First Trimester Pbmc Microrna Predicts Adverse Pregnancy Outcome

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Problem

Prior to the end of the first trimester, pathogenic mechanisms may commit pregnancies to adverse outcome such as pre-eclampsia and miscarriage. A long-term search for biomarkers predicting these adverse outcomes has not identified any that reliably succeed prior to the beginning of the second trimester. MicroRNAs, with their important role as regulators of signaling and metabolic pathways within living cells, may offer a new approach.

Methods

Optimal maternal PBMC microRNA markers were investigated using a series of sequential experiments, and 30 microRNAs were selected based on these results. Quantitative RT-PCR was then performed on these 30 microRNAs for 39 patients [19 healthy deliveries, 12 pre-eclampsia (seven late onset and five early onset) and eight miscarriages] during the first trimester of pregnancy. Results were scored, and their predictive values assessed.

Results

MicroRNA quantification in the early first trimester (mean 34.9 ± 19.2 days post-implantation) predicted miscarriage and late pre-eclampsia with a P value of $P < 0.0001$ and achieved an AUC of 0.90 for miscarriage and 0.90 for late pre-eclampsia.

Conclusion

MicroRNA quantification of maternal blood cells offers the clinician a single test result that is simple to interpret and available much earlier in pregnancy than previously obtainable. In addition, it is the only early pregnancy marker, to date, that can successfully predict late pre-eclampsia. Although the studies that we report are preliminary, we hope that future research will build upon our discoveries and enhance the power of maternal cell microRNA to predict adverse pregnancy outcome in the clinic.

Introduction

Reproductive disorders are a vexing problem facing couples attempting to have children. These disorders may involve a variety of conditions ranging from infertility and miscarriage to pre-eclampsia. Pre-eclampsia, in particular, is a pregnancy disorder affecting an estimated 5–8% of all pregnancies worldwide, making it the most frequently encountered major obstetrical complication. The condition

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is recognized clinically after 20 weeks of gestation with the new appearance of hypertension and proteinuria. In countries with limited access to medical care, it is estimated that the disorder is responsible annually for greater than 60,000 deaths worldwide.[1] In developed countries, therapeutic intervention is often concluded with premature delivery. While this intervention protects the mother, it results in significant morbidity and mortality to the neonate.[2] Delivery of the placenta is the only known curative treatment for the condition supporting the idea that the placenta is a principal source of pathogenesis.

To date, several markers have been investigated for their ability to predict pre-eclampsia prior to the onset of symptoms. Rademacher et al. demonstrated that inositol phosphoglycan P-type in the urine of pregnant women could act as a predictive screening test 2 weeks before the diagnosis of pre-eclampsia.[3] Karimunchi found that lower serum sFlt1 concentrations were associated with women who had high mid-trimester blood pressure.[4] Other even earlier markers that have investigated include soluble endoglin (sEng), pregnancy-associated plasma protein-A (PAPP-A) and placental protein 13 (PP13).[5,6] Khalil and colleagues originally suggested that PP13 was effective in the first trimester marker; however, in a second larger study, PP13 concentrations were not able to predict pre-eclampsia risk until 13 weeks.[7,8] Unfortunately, several studies have suggested that maladaptive trophoblast invasion may already have committed a pregnancy's fate as early as 11 weeks.[9–11] To date, no biomarkers have demonstrated the ability to predict pre-eclampsia before 10 weeks, a likely time frame for possible preventative treatment.

The recently described regulatory non-coding RNAs intrigued us, offering a potential for marker discovery. MicroRNAs are a newly discovered class of RNA species comprising a 22- to 24-base non-coding polynucleotide. Transcribed as RNA polynucleotides of much greater length, they undergo two sequential nucleolytic events, one in the nucleus and a second in the cytoplasm generating the mature microRNA sequence. Incorporated into the RNA-induced silencing complex (RISC), microRNAs target mRNA through complementary interactions. RISC is thought to inhibit translation of the targeted mRNA through a variety of distinct mechanisms.[12] The final product is joined to a cluster of proteins forming a ribonucleoprotein complex. Guided by sequence complementarity between the microRNA and a target mRNA, the ribonucleoprotein acts to inhibit translation of the targeted mRNA sequence into protein thereby regulating mRNA expression.[13,14]

MicroRNAs are now widely understood to have a central role in regulating gene expression. Because individual microRNA molecules recognize sequences common to genes within an individual pathway, they act as a unifying regulatory component balancing the individual elements of the pathway. MicroRNAs have been identified functioning upstream of immune response pathways. Thus, quantification of microRNAs involved in immune regulation may provide useful information regarding immune responses.[15] Because microRNA functions upstream, their quantification might provide earlier predictive information than currently studied biomarkers.

We chose to investigate the utility of microRNA quantification of peripheral blood cells collected during the first trimester as marker for pregnancy monitoring. Although Luque et al. concluded that maternal first trimester serum microRNA does not appear to have any predictive value for early pregnancy,[16] we were struck by the difference in prognostic power between quantification of peripheral blood cell and plasma microRNA. Our experiments suggest that quantification of microRNA in maternal cells can effectively predict pregnancy outcome before 10 weeks, a time frame when preventative treatment may be possible.

Materials & methods

Studies were nested from within a larger clinical database. MicroRNA analyses were performed retrospectively. No findings were made available to clinicians. Informed consent for use of patient samples in research was acquired from each patient. Blood samples were collected for routine laboratory studies, and residual material used for our studies. PBMCs preserved in TRIzol (Invitrogen, Carlsbad, CA, USA) and kept at −20°C.

Pregnancy Outcome Criteria

Healthy delivery

A 'healthy' delivery was defined as the delivery of a singleton normal karyotype baby with the following pregnancy criteria: (i) delivered at 37- to 40-week gestation, (ii) birthweight of ≥6 lbs, (iii) normal maternal blood pressure throughout pregnancy or

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(iv) twin delivery with gestational age ≥35 weeks with birthweights of ≥5.1 lbs and (v) no other pregnancy or delivery complications.[17]

Pre-eclampsia

'Pre-eclampsia' was defined as a pregnancy condition in which the pregnancy of a singleton/twin/triplet normal karyotype baby expressed the following characteristics: (i) IUGR (<90% normal weight for gestational age); (ii) blood pressure >140 systolic and/or >90 diastolic (two separate readings taken at least 6 hr apart); (iii) ≥300 mg of protein in a 24-hr urine sample.[18] 'Early-onset pre-eclampsia' was defined as onset at <34-week gestation. 'Late-onset pre-eclampsia' was defined as onset at ≥34-week gestation.[19]

Miscarriage

A 'miscarriage' was defined as a failed pregnancy of <24-week gestation age that had reached a minimum β-HCG level of 25 and/or demonstrated a visible uterine pregnancy sac via ultrasound.[20] All known karyotypically abnormal miscarriages were excluded from this study.

IVIg therapy

Intravenous immunoglobulin (IVIg) was administered at 400 mg/kg body weight for a history of immunologic recurrent miscarriage and/or infertility with one or more of the following test abnormalities present: elevated Th1:Th2 (>30.6), elevated% $CD56^+$ cells (>12%) and/or elevated% NK cytotoxicity (>15%).[21–23] In cases where an abnormality was present, IVIG was administered at least once during the IVF cycle or at a positive pregnancy test. An additional IVIG was given during the first trimester of pregnancy and then at 4 week intervals, if these levels were still elevated following repeated monthly% $CD56^+$ cell and/or NK cytotoxicity assessment. In all cases, blood for the experiments was drawn as a part of routine blood studies performed on patients, and microRNA results in no way influenced treatment.

Quantitative Real-Time PCR analysis (qRT-PCR)

In those experiments, where microRNA was quantified using real-time polymerase chain reaction, cycle threshold ('Ct') represents the amplification cycle at which a reliable signal is first detected. Each cycle represents a doubling of the target polynucleotide. Total RNA was isolated from TRIzol (Invitrogen). RNA quantity was determined using Nanodrop (Thermo Scientific, Waltham, MA, USA), and the quality was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). MicroRNA reverse transcription and pre-PCR amplification contained 10 ng of total RNA, pooled TaqMan assays at a final concentration of 0.2× for each assay and master mix of TaqMan MicroRNA Reverse Transcription Kit.[24] All reagents were purchased from Applied Biosystems, Inc. (Foster City, CA, USA). The reaction mixture was mixed with RNA and incubated as follows: 16°C for 30 min, 42°C for 30 min and then 85°C for 5 min. The 30 primer set that was used for the RT reaction included: RNU48, 340-5p, 424-5p, 33a-5p, 7-5p, 1229, 1267, 671-3p, 1, 133b, 144-3p, 582-5p, 30e-3p, 199a-5p, 199b-5p, 210, 221-5p, 575, 301a-3p, 148a-3p, 193a-3p, 219-5p, 132, 513a-5p, 1244, 16, 146a, 155, 181a, 196a and 223 (Applied Biosytems).

MicroRNA concentration was normalized against RNU48. The cycle threshold (Ct) values, corresponded to the PCR cycle number at which fluorescence emission reached a threshold above baseline emission, were determined, and the relative microRNA or mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems User Bulletin No. 2).[25] These methods were used in Experiments 1 and 4. Greater than 9000 individual qRT-PCRs were conducted using the Fluidigm BioMarkTM HD system in Experiment 4.[26]

Microarray Analysis

Agilent Technologies Human microRNA array kit version 2.4 was utilized according to manufacturer's instructions to perform microarray analysis (manual version G4170-90011) (Agilent Technologies, San Jose, CA, USA).[27] The microRNA microarray data were normalized using the Agilent GeneSpring GX v11.5.1.[28]

Experiment 1: qPCR 7 microRNA quantification before and after IVIg

Our initial microRNA samples were drawn 1–3 weeks before IVIg therapy and at 1–3 weeks after IVIg therapy in the first half of pregnancy. All available patients that had Trizol-preserved samples available during these time points were included in the study (16 patients). qRT-PCR was carried out using ABI 7900 HT real-time PCR system in a 384-well plate format. The PCR was performed at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and

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60°C for 1 min. The data were analyzed with ABI RQ Manager software (Foster City, CA, USA) after exportation as a SDS file. MicroRNA was quantified by qRT-PCR for the following seven microRNAs: hsa-miR-16, 132, 146a, 155, 181a, 196a and 223. These microRNAs were identified as differentially expressed in systemic inflammatory disease, and therefore, it was hoped that they might undergo differential expression following an anti-inflammatory intervention.[29–34]

Experiment 2: MicroArray profiling analysis of Group A and B IVIg response groups

Due to the bimodal distribution of patient miR-132 response discovered after IVIg therapy, four randomly selected patients from Experiment 1 with an initial miR-132 Ct level <21 were designated 'Group A' and four randomly selected patients from Experiment 1 with an initial miR-132 Ct level ≥ 21 were designated 'Group B'. These blood samples were drawn at 1–3 weeks before IVIg therapy and at 1–3 weeks after IVIg therapy. MiRNA microarray profiling was performed on 852 human microRNAs using Agilent's Human miRNA Microarray (V2), 8 × 15 K (Santa Clara, CA, USA) according to manufacturer's recommended protocol. Feature extraction was performed using Agilent FE software. The raw data were normalized using quantile normalization method with GeneSpring software (Santa Clara, CA, USA). Statistical analysis was performed using the *t*-test (Graphpad Software®, La Jolla, CA, USA). Two additional groups were created as controls by randomizing patients in groups A and B and comparing the results.

Experiment 3: MicroArray analysis of to assess microRNA correlations with pregnancy outcome

Additional patients with both known pregnancy outcomes and preserved first trimester microRNA samples were identified from our collection of eligible candidates. 11 patients were randomly selected for further microarray analysis (this number because it was efficiently performed when using a large microarray). These 11 pregnancies included five healthy deliveries, three compromised deliveries and three miscarriages (8-week, 8-week and 12-week losses; see Table III for patient details). Again, PBMC samples from these patients were assessed by an Agilant microarray comprising 852 microRNAs. MicroRNA results were compared between these outcome groups. Furthermore, two additional groups were created as controls by randomizing patients from the original outcome groups together and comparing the results (as in Experiment 2).

Experiment 4: miRNA expression analysis using 48.48 Fluidigm Biomark dynamic array for 30 selected miRNAs with a larger pregnancy outcome population without IVIg

Based on results from the previous three experiments, significant microRNAs were selected for qRT-PCR microRNA analysis: 7 microRNAs from Experiment 1 were combined with 23 microRNAs demonstrating strong associations with pregnancy outcome in Experiment 3 (most positive differences in the 'healthy' group and the most diminished in the 'compromised' group). Patients were selected that either used no IVIG or whose most recent IVIg treatment was at least 35 days from the blood draw. Thirty-nine patients met these inclusion criteria: 19 healthy deliveries, eight miscarriages, seven pregnancies with late-onset pre-eclampsia, five pregnancies with early-onset pre-eclampsia (See Table IV for patient details)

Quantitative RT-PCR microRNA analysis was then performed on these samples. After pre-amplification PCR, the product was diluted 1:5 with 1xTE and stored in −80°C until needed. qPCR was carried out using the 48 × 48 dynamic array (Fluidigm Corporation, San Francisco, CA, USA) following the manufacturer's protocol. Briefly, a 5-μL sample mixture was prepared for each sample containing 1 × TaqMan Universal Master Mix (No UNG), 1 × GE Sample Loading Reagent (Fluidigm PN 85000746) and each of diluted pre-amplified cDNA. 5 μL of assay mix was prepared with 1 × each of TaqMan miRNA assay and 1 × assay loading reagent (Fluidigm PN 85000736). An IFC controller was used to prime the fluidics array (chip) with control line fluid and then with samples and assay mixes in the appropriate inlets. After loading, the chip was placed in the BioMark Instrument for PCR at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The data were analyzed with Real-Time PCR Analysis Software in the BIOMARK instrument (Fluidigm Corporation). Four of the 30 microRNAs demonstrated very low readings so were considered technically unsuitable for scoring analysis (miR-33a-5p, 219-5p, 424-5p and 513-5p).

Identifying MicroRNAs to Use as Pregnancy Risk Markers

The microRNA quantifications for the 39 patient samples were sorted from highest to lowest Ct value

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for each of the twenty-six analytically suitable microRNAs. If a patient expressed a Ct value for a particular microRNA within the top 8 of 39 patients (approximately 20%), this patient received a score of '1'. Points for each patient were summed deriving a total risk factor score. ROC curves correlating risk factor score and pregnancy outcome were prepared using MedCalc Statistical Software version 12.7.7.[35]

Ethics Statement

Studies were nested within a larger clinical database. MicroRNA analyses were performed entirely retrospectively. Analyses involved the preservation of residual, unused patient samples that were to be discarded. The preserved material was not analyzed during the period in which any patient intervention was possible. No findings were made available to clinicians. Written informed consent for use of patient samples in research was acquired from each patient for the future use of their biological material and maintained in individual patient records. Data were anonymized prior to analysis. Patient identifying information was maintained in accordance with HIPAA requirements. No IRB study number was required due to the completely retrospective, observational and confidential nature of all the data in this study.

Results

Experiment 1 Results: qRT-PCR MicroRNA Identification of miR-132 A and B IVIg Response Groups

Expression of 7 microRNAs by RT-PCR was assessed for 16 pregnant patients before and after IVIg therapy: hsa-miR-16, 132, 146a, 155, 181a, 196a and 223. Two samples were taken, the first a mean of 13.6 ± 6.0 days prior to IVIg therapy and the second a mean of 11.7 ± 5.8 days following IVIg therapy (74.3 ± 64.6 days pregnant and 98.2 ± 63.8 days pregnant, respectively). All patients were being seen for recurrent miscarriage or infertility (1.4 ± 1.9 prior miscarriages; 1.7 ± 1.4 prior IVF failures, a mean of 36.3 ± 4.6 years old).

Although a consistent pattern of differential expression for the majority of the microRNAs was not found (data not shown), initial quantification prior to IVIg therapy suggested a bimodal distribution of hsa-miR-132 levels at Ct 21. Those with an initial hsa-miR-132 Ct≤21 (mean 17.1 ± 0.8) were designated 'Group A', and those with an initial Ct>21 (mean 25.9 ± 2.2) were designated 'Group B'. Five Group A patients and ten Group B patients were followed to delivery. All compromised pregnancies were found in the Group B patients (Table I).

Experiment 2 Results: MicroArray Analysis of hsa-miR-132 Groups

Eight patients, divided into two groups of four each by their initial hsa-Mir-132 Ct (average age 38.8 ± 4.0 years), groups 'A' and 'B' respectively, were then assessed by Agilant microarray before and after IVIg therapy. Similar to patients in Experiment 1, all patients in Experiment 2 were being seen for recurrent miscarriage or infertility (average 1.8 ± 2.4 prior miscarriages; 1.6 ± 1.4 prior IVF failures). The first blood draw was performed at an average of 43.6 ± 23.2 days pregnant, and the second blood draw was performed at an average of 55.2 ± 24.7 days pregnant. Blood was drawn an average of 15.3 ± 12.4 days prior to IVIg therapy and 16.3 ± 9.8 days following IVIg therapy (an average of 31.5 ± 17.5 days between microRNA blood draws).

The mean Ct signals from the pre-IVIg sample were subtracted from those of the post-IVIg sample for each microRNA, and the differences were sorted in descending order for both groups A and B. (Table II; the figure is truncated to show only the topmost and bottommost 10 differences in each group). Six microRNAs within the topmost 10 differences in the Group A were also found in the bottommost 10 in the Group B. A single microRNA amongst most negative 10 differences in Group A is found amongst the 10 most positive differences in Group B.

Control groups were created by randomizing the eight specimens into two groups: 'C' and 'D', respectively. An analysis was performed on control groups C and D using similar procedures to the experimental groups, and no associations were identified (data not shown).

Experiment 3 Results: MicroRNA MicroArray Analysis to Assess Correlations with Pregnancy Outcome

As specimens with known pregnancy outcomes became available, it was possible to repeat Experiment

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Table I Delivery outcomes in Group A and B patients

Patient#	Group A	Initial miR132 Ct level	Delivery outcome	Comments
1.	A	17.9	Healthy	Delivered: 39 weeks. 7 lbs 9 oz
2.	A	18.1	Healthy	Delivered: 36 weeks 6 days. 7 lbs 6 oz
3.	A	16.1	Healthy	Delivered: 3/14/12. 40 weeks 2 days. 7 lbs 11 oz
4.	A	17.1	Healthy	Delivered: 40 weeks 2 days. 6 lbs 12 oz
5.	A	16.8	Healthy	Delivered: 40 weeks. 7 lbs 11 oz
6.	A	16.5	Healthy	Delivered: 39 weeks. 7 lbs 11 oz
1.	B	25.4	Healthy	Delivered: 38 weeks 3 days. 8 lbs 3 oz
2.	B	27.3	Healthy	Delivered: 40 weeks. 7 lbs 2 oz
3.	B	21.7	Pre-eclampsia	Delivered: 38 weeks 2 days. 6 lbs 14 oz. Pre-eclampsia, anemia
4.	B	30.0	Healthy	Delivered: 39 weeks. 7 lbs 8 oz
5.	B	26.9	Pre-eclampsia	Delivered: 37 weeks. 5 lbs 5 oz. Pre-eclampsia
6.	B	25.0	Healthy	Delivered: 39 weeks. 7 lbs 5 oz
7.	B	24.9	Fetal distress	Delivered: 39 weeks. 7 lbs 2 oz. Fetal distress, low fetal heart rate.
8.	B	26.9	Premature	Delivered: 37 weeks 2 days. 6 lbs 12 oz
9.	B	28.2	Healthy	Delivered: 41 weeks. 8 lbs 15 oz
10.	B	25.1	Healthy	Delivered: 39 weeks. 8 lbs 2 oz

Patients with initial miR-132 Ct ≤21 (mean 17.1 ± 0.8) were designated 'Group A'.
Patients with initial miR Ct >21 (mean 25.9 ± 2.2) were designated 'Group B'. Blood was drawn an average of 13.6 ± 6.0 days prior to IVIg therapy at an average of 74.3 ± 64.6 days pregnant. MicroRNA was quantified by RT-PCR. Six Group A patients and ten Group B patients were followed up to delivery. All compromised pregnancies were found in the Group B patients.

2 where patient groups were segregated by pregnancy outcome. Eleven patients were again assessed by Agilant microarray. Patient population was similar to that used in Experiments 1 and 2 (mean age 38.8 ± 6.2 years, 1.8 ± 1.7 prior miscarriages, 0.2 ± 0.4 prior IVF failures). Samples were drawn in the first trimester from five patients with normal outcomes and three with 'compromised' (pre-eclampsia-like) pregnancies and three with miscarriages (See patient details in Table III). Two samples were analyzed for each patient, one sample drawn a week before IVIg (mean 45.1 ± 19.2 days pregnant) and a second drawn a week after IVIg (mean 60.0 ± 19.5 days pregnant). Differences, as above, were calculated and sorted in descending order as in Experiment 2. MicroRNAs with the most positive differences in the

Table II MicroArray analysis of Group A and B IVIg responses

	Mean differences Group A	MicroRNA	Mean differences Group B	MicroRNA
1.	2.52122	hsa-miR-136	−5.94462	hsa-miR-142-5p
2.	2.208455	hsa-miR-33a	−6.23567	hsa-miR-376b
3.	2.083515	hsa-miR-219-5p	−6.60811	hsa-miR-32
4.	2.082454	hsa-miR-153	−6.60915	hsa-miR-219-5p
5.	1.955594	hsa-miR-545	−6.66695	hsa-miR-33a
6.	1.757013	hsa-miR-32	−6.95672	hsa-miR-545
7.	1.301357	hsa-miR-1537	−7.18912	hsa-miR-1183
8.	1.270745	hsa-miR-1	−7.47155	hsa-miR-1537
9.	1.195371	hsa-miR-590-5p	−7.84218	hsa-miR-153
10..	1.140525	hsa-miR-301a	−8.09536	hsa-miR-1322

The table represents a truncation of nearly 900 microRNAs interrogated by microarray arranged in descending order of the differences between the post-IVIg and pre-IVIg signals.
The table comprises microRNAs within the topmost 10 differences for Group A and the bottommost 10 differences for Group B.
The colored cells represent microRNAs common to both groups.
The marked microRNAs behave in a diametrically opposite manner in the two groups.

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Table III Patient history and pregnancy outcome information for 11 patients in Experiment 3

	Healthy delivery (5 patients)	Pre-eclampsia (3 patients)	Miscarriage (3 patients*)
Age (years)	37.6 ± 5.1	43.7 ± 8.7	36.7 ± 4.5
#Prior live births	0.2 ± 0.4	0.3 ± 0.6	0.0 ± 0.0
#Prior miscarriages	1.6 ± 1.8	2.0 ± 1.7	2.0 ± 2.0
#Prior IVF failures	3.2 ± 4.1	7.0 ± 4.4	2.0 ± 0.0
Gestational age sample#1 (#days from LMP)	44.8 ± 26.2	49.0 ± 14.8	27.0 ± 33.1
Gestational age sample #2	64.0 ± 20.2	66.7 ± 13.9	46.7 ± 23.0
# singleton deliveries	4	3	
Singleton gestational age (weeks)	40.0 ± 0.8	37.7 ± 2.1	9.3 ± 2.3
Singleton infant weight (g)	3626 ± 488	2797 ± 448	NA
Triplet delivery	1		
Gestational age (weeks)	35.0		
Infant weight (g)	Approx. 1800 g each ×3		

*Three miscarriages occurred at 8-, 8- and 12-week gestational age, each with no known karyotypic abnormalities present.

'healthy' group were amongst the most diminished in the group with 'compromised' pregnancies. As in Experiment 2, patients were also randomized to form two control groups, and no similar patterns were found.

Experiment 4 Results: RT-PCR Analysis 30 miRNAs

Experiment 4 assessed the potential of maternal cell microRNA quantification to predict pregnancy outcome when collected as a single blood sample during the first trimester. Thirty microRNAs were selected from the previous experiments. Seven microRNAs from Experiment 1 combined with 23 microRNAs demonstrating the strongest associations with pregnancy outcome (most positive differences in the 'healthy' group and the most diminished in the 'compromised' group) from Experiment 3 were selected. qRT-PCR for these 30 microRNAs was performed on 39 patients [19 healthy deliveries, 12 pre-eclampsias, (seven late onset and five early onset), and eight miscarriages (See Table IV for population details)]. These 39 patients were either untreated or had their most recent IVIg treatment at least 35 days from the blood draw.

Identifying MicroRNAs Used as Pregnancy Risk Markers

The microRNA Ct list of 39 patient samples was sorted from highest to lowest Ct value for each of the thirty microRNAs. If a patient expressed a Ct value for a particular microRNA within the top 8 of 39 patients, this patient received a score of '1'. Points for each patient were summed deriving a total risk factor score.

A scoring system was devised where any sample residing within the top eight highest levels was given a point. Four microRNAs demonstrated very low readings so were considered technically unsuitable for analysis and excluded from the scoring system (miR-33a-5p, 219-5p, 424-5p and 513-5p). The results for each patient were summed. The results displayed in Table V are sorted by pregnancy outcome. Specific pregnancy outcome details are listed in Table IV. MicroRNA patterns are reported that predict the development of late pre-eclampsia with a P value of $P < 0.0001$ and fitted ROC area of 0.90 (Fig. 1).

MicroRNA patterns predict the development of miscarriage with a P value of $P < 0.0001$ and fitted ROC area of 0.90 (Fig. 2).

Discussion

Our studies were originally fashioned by the progressing availability of Trizol-preserved PBMCs from pregnant women undergoing therapy for infertility and recurrent miscarriage. Because we were aware of studies quantifying PBMC microRNAs of patients with systemic inflammatory conditions, we hypothesized that IVIg might perturb the same microRNAs highlighted in these studies. Only later, when correlations with inflammation-related microRNAs and

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Table IV Patient history and pregnancy outcome information for 39 patients in Experiment 4

Patient History	Healthy (19 cases)	Miscarriage (8 cases**)	Pre-eclampsia: All (12 cases)	Late Pre-eclampsia (7 cases)	Early Pre-eclampsia (5 cases)
Age (years)	36.3 ± 4.7	37.0 ± 5.4	36.7 ± 3.5	37.7 ± 3.8	35.2 ± 2.8
#Prior live births	0.5 ± 0.6	0.9 ± 1.1	0.3 ± 0.5	0.3 ± 0.5	0.2 ± 0.4
#Prior miscarriages	1.4 ± 1.4	2.9 ± 1.6	2.1 ± 1.6	2.1 ± 1.3	2.0 ± 2.0
#Prior IVF failures	1.3 ± 1.3	1.8 ± 2.4	1.0 ± 1.1	1.0 ± 1.0	1.0 ± 1.4
Gest age of sample (#days from LMP)	50.9 ± 15.9	50.3 ± 21.1	65.8 ± 17.8	60.6 ± 12.6	73.0 ± 22.9
#Days post-implantation (LMP date, 21 days)	29.9 ± 15.9	29.3 ± 21.1	44.8 ± 17.8	47.9 ± 12.6	52.0 ± 22.9
Singleton deliveries	16	–	10	7	3
Gestational age (weeks)	39.1 ± 1.1	6.9 ± 3.1	35.6 ± 2.9	37.0 ± 2.0	32.3 ± 1.5
Infant weight (g)	3243 ± 314	–	2421 ± 554	2710 ± 225	1747 ± 506
C-section (%)	50% (8/19)	–	75% (9/12)	57% (4/7)	100% (5/5)
Twin deliveries	3	–	2	0	2
Gestational age (weeks)	36.7 ± 1.2	–	29.0 ± 1.4	–	29.0 ± 1.4
Infant weight (g)	2689 ± 151	–	1035 ± 268	–	1035 ± 268
C-section (%)	100% (3/3)	–	100% (2/2)	–	100% (2/2)

**All miscarriages occurred at ≤10 weeks pregnant except for one miscarriage that occurred at 14 weeks pregnant. No known karyotypic abnormalities present.

pregnancy outcome were found, we specifically investigated pregnancy-related markers.

In Experiment 1, we found that a single microRNA amongst those selected for study, hsa-miR-132, split the patients into two IVIG response groups. MicroRNA levels clustered into two distinct populations on their initial sampling (Table I). We wondered whether the clusters represented a more extensive difference that might involve a wider range of differentially expressed microRNAs.

As delivery information became available for pregnancy samples used in Experiment 1, we found that the hsa-miR-132 dichotomy appeared to correlate with pregnancy outcome. The group with a high initial microRNA level (Group A) was free of pregnancy complications, while those with low levels (Group B) were evenly divided between those free of complications and those with compromised pregnancies (Table I).

In Experiment 2, a microarray comprising nearly 1000 microRNAs could be semiquantified. Two groups defined by their initial hsa-miR-132 expression level were identified, and the differences between pre- and post-IVIg intervention arranged in descending order for each of the two groups. Not only did the study support the hypothesis, it supported it remarkably well. Six microRNAs within the topmost 10 of differences in the first group were also found in the bottommost 10 in the second group (Table II). It is very interesting that an anti-inflammatory perturbation exposed so profound yet cryptic a dichotomy. When two control groups were created by randomizing the eight A and B specimens into two mixed groups and the process repeated, the microRNAs behavior patterns could no longer be identified.

In Experiment 3, with the accumulation of additional patient samples where clinical outcome was known, a second microarray study comparing patients with complicated versus healthy outcomes could be constructed. The study was limited to those patients whose initial sample was collected 6 weeks after their last menstrual period (3 weeks after the estimated time of implantation; see Table III). A similar pattern of microRNAs with the highest differences in one group identified amongst the lowest in the second was again seen. Again, when the two groups were randomized into control groups, the patterns could no longer be found.

The finding that clinical outcome could be predicted during the early first trimester with interrogation of maternal cells not acquired from the placental site was quite unexpected. Current studies within the field have been confined to the examination of shed and secreted placental substances recovered

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Table v MicroRNA pregnancy risk score

Patient	Outcome	#Days post-implantation (LMP date, 21 days)	Score
Patient #1	PL	31	10
Patient #2	PL	27	13
Patient #3	PL	62	23
Patient #4	PL	36	21
Patient #5	PL	29	6
Patient #6	PL	36	1
Patient #7	PL	44	23
Patient #8	PE	59	4
Patient #9	PE	70	3
Patient #10	PE	42	1
Patient #11	PE	72	8
Patient #12	PE	17	1
Patient #13	M	13	2
Patient #14	M	20	6
Patient #15	M	71	11
Patient #16	M	21	8
Patient #17	M	32	2
Patient #18	M	16	10
Patient #19	M	11	9
Patient #20	M	50	8
Patient #21	H	36	2
Patient #22	H	32	2
Patient #23	H	48	1
Patient #24	H	47	0
Patient #25	H	60	6
Patient #26	H	36	2
Patient #27	H	62	1
Patient #28	H	33	1
Patient #29	H	36	1
Patient #30	H	29	5
Patient #31	H	14	2
Patient #32	H	14	4
Patient #33	H	18	3
Patient #34	H	6	1
Patient #35	H	27	1
Patient #36	H	21	3
Patient #37	H	62	2
Patient #38	H	18	0
Patient #39	H	2	1

PL, late-onset pre-eclampsia, PE, early-onset pre-eclampsia, M, miscarriage, H, healthy delivery.

from plasma during the first trimester. Interrogation of material of placental origin has been favored over approaches directed toward maternal cellular material. Redman and Sargent suggest events of interest at early time points are placental.[9]

However, exposure of peripheral blood cells to the placenta during the second and third trimesters of pregnancy is only transient. Contact is largely absent during most of the first trimester. Plugged spiral arteries enforce shunting of blood cells through the inner myometrium.[16] As decidual elements are closely involved in the pathologic events leading to inadequate spiral artery transformation, we speculated that the microRNAs may be differentially altered between healthy and compromised pregnancies following IVIg challenge. These differentially altered microRNAs might play significant roles in the decidual pathology of compromised pregnancy.

To investigate this question, we constructed a panel of 30 microRNAs from those that were identified in experiments 2 and 3 as well as microRNAs investigated in the first the first experiment. We hoped that we might find microRNAs patterns predictive of pregnancy outcome when sampled during the first trimester.

Quantitative RT-PCR was then performed on 39 patients who were either untreated or whose most recent treatment with IVIg was at least 35 days from the blood draw (Experiment 4). Nineteen healthy deliveries, 12 pre-eclampsias (seven late onset and five early onset) and eight miscarriages were assessed (Table IV). Results for each microRNA were arranged from highest (lowest Ct) to lowest level (highest Ct). We devised a scoring system where we arranged quantitative results in descending order. We scored a point to each patient sample where the result fell within the topmost eight results (approximately 20%). The scores for each patient were summed (Table V). A high total microRNA score could predict impending adverse pregnancy outcome with great accuracy [miscarriage ($P < 0.0001$) and late pre-eclampsia ($P < 0.0001$)]. MicroRNA patterns were found to successfully predict the development of late pre-eclampsia with fitted ROC area of 0.90 (Fig. 1) and for miscarriage with a fitted ROC area of 0.90 (Fig. 2).

It is interesting that non-placental microRNA patterns from first trimester maternal cells can predict pregnancy outcome, especially late-onset pre-eclampsia. A coherent explanation for the surprising observations awaits additional investigation. In addition, because these regulatory networks are maternally defined, this opens up the possibility that additional information about pregnancy risk can be determined preconception, even when no placental tissues are present.

Conclusion

Prior to the results reported here, the search for biomarkers predicting adverse pregnancy outcome has

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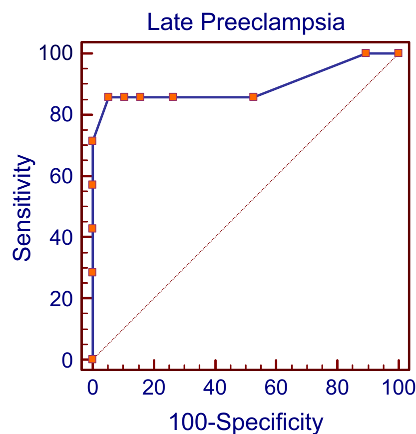
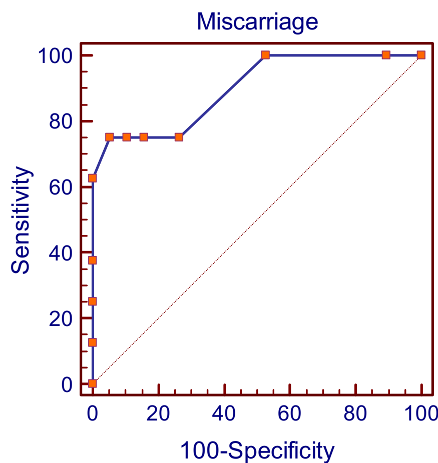


Area under the ROC curve (AUC)	0.90
P Value	<0.0001
Diagnostic Threshold Value	>5
Sensitivity (Threshold >5)	86
Specificity (Threshold >5)	95

Fig. 1 ROC curve analysis for microRNA risk score and late pre-eclampsia outcome.

not identified any with the potential for diagnosis prior to the beginning of the second trimester. MicroRNAs, however, may offer a new approach. Not only may they improve our understanding of the feto-maternal dialogue, it is the only early pregnancy marker, to date, that can successfully predict late pre-eclampsia. Although the studies that we report are preliminary, we hope that future research



Area under the ROC curve (AUC)	0.90
P Value	<0.0001
Diagnostic Threshold Value	>5
Sensitivity (Threshold >5)	75
Specificity (Threshold >5)	95

Fig. 2 ROC curve analysis for microRNA risk score and miscarriage.

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will build upon our discoveries and enhancing the power of maternal cell microRNA to predict pregnancy disease in the clinic.

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