correlation between variables including absolute and/or relative microRNA quantification and Doppler ultrasonography parameters (the pulsatility index in the umbilical artery, the pulsatility index in the middle cerebral artery, and the cerebroplacental ratio) was calculated using the Spearman’s rank correlation coefficient (ρ). If it varies from −0.5 to 0, there is a weak negative correlation. The significance level was established at a P value of P < 0.05.

3. Results

3.1. Circulating C19MC MicroRNAs Differentiate between Complicated and Normal Pregnancies. Overall, increased plasmatic levels of miR-516-5p (P = 0.008), miR-517* (P = 0.003), miR-520a* (P < 0.001), miR-525 (P = 0.003), and miR-526a (P = 0.004) were observed in women with pregnancy-related complications (gestational hypertension, preeclampsia and fetal growth restriction) compared to normal pregnancies.

Similarly, the difference in gene expression of circulating microRNAs between pregnancy-related complications and the control cohort (normal pregnancies) achieves statistical significance for miR-516-5p (P < 0.001), miR-517* (P = 0.005), miR-520a* (P = 0.001), miR-525 (P = 0.001), and miR-526a (P = 0.004).

3.2. Upregulation of Circulating C19MC MicroRNAs in Pregnancies with Established Preeclampsia. Consecutive detailed group analysis confirmed a difference in the levels of extracellular microRNAs in 5/5 C19MC microRNAs (miR-516-5p, P = 0.037; miR-517*, P = 0.015; miR-520a*, P = 0.003; miR-525, P = 0.026; and miR-526a, P = 0.032).

While plasmatic 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 (miR-516-5p, P = 0.037; miR-517*, P = 0.033; miR-520a*, P = 0.001; miR-525, P = 0.026; and miR-526a, P = 0.030) (Figures 1(a)–1(e)).

Parallel, significant difference in microRNA gene expression was found between groups of preeclampsia, gestational hypertension, fetal growth restriction, and controls (miR-516-5p, P = 0.005; miR-517*, P = 0.028; miR-520a*, P = 0.011; miR-525, P = 0.01; miR-526a, P = 0.034). Again, while the expression of microRNAs between the control cohort, gestational hypertension, and fetal growth restriction did not differ, the highest expression was detected in the group of patients with preeclampsia (miR-516-5p, P = 0.009; miR-517*, P = 0.043; miR-520a*, P = 0.009; miR-525, P = 0.01; miR-526a, P = 0.035) (Figures 1(f)–1(j)).

3.3. The Association Study of Circulating C19MC MicroRNAs and the Severity of the Disease with respect to Clinical Signs and Requirements for the Delivery. Plasmatic concentrations and/or expression profile of C19MC microRNAs were analysed in relation to the severity of the disease with respect to the degree of clinical signs (mild and severe preeclampsia) and requirements for the delivery (before and after 34 weeks of gestation). No effect of the severity of the disease either on plasmatic C19MC microRNA concentrations (miR-516-5p, P = 0.396; miR-517*, P = 0.226; miR-520a*, P = 0.08; miR-525, P = 0.237; and miR-526a, P = 0.201) or C19MC microRNA expression levels (miR-516-5p, P = 0.476; miR-517*, P = 0.58; miR-520a*, P = 0.239; miR-525, P = 0.397; miR-526a, P = 0.646) was observed.

Further, the association between C19MC microRNA plasmatic levels and/or gene expression and the occurrence of previous hypertension in the cohort of patients with preeclampsia was determined. No difference between the group of preeclampsia superposed on chronic hypertension and/or gestational hypertension and the group of patients with unexpected onset of preeclampsia was revealed (absolute quantification: miR-516-5p, P = 0.885; miR-517*, P = 0.538; miR-520a*, P = 0.342; miR-525, P = 0.909; miR-526a, P = 0.273; relative quantification: miR-516-5p, P = 0.721; miR-517*, P = 0.621; miR-520a*, P = 0.885; miR-525, P = 0.568; miR-526a, P = 0.201).

3.4. The Association Study of Circulating C19MC MicroRNAs and the Severity of the Disease with respect to Doppler Ultrasonography Monitoring. The association between the plasmatic concentration and gene expression levels of C19MC microRNAs and Doppler ultrasonography parameters (the pulsatility index in the umbilical artery the pulsatility index in the middle cerebral artery, and the cerebroplacental ratio) was studied in the cohort of pregnancies complicated with preeclampsia and/or fetal growth restriction.

No difference within the group of complicated pregnancies with normal and abnormal values of flow rate in the umbilical artery was found out with the exception of miR-526a, which was upregulated in the group of patients with abnormal blood flow velocity waveforms (absolute quantification: P = 0.038; relative quantification: P = 0.05).

Further, the statistical analysis showed no effect of the pulsatility index in the middle cerebral artery and the cerebroplacental ratio on the plasmatic concentrations (A. cerebi media: P = 0.479, P = 0.826, P = 0.528, P = 0.625, P = 0.154; CPR: P = 0.426, P = 0.479, P = 0.443, P = 0.867, and P = 0.181) and expression levels (A. cerebi media: P = 0.826, P = 0.931, P = 0.427, P = 0.639, and P = 0.297; CPR: P = 0.517, P = 0.288, P = 0.198, P = 0.984, and P = 0.195) of all microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) that were identified to be upregulated in plasma samples derived from preeclampsia with or without fetal growth restriction.

The correlation between variables including absolute and/or relative quantification of particular microRNA in maternal plasma and the values of flow rate in the umbilical artery and the fetal blood vessel was calculated using Spearman’s rank correlation coefficient. The pulsatility index in the umbilical artery did not show any correlation with microRNA plasmatic concentrations and/or microRNA gene expression. However, a weak negative correlation between the
Figure 1: Continued.
concentrations (miR-520a) between cerebroplacental ratio and microRNA plasmatic was observed. Furthermore, a weak negative correlation inflammatory response (Table 2). The data were collected from
connected to the regulation of the immune system and inflam-
extracellular C19MC microRNAs in patients with established
relationship analysis of predicted targets of the five elevated
MicroRNAs in Preeclampsia.

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plasmatic levels and/or gene expression between pregnancies with mild and severe preeclampsia, pregnancy-related complication with the need for the delivery before 34 weeks of gestation, and those who delivered after this critical period and pregnancies with abnormal and normal blood flow velocity waveforms. Nevertheless, the levels of miR-526a were significantly increased in the group of patients with abnormal values of flow rate in the umbilical artery.

On the other hand, the dependence between the levels of plasmatic C19MC microRNAs and the pulsatility index in the middle cerebral artery and the values of cerebroplacental ratio was demonstrated. The relation between the increased levels of plasmatic C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) and decreased values of flow rate in the middle cerebral artery reached a statistical significance in complicated pregnancies.

Similarly, the relationship between increased levels of plasmatic C19MC microRNAs (miR-520a* and miR-526a) and decreased values of cerebroplacental ratio was revealed.

In conclusion, microRNAs play a fundamental role in a variety of physiological and pathological processes involving
Table 2: (a) Function and functional relationship analysis of target genes of differentially expressed extracellular C19MC microRNAs in preeclampsia in relation to immune system response. (b) Function of target genes of differentially expressed extracellular C19MC microRNAs in preeclampsia in immune system response.

(a)

| microRNA | miR-516-5p | miR-517* | miR-526a | miR-525 | miR-520a* |
|----------|-------------|----------|----------|---------|-----------|
| Number of predicted target genes | 349 | 179 | 212 | 340 | 352 |
| Unique target genes | | | | | |
| Shared with miR-525 | | | | | |

| Gene official symbol | Gene full name | The role in immune system response |
|----------------------|----------------|-----------------------------------|
| ACVR2B               | Activin A receptor, type IIB | Activins belong to the TGF-β superfamily |
| AHSA2                | AHA1, activator of heat shock 90 kDa protein ATPase homolog 2 (yeast) | Hsp90 is an inducible molecular chaperone protecting stressed cells |
| ATRN                 | Attractin | Involvement in initial immune cell clustering during inflammatory responses that may regulate the chemotactic activity of chemokines |
| BCAP29               | B-cell receptor-associated protein 29 | Involvement in CASP8-mediated apoptosis |
| CCR2                 | Chemokine (C-C motif) receptor 2 | Binds monocyte chemoattractant protein-1 involved in monocyte infiltration during inflammation |
| CD109                | CD109 molecule | Encodes GPI-linked glycoprotein that negatively regulates signaling of TGF-β |
| CD1A                 | CD1a molecule | Encodes glycoproteins structurally related to MHC proteins mediating the presentation of lipid and glycolipid antigens |
| CD2                  | CD2 molecule | A surface antigen of thymocytes, T, and NK cells |
| CD24                 | CD24 molecule | Encodes a sialoglycoprotein expressed on mature granulocytes and B cells |
| CD300LB              | CD300 molecule-like family member b | A nonclassical activating receptor of the Ig superfamily expressed on myeloid cells |
| CD302                | CD302 molecule | A C-type lectin receptor involved in cell adhesion, migration, endocytosis, and phagocytosis |
| CD46                 | CD46 molecule, complement regulatory protein | Has cofactor activity for inactivation of complement components C3b and C4b by serum factor I |
| CD93                 | CD93 molecule | Involvement in intercellular adhesion and in the clearance of apoptotic cells |
| CFLAR                | CASP8 and FADD-like apoptosis regulator | Regulator of apoptosis structurally similar to caspase-8 |

All the targets were predicted by a bioinformatics tool MirTarget2 using miRDB online database.

(b)
| Gene official symbol | Gene full name                                                                 | The role in immune system response                                                                 |
|---------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| DNAJC21             | DnaJ (Hsp40) homolog, subfamily C, member 21                                    | A molecular chaperone protein protecting against cellular stress                                   |
| DNAJC25             | DnaJ (Hsp40) homolog, subfamily C, member 25                                    | A molecular chaperone protein protecting against cellular stress                                   |
| FAS                 | FAS cell surface death receptor (FAS)                                           | Plays a central role in regulation of programmed cell death                                        |
| FLT1                | Fms-related tyrosine kinase 1                                                  | A member of vascular endothelial growth factor receptor (VEGFR) playing an important role in angiogenesis and vasculogenesis |
| HSF5                | Heat shock transcription factor family member 5                                  | A transcriptional activator of heat shock genes                                                   |
| HSP90AA1            | Heat shock protein 90 kDa alpha (cytosolic), class A member 1                   | An inducible molecular chaperone protecting stressed cells                                        |
| IGFI R              | Insulin-like growth factor 1 receptor                                           | Antiapoptotic agent enhancing cell survival                                                      |
| IGFBP1              | Insulin-like growth factor binding protein 1                                    | Prolongs the half-time of IGFs in plasma that regulate cell growth and development                 |
| IL10RA              | Interleukin 10 receptor, alpha                                                  | Involvement in inhibition of the synthesis of proinflammatory cytokines                           |
| IL17RE              | Interleukin 17 receptor E                                                       | Participation in MAPK pathway                                                                   |
| IL6ST               | Interleukin 6 signal transducer (gp130, oncostatin M receptor)                 | A signal transducer shared by IL-6, LIF, and oncostatin M                                        |
| IL9R                | Interleukin 9 receptor                                                           | Mediates IL-9 effects like stimulation of cell proliferation and prevention of apoptosis         |
| IRAK1               | Interleukin-1 receptor-associated kinase 1                                      | Responsible for IL-1 induced upregulation of the transcription factor NF-kappa B                 |
| IRAK3               | Interleukin-1 receptor-associated kinase 3                                      | Functions as a negative regulator of Toll-like receptor signaling                                |
| LILRA2              | Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 2 | An activatory cell-surface receptor expressed on monocytes, B cells, dendritic, and NK cells     |
| LILRB5              | Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 5 | An inhibitory cell-surface receptor expressed on immune cells                                   |
| MMD2                | Monocyte to macrophage differentiation-associated 2                             | Modulates Ras signaling                                                                         |
| MTDH                | Metadherin                                                                     | Involvement in HIF-1 alpha mediated angiogenesis and RNA-induced silencing complex and miRNA functions |
| PAPPA               | Pregnancy-associated plasma protein A, pappalysin-1                             | Involvement in local proliferative processes such as wound healing                               |
| PDCD6IP             | Programmed cell death 6 interacting protein                                     | Protects against cell death                                                                     |
| PPARA               | Peroxisome proliferator-activated receptor alpha                                | Affects the expression of genes involved in cell proliferation, cell differentiation, and in immune and inflammation responses |
| SOCS2               | Suppressor of cytokine signaling 2                                              | A negative regulator of JAK/STAT cytokine signaling pathway                                      |
| TLR2                | Toll-like receptor 2                                                            | Plays a fundamental role in activation of innate immunity, stimulates NF-kappa B                 |
| TLR7                | Toll-like receptor 7                                                            | Plays a fundamental role in activation of innate immunity                                        |
| TNFRSF19            | Tumor necrosis factor receptor superfamily, member 19                            | Interacts with TRAF family members, induces apoptosis by a caspase-independent mechanism         |
pregnancy-related complications. Current study demonstrated for the first time that circulating C19MC microRNAs might play a role in the pathogenesis of preeclampsia, but not in the pathogenesis of gestational hypertension and fetal growth restriction. The study brought interesting finding that the upregulation of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) is a characteristic phenomenon of established preeclampsia.

5. Conclusion
The study brought the interesting finding that the upregulation of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) is a characteristic phenomenon of established preeclampsia.

Conflict of Interests
The authors report no conflict of interests.

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References
[1] C. W. G. Redman and I. L. Sargent, “Preeclampsia and the systemic inflammatory response,” Seminars in Nephrology, vol. 24, no. 6, pp. 565–570, 2004.
[2] D. M. Nelson, “Apoptotic changes occur in syncytiotrophoblast of human placental villi where fibrin type fibrinoid is deposited at discontinuities in the villous trophoblast,” Placenta, vol. 17, no. 7, pp. 387–391, 1996.
[3] C. B. M. Oudejans, M. L. Tjoa, B. A. Westerman, M. A. M. Mulders, I. J. Van Wijk, and J. M. G. Van Vugt, “Circulating trophoblast in maternal blood,” Prenatal Diagnosis, vol. 23, no. 2, pp. 111–116, 2003.
[4] B. Huppertz and J. C. P. Kingdom, “Apoptosis in the trophoblast—role of apoptosis in placental morphogenesis,” Journal of the Society for Gynecologic Investigation, vol. 11, no. 6, pp. 353–362, 2004.
[5] A. F. Orozco, F. Z. Bischoff, C. Horne, E. Popek, J. L. Simpson, and D. E. Lewis, “Hypoxia-induced membrane-bound apoptotic DNA particles: potential mechanism of fetal DNA in maternal plasma,” Annals of the New York Academy of Sciences, vol. 1075, pp. 57–62, 2006.
[6] I. Hromadnikova, “Extracellular nucleic acids in maternal circulation as potential biomarkers for placental insufficiency,” DNA and Cell Biology, vol. 31, no. 7, pp. 1221–1232, 2012.
[7] C. W. Redman and I. L. Sargent, “Latest advances in understanding preeclampsia,” Science, vol. 308, no. 5728, pp. 1592–1594, 2005.
[8] T. Y. Khong, F. De Wolf, W. B. Robertson, and I. Brosens, “Inadequate maternal vascular response to placenta in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants,” British Journal of Obstetrics and Gynaecology, vol. 93, no. 10, pp. 1049–1059, 1986.
[9] WHO, “World Health Organization International Collaborative Study of Hypertensive Disorders in Pregnancy. Geographic variation in the incidence of hypertension in pregnancy,” American Journal of Obstetrics and Gynecology, vol. 158, no. 1, pp. 80–83, 1988.
[10] J. E. Bamfo and A. O. Odibo, “Diagnosis and management of fetal growth restriction,” Journal of Fetal Medicine, vol. 2011, Article ID 640715, 15 pages, 2011.
[11] ACOG Committee on Practise Bulletins-Obstetrics, “Diagnosis and management of preeclampsia and eclampsia,” Obstetrics and Gynecology, vol. 99, no. 1, pp. 159–167, 2002.
[12] S. J. Germain, G. P. Sacks, S. R. Soorana, I. L. Sargent, and C. W. Redman, “Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles,” Journal of Immunology, vol. 178, no. 9, pp. 5949–5956, 2007.
[13] S. Kh. Khan, D. Wojdyla, L. Say, A. M. Gülmezoglu, and P. F. Van Look, “WHO analysis of causes of maternal death: a systematic review,” The Lancet, vol. 367, no. 9516, pp. 1066–1074, 2006.
[14] A. Reddy, X. Y. Zhong, C. Rusterholz et al., “The effect of labour and placental separation on the shedding of syncytiotrophoblast microparticles, cell-free DNA and mRNA in normal pregnancy and pre-eclampsia,” Placenta, vol. 29, no. 11, pp. 942–949, 2008.
[15] L. C. Y. Poon, R. Akolekar, R. Lachmann, J. Beta, and K. H. Nicolaides, “Hypertensive disorders in pregnancy: screening by biophysical and biochemical markers at 11–13 weeks,” Ultrasound in Obstetrics and Gynecology, vol. 35, no. 6, pp. 662–670, 2010.
[16] R. Akolekar, A. Syngelaki, R. Sarquis, M. Zvanca, and K. H. Nicolaides, “Prediction of early, intermediate and late pre-eclampsia from maternal factors, biophysical and biochemical markers at 11–13 weeks,” *Prenatal Diagnosis*, vol. 31, no. 1, pp. 66–74, 2011.

[17] P. von Dadelszen, L. A. Magee, and J. M. Roberts, “Subclassification of pre-eclampsia,” *Hypertension in Pregnancy*, vol. 22, no. 2, pp. 143–148, 2003.

[18] B. Huppertz, “Placental origins of preeclampsia: challenging the current hypothesis,” *Hypertension*, vol. 51, no. 4, pp. 970–975, 2008.

[19] H. Valensise, B. Vasapollo, G. Gagliardi, and G. P. Novelli, “Early and late pre-eclampsia: two different maternal hemodynamic states in the latent phase of the disease,” *Hypertension*, vol. 52, no. 5, pp. 873–880, 2008.

[20] Y. M. Dennis Lo, N. Corbetta, P. F. Chamberlain et al., “Presence of fetal DNA in maternal plasma and serum,” *The Lancet*, vol. 350, no. 9076, pp. 485–487, 1997.

[21] M. Smid, A. Vassallo, F. Lagona et al., “Quantitative analysis of fetal DNA in maternal plasma in pathological conditions associated with placental abnormalities,” *Annals of the New York Academy of Sciences*, vol. 945, pp. 132–137, 2001.

[22] E. Caramelli, N. Rizzo, M. Concu et al., “Cell-free fetal DNA concentration in plasma of patients with abnormal uterine artery Doppler waveform and intrauterine growth restriction—a pilot study,” *Prenatal Diagnosis*, vol. 23, no. 5, pp. 367–371, 2003.

[23] J.-M. Costa, A. Benachi, and E. Gautier, “New strategy for prenatal diagnosis of X-linked disorders,” *New England Journal of Medicine*, vol. 346, no. 19, p. 1502, 2002.

[24] R. J. Rijnders, C. E. van der Schoot, B. Bossers, M. A. de Vroeede, and G. C. Christiaens, “Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia,” *Obstetrics and Gynecology*, vol. 98, no. 3, pp. 374–378, 2001.

[25] B. H. W. Faas, E. A. Beuling, G. C. M. L. Christiaens, A. E. G. K. Von Dem Borne, and C. E. Van Der Schoot, “Detection of fetal RHD-specific sequences in maternal plasma,” *The Lancet*, vol. 352, no. 9135, pp. 1196, 1997.

[26] Y. M. D. Lo, N. M. Hjelm, C. Fidler et al., “Prenatal diagnosis of fetal RHD status by molecular analysis of maternal plasma,” *New England Journal of Medicine*, vol. 339, no. 24, pp. 1734–1738, 1998.

[27] I. Hromadnikova, L. Vechtova, K. Vesela, B. Benesova, J. Doucha, and R. Vlk, “Non-invasive fetal RHD and RHCE genotyping using real-time PCR testing of maternal plasma in RhD-negative pregnancies,” *Journal of Histochemistry and Cytochemistry*, vol. 53, no. 3, pp. 301–305, 2005.

[28] K. C. A. Chan, C. Ding, A. Gerovassili et al., “Hypermethylated RASSF1A in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis,” *Clinical Chemistry*, vol. 52, no. 12, pp. 2211–2218, 2006.

[29] Y. M. D. Lo, T. N. Leung, M. S. C. Tein et al., “Quantitative abnormalities of fetal DNA in maternal serum in pre-eclampsia,” *Clinical Chemistry*, vol. 45, no. 2, pp. 184–188, 1999.

[30] T.-W. Lau, T. N. Leung, L. Y. S. Chan et al., “Fetal DNA clearance from maternal plasma is impaired in pre-eclampsia,” *Clinical Chemistry*, vol. 48, no. 12, pp. 2141–2146, 2002.

[31] B. M. Byrne, A. Crowley, F. Taulo, J. Anthony, J. J. O’Leary, and C. O’Herlihy, “Fetal DNA quantitation in peripheral blood is not useful as a marker of disease severity in women with pre-eclampsia,” *Hypertension in Pregnancy*, vol. 22, no. 2, pp. 157–164, 2003.

[32] A. Sekizawa, M. Jimbo, H. Saito et al., “Cell-free fetal DNA in the plasma of pregnant women with severe fetal growth restriction,” *American Journal of Obstetrics and Gynecology*, vol. 188, no. 2, pp. 480–484, 2003.

[33] D. W. Y. Tsui, K. C. A. Chan, S. S. C. Chim et al., “Quantitative aberrations of hypermethylated RASSF1A gene sequences in maternal plasma in pre-eclampsia,” *Prenatal Diagnosis*, vol. 27, no. 13, pp. 1212–1218, 2007.

[34] I. Hromadnikova, M. Benesova, L. Zejskova et al., “The effect of DYS-14 copy number variations on extracellular fetal DNA quantification in maternal circulation,” *DNA and Cell Biology*, vol. 28, no. 7, pp. 351–358, 2009.

[35] I. Hromadnikova, L. Zejskova, K. Kotlabova et al., “Quantification of extracellular DNA using hypermethylated RASSF1A, SRY, and GLO sequences—evaluation of diagnostic possibilities for predicting placental insufficiency,” *DNA and Cell Biology*, vol. 29, no. 6, pp. 295–301, 2010.

[36] B. L. Pineles, R. Romero, D. Montenegro et al., “Distinct subsets of microRNAs are expressed differentially in the human placentas of patients with preeclampsia,” *American Journal of Obstetrics and Gynecology*, vol. 196, no. 3, pp. 261.e1–261.e6, 2007.

[37] X.-M. Zhu, T. Han, I. L. Sargent, G.-W. Yin, and Y.-Q. Yao, “Differential expression profile of microRNAs in human placentas from preeclamptic pregnancies vs normal pregnancies,” *American Journal of Obstetrics and Gynecology*, vol. 200, no. 6, pp. 661.e1–661.e7, 2009.

[38] Y. Hu, P. Li, S. Hao, L. Liu, J. Zhao, and Y. Hou, “Differential expression of microRNAs in the placentae of Chinese patients with severe pre-eclampsia,” *Clinical Chemistry and Laboratory Medicine*, vol. 47, no. 8, pp. 923–929, 2009.

[39] S. S. C. Chim, T. K. F. Shing, E. C. W. Hung et al., “Detection and characterization of placental microRNAs in maternal plasma,” *Clinical Chemistry*, vol. 54, no. 3, pp. 482–490, 2008.

[40] E. C. Lai, “Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation,” *Nature Genetics*, vol. 30, no. 4, pp. 363–364, 2002.

[41] D. P. Bartel, “MicroRNAs: genomics, biogenesis, mechanism, and function,” *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[42] T. Gunel, Z. G. Zeybek, P. Akçakaya et al., “Serum microRNA expression in pregnancies with preeclampsia,” *Genetics and Molecular Research*, vol. 10, no. 4, pp. 4034–4040, 2011.

[43] Q. Yang, J. Lu, S. Wang, H. Li, Q. Ge, and Z. Lu, “Application of next-generation sequencing technology to profile the circulating microRNAs in the serum of preeclampsia versus normal pregnant women,” *Clinica Chimica Acta*, vol. 412, no. 23-24, pp. 2167–2173, 2011.

[44] L. Wu, H. Zhou, H. Lin et al., “Circulating microRNAs are elevated in plasma from severe preeclamptic pregnancies,” *Reproduction*, vol. 143, no. 3, pp. 389–397, 2012.

[45] J.-F. Mouillet, T. Chu, C. A. Hubel, D. M. Nelson, W. T. Parks, and Y. Sadovsky, “The levels of hypoxia-regulated microRNAs in plasma of pregnant women with fetal growth restriction,” *Placenta*, vol. 31, no. 9, pp. 781–784, 2010.

[46] H. Seitz, H. Royo, M.-L. Bortolin, S.-P. Lin, A. C. Ferguson-Smith, and J. Cavaillé, “A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain,” *Genome Research*, vol. 14, no. 9, pp. 1741–1748, 2004.
[47] Y. Liang, D. Ridzon, L. Wong, and C. Chen, “Characterization of microRNA expression profiles in normal human tissues,” BMC Genomics, vol. 8, article 166, 2007.

[48] I. Bentwich, A. Avniel, Y. Karov et al., “Identification of hundreds of conserved and nonconserved human microRNAs,” Nature Genetics, vol. 37, no. 7, pp. 766–770, 2005.

[49] S. Lin, W. K. C. Cheung, S. Chen et al., “Computational identification and characterization of primate-specific microRNAs in human genome,” Computational Biology and Chemistry, vol. 34, no. 4, pp. 232–241, 2010.

[50] D. M. Morales-Prieto, S. Ospina-Prieto, W. Chaiwangyen, M. Schoenleben, and U. R. Markert, “Pregnancy-associated miRNA-clusters,” Journal of Reproductive Immunology, vol. 97, no. 1, pp. 51–61, 2013.

[51] K. Kotlabova, J. Doucha, and I. Hromadnikova, "Placental-specific microRNA in maternal circulation—identification of appropriate pregnancy-associated microRNAs with diagnostic potential," Journal of Reproductive Immunology, vol. 89, no. 2, pp. 185–191, 2011.

[52] M.-L. Bortolin-Cavaille, M. Dance, M. Weber, and J. Cavaille, "C19MC microRNAs are processed from introns of large Pol-II, non-protein-coding transcripts," Nucleic Acids Research, vol. 37, no. 10, pp. 3464–3473, 2009.

[53] MiRNAMap 2.0 [Internet], Department of Biological Science and Technology, Institute of Bioinformatics National Chiao Tung University, Hsinchu, Taiwan, 2013, http://mirnamap.mbc.nctu.edu.tw.

[54] I. Hromadnikova, K. Kotlabova, J. Doucha, K. Dlouha, and L. Krofta, “Absolute and relative quantification of placenta-specific microRNAs in maternal circulation with placental insufficiency—related complications,” Journal of Molecular Diagnostics, vol. 14, no. 2, pp. 160–167, 2012.

[55] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2-\Delta\Delta CT method," Methods, vol. 25, no. 4, pp. 402–408, 2001.

[56] M. A. Maccani, J. F. Padbury, and C. J. Marsit, “miR-16 and miR-21 expression in the placenta is associated with fetal growth,” PLoS ONE, vol. 6, no. 6, Article ID e21210, 2011.
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