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Candidate Gene and MicroRNA Expression in Fetal Membranes and Preterm Delivery Risk

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Abstract

We investigated candidate gene and microRNA (miRNA) expression in amnion and chorion in relation to risk of preterm delivery (PTD). Amnion and chorion were separated from placenta and collected at delivery from participants who delivered at term (N = 10) and from participants who delivered preterm following spontaneous labor (sPTL-PTD; N = 10), premature rupture of membranes (PPROM-PTD; N = 10), and preeclampsia (PE-PTD; N = 10). Expression of genes (metalloproteinase [MMP] 2, MMP-9, and tissue inhibitors of MMP-1) and miRNAs (miR-199a*, -202*, -210, -214, -223, and -338) was profiled using quantitative real-time polymerase chain reaction approaches. Adjusted multinomial logistic regression models were used to calculate relative risk ratios (RRR), 95% confidence intervals, and *P* values. Among controls, the expression of miR-199a*, -202*, and -214 was lower in the amnion compared with their expression in the chorion, whereas the expression of miR-210 was higher in the amnion compared with its expression in the chorion (all *P* values < .05). In the amnion, MMP-9 expression was associated with PTD risk (overall *P* value = .0092), and MMP-9 expression was positively associated with the risk of PPROM-PTD (RRR: 31.10) and inversely associated with the risk of PE-PTD (RRR:6.55e-6), although individual associations were not statistically significant. In addition, in the amnion, the expression of miR-210 (RRR: 0.45; overall *P* value = .0039) was inversely associated with the risk of PE-PTD, and miR-223 was inversely associated with all subtypes of PTD (overall *P* value = .0400). The amnion and chorion differ in their miRNA expression. The expression of MMP-9, miR-210, and -223 in the amnion is associated with PTD risk.

Keywords

gene expression, microRNA expression, amnion, chorion, preterm delivery

Introduction

The pathogenesis of preterm delivery (PTD) is related to, at least in part, a complex set of biochemical processes that lead to an untimely degradation of the extracellular matrix (ECM) of the amnion, chorion, or both fetal membranes.[1-3] However, biological mechanisms underlying ECM remodeling and related untimely rupture of fetal membranes that contribute to PTD are incompletely understood. Matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, and their regulation by specific tissue inhibitors of MMPs (TIMPs) are involved in remodeling and physiological homeostasis of ECM that takes place during placentation (eg, trophoblast invasion) and parturition (ie, cervical ripening, rupture of fetal membranes, and placental detachment).[4-6] Prior studies have documented the associations of MMPs and TIMPs expression in the placenta and fetal membranes with PTD, although findings were not consistent.[6,7]

MicroRNAs (miRNAs) are posttranscription regulatory mechanisms that play important roles in physiologic and pathologic processes.[8-10] Placental expression of miRNAs has been related to pregnancy complications, such as preeclampsia (eg, miR-210), chorioamnionitis (miR-223, -338, and -214), and preterm labor (eg, miR-199a, -202, and -214) that lead to PTD.[11-14] However, significant gaps remain in the understanding of PTD-related posttranscription regulation in the amnion and chorion, respectively. Such investigations have the

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potential to further our understanding of genetic factors and gene–environment interactions that underlie the heterogeneous mechanisms that contribute to untimely perinatal events that lead to PTD. Therefore, we conducted an investigation that involves candidate gene (MMP-2, MMP-9, and TIMP-1) and candidate miRNA (miR-199a*, -202*, -210,-214, -223, and -338) expression profiling experiments in fetal membranes collected from term pregnancies and pregnancies that ended in PTD following premature rupture of membranes (PPROM-PTD), spontaneous preterm labor (sPTL-PTD), or preeclampsia (PE-PTD).

Materials and Methods

Study Setting and Study Population

The current study was based on data collected as part of the Placenta MicroArray study. The Placenta MicroArray study was designed to examine differential placental gene expression in relation to pregnancy complications. Eligible study participants were women who delivered at Swedish Medical Center, Seattle, Washington. Study personnel identified and recruited potential participants from labor and delivery ward admission records.

For the current study, potential participants were identified among PTD cases and term controls. We used estimated date of confinement (EDC) in defining PTD. We assessed EDC using maternal report of last menstrual period (LMP) combined with ultrasound dating completed $\leq$20 weeks' gestation. If both LMP and ultrasound date were available and the 2 agree within 14 days, we used the former to assign gestational age. If they differed by >14 days, we used the ultrasound-determined date. We distinguished among 3 subgroups of PTD based on the antecedent reason for the PTD: (1) sPTL-PTD—physician diagnosis of spontaneous onset of labor before rupture of fetal membranes and delivery before 37 weeks' gestation, (2) PPROM-PTD—physician diagnosis of spontaneous rupture of fetal membranes prior to the onset of labor and delivery before 37 weeks' gestation, and (3) Medically indicated PE-PTD—pregnancies delivered before 37 weeks' gestation as a result of medical intervention following preeclampsia. Participants with history of preexisting and gestational diabetes, chronic hypertension, or clinical chorioamnionitis were excluded. We included 10 participants from each of the 3 PTD subgroups (sPTL-PTD, PPROM-PTD, and PE-PTD) and 10 control participants who delivered at term. Study protocols were approved by Swedish Medical Center Institutional Review Board, and participants provided informed consent.

Data Collection

Medical records were reviewed to collect information on maternal sociodemographic characteristics, family/medical histories, occupation, reproductive and medical histories, height, weight, and other maternal characteristics. In addition, information on course and outcomes of the pregnancy, including presence or absence of pregnancy complications and mode of delivery, was abstracted from medical records. At delivery, placentas were collected, weighed, double bagged, and transported in coolers to our dedicated placenta-processing laboratory, where the amnion was separated from the chorion. Samples were then stored at -80°C until further processing.

Placental Sample Processing and RNA Extraction

After the amnion and chorion were separated, tissue biopsies (~ 0.5 cm^3 each) were collected. Biopsies were placed in cryotubes containing RNAlater (Qiagen Inc, Valencia, California) at 10 μL per 1 mg of tissue and stored at -80°C. The samples were homogenized using a Tissue Tearor (BioSpec Products Inc, Bartlesville, Oklahoma) or Mini-Beadbeater-8 (BioSpec Products Inc) in a lysis buffer from the RNeasy Fibrous Midi Kit (Qiagen Inc) with added β-mercaptoethanol to disrupt any proteins that might be destroying nucleic acid. For mRNA expression profiling, total RNA was extracted using a standardized protocol adapted from the RNeasy Fibrous Tissue Midi Handbook (Qiagen Inc). Total RNA concentration was calculated by determining absorbance at 260 nm (Spectramax Plus 384 spectrophotometer; Molecular Devices, Sunnyvale, California). Protein contamination was monitored by the ratio of absorbance at 260 nm to absorbance at 280 nm (A260/A280). All samples had A260/A280 ratio greater than 1.8. For miRNA expression profiling, total RNA was extracted using the mirVana miRNA isolation kit (Life Technologies, Carlsbad, California). RNA concentration and quality were assessed on the Agilent Bioanalyzer (Agilent, Santa Clara, California). All samples were diluted to 0.25 μg/μL in sterile water and aliquoted for storage at -80°C. All RNA samples, including reference RNA, underwent a quality control check and were labeled using the same standardized protocols.

Messenger RNA and miRNA Expression Measurement

We used quantitative real-time polymerase chain reaction (qRT-PCR) experiments and Taqman gene expression assays from Applied Biosystems (Foster City, California) to measure placental expression of 3 messenger RNAs (mRNAs; MMP-2, MMP-9, and TIMP-1) and 6 miRNAs (miR-199a*, -202*, -210,-214, -223, and -338). Quantitative real-time polymerase chain reaction was performed in duplicates using assays developed by Applied Biosystems. Reactions were run on an ABI PRISM 7000 real-time polymerase chain reaction machine (Applied Biosystems, Foster City, California) using the default cycling conditions. Threshold cycle (C_t) values of the duplicates differing by greater than 0.2 times the standard deviation were retested. The C_t values of the duplicates differing by less than 0.2 times the standard deviations were averaged for analysis. Raw measurements were normalized using SDHA and RNU44 expression measurement for the mRNA and miRNA expression profiling experiments, respectively. The qRT-PCR

Table 1. Characteristics of the Study Participants.

Characteristics	Term Controls (N =10), n (%)	sPTL-PTD Cases (N = 10), n (%)	PPROM-PTD Cases (N = 10), n (%)	PE-PTD Cases (N = 10), n (%)
Maternal age,[a] years	32.0 ± 5.0	29.9 ± 6.5	30.9 ± 3.0	32.9 ± 6.3
Non-Hispanic white	8 (80.0)	6 (60.0)	5 (50.0)	6 (60.0)
Nulliparous	2 (20.0)	3 (30.0)	2 (20.0)	4 (40.0)
Single marital status	1 (10.0)	4 (40.0)	3 (30.0)	2 (20.0)
Smoked during pregnancy	0 (0)	2 (20.0)	2 (20.0)	1 (10.0)
Prepregnancy BMI,[a] kg/m^2	23.7 ± 2.3	23.3 ± 2.9	22.6 ± 2.8	26.0 ± 4.8
Mode of delivery				
C-section	4 (40.0)	3 (30.0)	3 (30.0)	5 (50.0)
Vaginal	6 (60.0)	7 (70.0)	7 (30.0)	5 (50.0)
Gestational age at delivery,[a] weeks	39.8 ± 1.3	34.1 ± 1.9	34.8 ± 1.2	32.6 ± 2.3

Abbreviations: BMI, body mass index; PE, medically indicated following preeclampsia; PPROM, preterm premature rupture of membrane; PTD, preterm delivery; SD, standard deviation; sPTL, spontaneous preterm labor.
[a]Mean ± SD, otherwise number (%).

experiments were performed at the Center for Perinatal Studies, Swedish Medical Center, Seattle, Washington.

Statistical Analyses

The distributions of maternal sociodemographic, medical, and clinical characteristics according to PTD case (by subtype) or control status were examined. We compared the relative expression (relative to the housekeeping [HK] genes) of mRNAs and miRNAs in the amnion and the chorion using Student *t* test. The fold change was evaluated based on the raw expression (δ C_t value) scale $\left(2^{(\bar{y}_a - \bar{y}_c)}\right)$. We then used multinomial logistic regression models to evaluate the association between mRNA or miRNA raw expression (δ C_t values) and the risk of being in one of the PTD subtype groups (sPTL-PTD, PPROM-PTD, or PE-PTD) compared to controls.[15] The relative risk ratios (RRRs) and related 95% confidence intervals (CIs) and overall *P* values based on a likelihood ratio tests (comparing the model with mRNA/miRNA and adjustment variables to a model with only the adjustment variables) were used to determine whether the expression of mRNA/miRNA was associated with each subtype of PTD.[15] The models were adjusted for maternal age and mode of delivery (cesarean section or vaginal delivery). All statistical tests were 2 tailed, and statistical significance was defined at $\alpha = 0.05$. STATA (STATA ver 11.0, College Station, Texas) and R software (www.R-project.org) were used for analyses.

Results

Average age of participants in the control and the case groups ranged between 30 and 33 years (Table 1). The majority of study participants were non-Hispanic white. The PE-PTD cases had higher proportions of nulliparous women (40%) compared with the control participants (20%) and other PTD cases (30% for sPTL-PTD cases and 20% for PPROM-PTD cases). Similarly, PE-PTD cases had higher mean prepregnancy body mass index (26.0 kg/m^2) compared to controls (23.7 kg/m^2) and other PTD cases (23.3 and 22.6 kg/m^2 for sPTL-PTD and PPROM-PTD cases, respectively). None of the participants had chorioamnionitis.

Among controls, we did not observe differences in the expression of MMP-2, MMP-9, or TIMP-1 in the amnion and chorion (all *P* values > .05; Table 2 and Supplemental Figure). However, we observed statistically significant differences in the expression of all candidate miRNAs, except miR-223 and 338, between the amnion and the chorion. The expression of miR-199a*, miR-202*, and miR-214 was lower in the amnion compared with their expression in the chorion, whereas the expression of miR-210 was higher in the amnion compared with its expression in the chorion (all *P* values < .05).

In the amnion, the expression of MMP-9 was associated with PTD risk (overall *P* value = .0092; Table 3). More specifically, MMP-9 expression was positively associated with PPROM-PTD risk (RRR: 31.10; 95% CI: 0.19-5.33e2) and inversely associated with PE-PTD risk (RRR: 6.55e-6, 95% CI: 3.97e-7-10.82), although these individual associations were not statistically significant. The expression of miR-210 (RRR: 0.45; 95% CI: 0.22-0.91; overall *P* value = .0039) in the amnion was inversely associated with PE-PTD risk. In addition, in the amnion, miR-223 was inversely associated with all subtypes of PTD (overall *P* value = .0400), although individual associations of miR-223 with PTD subtypes were not statistically significant. We did not observe association of mRNA or miRNA expression with PTD risk in the chorion.

Comments

In the current study, we found differences in the expression of several miRNAs in the amnion and chorion sampled from control pregnancies. We also found that amnion MMP-9 expression was positively associated with the risk of PPROM-PTD and inversely associated with the risk of PE-PTD. The expression of both miR-210 and miR-223 in the amnion was inversely associated with the risk of PE-PTD. In addition, the expression of miR-223 was inversely associated with the risk of PPROM-PTD and sPTL-PTD.

Table 2. Candidate mRNA/miRNA Expression in Amnion and Chorion of Term Pregnancies.

	Amnion (N = 10)		Chorion (N = 10)			
Transcript	Mean ($\bar{y}_a$)	SD	Mean ($\bar{y}_c$)	SD	Fold Change[a] Amnion/Chorion	P[b]
mRNA						
MMP-22	0.250	0.264	0.324	0.211	0.950	.1224
MMP-9	0.232	0.304	0.257	0.298	0.983	.8320
TIMP-12	0.027	0.020	0.029	0.017	0.931	.9101
miRNA						
miR-99a*	1.051	0.585	2.280	1.235	0.427	.0139
miR-202*	0.754	0.702	1.821	1.438	0.477	.0548
miR-210	9.854	8.946	2.899	1.271	124.069	.0368
miR-214	1.262	1.081	3.737	3.096	0.180	.0358
miR-223	6.754	10.848	2.249	2.265	22.706	.2281
miR-338	3.072	4.698	1.836	1.704	2.355	.4501

Abbreviations: miRNA, microRNA; MMP, metalloproteinase; mRNA, messenger RNA; TIMP, tissue inhibitors of MMPs.

[a]Fold change is provided based on the raw expression scale $\left(2^{(\bar{y}_a-\bar{y}_c)}\right)$.

[b]Student *t* test *P* value.

Table 3. Association Between Candidate mRNA/miRNA Expression in Amnion and Chorion and Risk of Preterm Delivery Across PTD Subtypes.

	Controls	sPTL-PTD, RRR (95% CI)	PPROM-PTD, RRR (95% CI)	PE-PTD, RRR (95% CI)	P[a]
Amnion					
mRNA					
MMP-22	Referent	9.054 (0.200-410.100)	0.150 (1.30e-3-17.120)	1.791 (0.031-102.600)	.2410
MMP-9	Referent	1.452 (4.94e-2-42.7)	31.100 (0.189-5.1e3)	6.55e-6 (3.97e-12-10.820)	.0092
TIMP-12	Referent	2.88e5 (7.56e-10-1.1e20)	1.066 (1.54e-21-7.37e20)	74.610 (3.11e-14-1.79e17)	.8365
miRNA					
miR-199a*	Referent	0.951 (0.227-3.984)	2.019 (0.554-7.537)	0.737 (0.175-3.104)	.4730
miR-202*	Referent	1.561 (0.380-6.413)	1.674 (0.418-6.704)	2.640 (0.635-10.980)	.5810
miR-210	Referent	0.867 (0.700-1.074)	0.848 (0.666-1.081)	0.449 (0.222-0.910)	.0039
miR-214	Referent	0.954 (0.414-2.200)	2.160 (0.743-6.279)	1.058 (0.519-2.153)	.2500
miR-223	Referent	0.605 (0.267-1.373)	0.862 (0.666-1.117)	0.671 (0.311-1.450)	.0400
miR-338	Referent	0.768 (0.173-1.338)	0.921 (0.714-1.189)	0.481 (0.473-1.248)	.2150
Chorion					
mRNA					
MMP-22	Referent	3.954 (0.048-3.28e2)	0.806 (8.5e-3-76.87)	0.500 (5.0e-3-49.70)	.8107
MMP-9	Referent	0.669 (1.59e-2-28.22)	1.309 (1.98e-2-86.49)	5.1e-4 (2.74e-8-9.57)	.1239
TIMP-12	Referent	2.34e2 (1.74e-17-3.13e21)	2.02e3 (4.86e-18-8.43e23)	3.28e-4 (3.72e-25-2.89e17)	.9193
miRNA					
miR-199a*	Referent	1.141 (0.818-1.591)	0.987 (0.655-1.487)	0.856 (0.472-1.557)	.5304
miR-202*	Referent	1.108 (0.786-1.563)	0.658 (0.224-1.927)	0.980 (0.614-1.564)	.5249
miR-210	Referent	1.275 (0.800-2.032)	1.445 (0.892-2.342)	1.301 (0.827-2.048)	.3904
miR-214	Referent	1.077 (0.880-1.318)	0.994 (0.778-1.269)	0.867 (0.610-1.233)	.4792
miR-223	Referent	0.999 (0.623-1.601)	0.671 (0.331-1.359)	0.302 (0.063-1.450)	.1082
miR-338	Referent	1.267 (0.785-2.043)	1.169 (0.728-1.878)	0.618 (0.209-1.832)	.3015

Abbreviations: 95% CI, 95% confidence interval; miRNA, microRNA; MMP, metalloproteinase; mRNA, messenger RNA; PE, medically indicated following preeclampsia; PPROM, preterm premature rupture of membrane; PTD, preterm delivery; RRR, relative risk ratio; sPTL, spontaneous preterm labor.

[a]Overall *P* value corresponding to log-likelihood ratios of multinomial logistic regression models adjusted for maternal age and mode of delivery with and without marker being evaluated.

The transcriptome of fetal membranes, during labor and delivery, is characterized by region- (eg, site of membrane rupture) and tissue-specific (eg, between amnion and chorion) differential expression of genes that are involved, among others, in hormonal and ECM remodeling pathways.[1,4-7,16] Investigators have previously demonstrated differences in expression of pregnancy-specific glycoprotein species (eg, the human chorionic gonadotropin α transcript) and expression of MMP-2, MMP-9, and TIMP-1 in the amnion and chorion.[4,16-19] Yonemoto et al have reported increased MMP-2 protein and MMP-2 and -9 proenzyme activities in the amnion, but not in the chorion, with term labor.[18] In the current study, we did not find statistically significant differences in the expression of MMP-2, MMP-9, or TIMP-1. However, we did find

significant differences in miRNA expression between the amnion and the chorion. Given that potential differences in distinct transcriptome and protein expression patterns in specific cell types or tissues may be due to epigenetic factors, such as DNA methylation and miRNA expression, our observations are in line with previous reports. For instance, a study by Eckmann-Scholz et al indicated that major tissue-specific (between chorion and amnion) differences exist in DNA methylation, a form of epigenetic regulation.[20] To our knowledge, this is the first study to report miRNA expression differences between the amnion and the chorion.

Our finding that MMP-9 expression in the amnion is positively associated with PPROM-PTD risk is in line with several previous observations. Regional activation of MMPs, in particular MMP-9, subsequent to triggers that include cytokines (eg, tumor necrosis factors α, interleukin [IL]-1β, and IL-6), hormones (eg, prostaglandins E2 and F2α), mechanical stretching, and neuropeptides and placental peptides (eg, corticotropin-releasing hormone) may trigger a cascade of events that reduce fetal membrane integrity and promote rupture.[21-23] Matrix metalloproteinase 9 is selectively expressed at the end of gestation by the amnion, trophoblasts, and decidual cells.[21] Matrix metalloproteinase 9 enzyme expression and the decline of membrane tensile strength have been correlated.[21] Investigators have also shown that MMP-9 levels are increased in the amniotic fluid of women with premature rupture of membranes (PROM) in humans and in amniotic fluid of laboratory animals after bacterial- or cytokine-induced labor and that this can lead to degradation of the amniochorion basement membranes and other ECM components resulting in PROM.[23]

In the current study, MMP-9 expression in the amnion was inversely associated with the risk of PE-PTD. Previously, Mayor-Lynn et al have reported MMP-9 downregulation, although not statistically significant, in preeclamptic placenta.[24] Matrix metalloproteinases play key roles in placentation, including trophoblast invasion and vascular alterations induced by hypertension (particularly MMP-2).[25,26] However, the specific role of MMP-9 in the amnion, in relation to preeclampsia pathogenesis, is not well known and deserves further investigations.

A number of regulatory mechanisms have been proposed for secretion of MMPs that include physiological inhibition by TIMPs.[27] Interactions of MMP-2 and MMP-9 with TIMP-1 and TIMP-2 are well established, and the imbalance favoring MMPs leads to cervical ripening and fetal membrane rupture.[28] Montagnana et al demonstrated that the expression of MMP-2 and TIMP-1 is elevated in preeclampsia and that modification of the fine balance between MMPs and their inhibitors is important in the structural and functional vascular changes in women with complicated pregnancies.[29] In the current study, we did not find associations of TIMP expression, in the amnion or chorion, with PTD risk. In post hoc exploratory analyses, we investigated associations of MMP-2–TIMP and MMP-9–TIMP ratios with the risk of PTD and did not find significant associations or apparent trends of associations between the 2 ratios and PTD risk ($P > .05$; data not shown).

Several studies have previously investigated miRNA expression in fetal membranes in relation to PTD risk. Kim et al stated that miRNA-mediated posttranscriptional regulation of gene expression machinery in the amnion, the amniotic microRNAome, plays an important role in parturition.[30] In contrast to inverse associations of miR-210 expression in the amnion with the risk of PE-PTD, observed in the current study, we, along with a number of other investigators, have previously reported upregulation of miR-210 expression in preeclamptic placenta.[11,12,31] Targets of miR-210 are involved in regulation of lipid metabolism, transcription regulation, innate immune response, response to stimuli, and complement activation and classic pathway.[32,33] Importantly, miR-210 target expression of hypoxia-inducible factor 1 to regulate endothelial response to hypoxia, formation of capillary-like structures, vascular endothelial growth factor-driven cell migration, cell differentiation, and survival, disturbances of which have been described in preeclampsia pathogenesis.[33] Our observation of inverse association between miR-210 expression and PE-PTD risk is intriguing and deserves further investigations. None of the previous studies investigated amnion specifically, and one possibility could be that amnion plays a uniquely different role in PE pathogenesis.

Recently, Zhu et al reported downregulation of miR-223 expression in preeclamptic placenta.[31] We observed similar inverse associations of miR-223 with PE-PTD risk along with inverse associations of miR-223 expression with the risk of sPTL-PTD and PPROM-PTD. Targets of miR-223 include a broad set of genes involved in inflammation, hematopoietic differentiation, immune cell function, and activation.[34] This is in line with our observation as many of these pathways are involved in pathogenesis that leads to sPTL-PTD or PPROM-PTD.

Our study findings and comparisons with previous reports should be interpreted in light of several potential limitations. First, there is no consensus regarding choice of the HK gene/miRNAs for mRNA/miRNA profiling experiments in placental research. Differences in HK gene selection can contribute to differences in findings between studies. Second, there may be biological heterogeneity across parts of the amnion and chorion that may not captured in our study and those of others. Third, we had relatively small number of study participants in each group, which limited our efforts to control for potential confounders and reduced statistical power to detect significant associations. Several gene/miRNA-PTD risk associations, such as the association of MMP-9 with PPROM-PTD in the amnion, were notable but not statistically significant. Fourth, samples were collected after delivery. Although we adjusted for mode of delivery, other factors, such as administration of medications (eg, epidurals), may influence gene/miRNA expression in differential fashion. Given the preliminary nature of our study, we did not adjust for multiple testing. However, concordance of our findings with previous reports[4,11,31] suggests that statistically significant findings observed in studies are not likely to be spurious. Finally, underlying placental pathologies, other than chorioamnionitis, may confound associations.

In sum, we found that MMP-9 and mRNAs (miR-210 and miR-223) in the amnion are differentially expressed in PTD. Future studies evaluating other determinants of pretranscriptional/posttranscriptional regulators of key enzymes (eg, MMP-9) such as endogenous biomarkers (eg, cortisol), pharmacological agents (eg, doxycycline), or others (eg, vitamin D) can enhance understanding of pathophysiological mechanisms leading to PTD.[21-23,35] Further, characterization of PTD-related differentially expressed mRNAs/miRNAs (such as MMP-9 and miR-210) may provide opportunities to identify potential candidate markers of preterm labor and/or PTD as well as targets for designing effective preventative and therapeutic agents.

Declaration of Conflicting Interests

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Supplemental Material

The online supplemental figure is available at http://rs.sagepub.com/supplemental.

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