Journal of Reproductive Immunology 110 (2015) 22–35

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jreprimm

First-trimester maternal cell microRNA is a superior pregnancy marker to immunological testing for predicting adverse pregnancy outcome

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ARTICLE INFO

Article history:
Received 12 March 2015
Accepted 16 March 2015

Keywords:
Preeclampsia
Miscarriage
Implantation
TNF-α/Il-10 ratio
T regulatory cell
Natural killer cell

ABSTRACT

Patients at risk of immune-mediated pregnancy complications have historically relied on the use of peripheral blood immunological assays for diagnosis and pregnancy monitoring. However, these tests often fail to identify many at-risk patients, achieving moderately predictive receiver operating characteristic (ROC) curve AUCs of 0.60–0.70. We previously demonstrated that a microRNA panel comprising 30 microRNAs successfully predicts pregnancy outcome in the first trimester. In our current study we constructed a smaller, more clinically useful seven-microRNA panel from the original panel of 30 microRNAs with equivalent sensitivity and specificity. To select optimal microRNAs for a smaller panel, quantitative RT-PCR on 30 microRNAs was first performed on 48 patients (191 samples) with concurrent immunological testing: TNFα/Il-10 ratio, IFNγ/Il-10, CD56+16+%, NK 50:1 cytotoxicity and T regulatory cells. MicroRNAs were separated into clusters associated with: Th1/Th2 response; T regulatory cell percent; pregnancy risk; treatment response. Seven most differentially expressed microRNAs were selected. The seven microRNA scoring system was then applied to 39 patient samples in the first trimester of pregnancy (19 healthy deliveries, 8 miscarriages, 12 preeclampsia [7 late-onset and 5 early-onset]) and 20 samples in the preconception period (2–10 weeks before conception). Predictive value was assessed. ROC curves for the seven-microRNA panel achieved AUC 0.92 for miscarriage and 0.90 for preeclampsia (blood drawn 34.9 ± 19.2 days post-implantation). For samples measured preconception, ROC curve analysis demonstrated AUC 0.81 for adverse pregnancy outcome. Maternal PBMC microRNA can identify high-risk patients likely to benefit from immunotherapy with improved sensitivity and specificity compared with standard immune assays.

1. Introduction

An immunological basis for "unexplained" implantation failure, miscarriage and late term pregnancy complications/failures has been supported in numerous studies (Beer et al., 1996; Beer and Kwak, 1997; Coulam et al., 1995; Kwak et al., 1998a, 1998b, 2000; Kwak-Kim et al., 2003; Ng et al., 2002; Saito et al., 2007; Steinborn et al., 2008; Thum, 2007; Winger et al., 2012). Such cases have often

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http://dx.doi.org/10.1016/j.jri.2015.03.005
0165-0378/

been associated with abnormalities in several different in vitro immune parameters: natural killer cell cytotoxicity, proportion of peripheral blood CD56+CD3− cells, and Th1:Th2 bias. When patients with these abnormalities are treated with immunotherapy, results have been encouraging (Carp, 2007; Clark et al., 2006; Winger et al., 2011a, 2011b). However, the immunological assays employed have low specificity and sensitivity (Lee et al., 2013; Thum et al., 2004). In addition, these assays specifically fail to predict the risk of late term pregnancy complications such as preeclampsia and preterm birth. Alternative testing approaches are needed.

In the search for new markers, we became interested in the use of microRNA, a group of small, non-coding RNAs. MicroRNAs are a newly discovered class of RNA species comprising a 22- to 24-base non-coding polynucleotide. Their recent recognition belies their evolutionarily ancient role coordinating metabolic and signaling pathways within a changing cellular environment. 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 within a short 7- to 8-base sequence, the "seed sequence." Over a thousand functional microRNAs have now been identified. They regulate translation and turnover of the mRNAs coding for many proteins and enzymes within common pathways, thereby providing a unifying control point for metabolic and signaling pathways (Krol et al., 2010). A number of microRNAs have been reported as differentially regulated in systemic inflammatory disorders. For example, Pauley et al. (2008) observed significant increases in the relative expression of the miRNAs 146a, 155, 132, 16, and let-7a in rheumatoid arthritis patients compared with normal controls. Sonkoly et al. (2008) demonstrated that microRNAs 181a and miR-223 can determine immune cell fate, while microRNA-155 can regulate adaptive immune response elements such as antigen presentation. Because microRNAs function upstream of many immune functions that are currently measured, they may provide earlier and more reliable information on pregnancy risk than the tests commonly used (Allantaz et al., 2012).

In our previous study, we created a scoring system using a panel of 30 microRNAs to predict pregnancy outcome. We found that early first-trimester PBMC microRNA could predict "compromised" pregnancy outcome (e.g., miscarriage or preeclampsia) with a high degree of sensitivity and specificity (Winger et al., 2014). Our study was, to our knowledge, the first study to reliably predict preeclampsia outcome using samples collected early during the first trimester (<6 weeks post-implantation). In our current study, we achieve three additional clinically relevant objectives: (1) to reduce the 30 microRNA panel into a smaller panel more suitable for clinical application; (2) to compare the clinical value of PBMC microRNA testing to the currently used immune assays using a quantitative receiver operating characteristic (ROC) curve approach, and (3) to demonstrate the utility of the panel for preconception diagnosis.

2. Materials and methods

2.1. Thirty-microRNA panel

In our previous study, we selected first-trimester PBMC samples from patients using Agilant microarray comprising 852 microRNAs. MicroRNA quantifications of different pregnancy outcome groups (healthy, preeclampsia, miscarriage) were compared. Twenty-three microRNAs that demonstrated the strongest associations with pregnancy outcome (the most positive differences, in the "healthy" group and the most diminished in the "compromised" group) were selected as being most clinically useful. These 23 microRNAs were combined with seven microRNAs already known to be differentially expressed in systemic inflammatory disease: hsa-miR-16, 132, 146a, 155, 181a, 196a, and 223 (Pauley et al., 2008; Shaked et al., 2009; Sonkoly et al., 2008). This created our original 30-microRNA panel.

In our current study we desired to construct a smaller, more clinically useful testing panel than one involving 30 microRNAs. Seven microRNAs was considered to be an ideal number as it can easily be configured with an additional control on an 8 × 12-well polymerase chain reaction (PCR) plate.

2.2. Selection of a seven-microRNA panel

To determine the seven most clinically useful microRNAs, we first selected the most differentially expressed microRNAs in various clinical situations from our original 30-microRNA panel. MicroRNAs were selected that could: (1) distinguish between Th1/Th2 and T regulatory cell response, (2) identify both pregnancy risk and treatment response, and (3) identify risk of adverse pregnancy outcome at a preconception time point. Experiments were performed using the pool of 191 PBMC specimens with a wide range of histories (20 healthy deliveries, 15 preeclampsia [8 late-onset, 7 early-onset], and 13 miscarriages). Samples had been collected at multiple different time points for each patient, 2–14 weeks preconception, 2–4 weeks post-implantation and 4–7 weeks post-implantation, in some cases before and after immunotherapy (Tables 1a and 1b). All PBMC samples were stored in Trizol for later extraction and analysis. Differential microRNA expression among the various clinical groups was calculated and the optimal microRNAs selected.

Once patient groups were defined, the most differentially expressed microRNAs for each comparison were selected in a quantitative fashion. The absolute value of the mean differences between the microRNA Ct levels of affected and unaffected patients was calculated (for example, between "preeclampsia" and "healthy" populations). This value was then divided by the average of the standard deviations of the two groups, generating a ratio that we arbitrarily defined as the "expression ratio" It was hypothesized that, the greater the expression ratio, the more likely the microRNA would be useful as a predictive marker.

The expression ratios for each microRNA were sorted from largest to smallest for each of the clinical conditions of interest: preconception risk, preeclampsia, miscarriage,

Table 1a
History of 48 patients used to create the seven microRNA scoring system (191 samples).

History	Healthy delivery (20 patients)	Late preeclampsia (8 patients)	Early preeclampsia (7 patients)	Miscarriage (13 patients)
Age (years)	36.3 ± 5.6	40.0 ± 7.3	36.1 ± 3.1	37.3 ± 4.2
No. of previous live births	0.2 ± 0.4	0.3 ± 0.5	0.4 ± 0.8	0.4 ± 0.9
No. of previous miscarriages	1.3 ± 1.7	2.1 ± 1.6	3.3 ± 1.9	2.2 ± 1.7
No. of previous IVF failures	1.3 ± 41.7	3.6 ± 4.2	0.9 ± 1.6	1.8 ± 2.0
Gestational age at delivery (weeks)	39.3 ± 1.2	37.3 ± 2.0	27.9 ± 6.6	6.9 ± 3.4[a]
Infant weight (g)	3321 ± 192	2653 ± 322	1750 ± 884 (one set of twins)	NA
% of patients used IVIg therapy	95% (19/20)	100% (8/8)	86% (6/7)	92% (12/13)
Timing of blood draws with sample sizes				
Preconception (mean cycle day ± SD)	11 samples (8.1 ± 7.0)[b]	5 samples (7.3 ± 5.9)[b]	0 samples (NA)	9 samples (NA)
2–4 weeks post-implantation	10 samples	6 samples	6 samples	10 samples
4–7 weeks post-implantation	19 samples	6 samples	7 samples	5 samples
Other	40 samples	38 samples	8 samples	11 samples

IVIg intravenous immunoglobulin.
[a] All miscarriages occurred at ≤12 weeks' gestation except for one miscarriage that occurred at 14 weeks' gestation. No known karyotypic abnormalities were present.
[b] All preconception samples with dates known were drawn in the late menstrual/late proliferative phase except for one sample in the healthy group that was drawn in the secretory phase (cycle day 24).

"before therapy," and "after therapy." Those microRNAs with the highest "expression ratio" were entered into a Venn diagram from which the final seven microRNAs would be selected.

2.3. Quantitative real-time PCR analysis

MicroRNA was quantified using real-time polymerase chain reaction. The term "cycle threshold" ("Ct") represented the amplification cycle at which a reliable signal generated by the amplified product is first detected. Each cycle of amplification represented a doubling of the target polynucleotide. Total RNA was isolated from the Trizol™-preserved samples and quantity determined using Nanodrop™ (Thermo Scientific, Waltham, MA, USA) and the quality was assessed using 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 a master mix of TaqMan™ MicroRNA Reverse Transcription Kit (TaqMan, 2013). 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, hsa-miR-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 Biosystems). MicroRNA concentrations were normalized against RNU48. Cts were determined and the relative microRNA or mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems User Bulletin No. 2, 2013).

Table 1b
Thirty-nine first-trimester pregnant samples on which the seven microRNA scoring system was applied and receiver operating characteristic (ROC) curves derived.

Patient history	Healthy (19 cases)	Miscarriage (8 cases)	Preeclampsia: all (12 cases)	Late preeclampsia (7 cases)	Early preeclampsia (5 cases)
Age (years)	36.3 ± 4.7	37.0 ± 5.4	36.7 ± 3.5	37.7 ± 3.8	35.2 ± 2.8
No. of previous live births	0.5 ± 0.6	0.9 ± 1.1	0.3 ± 0.5	0.3 ± 0.5	0.2 ± 0.4
No. of previous miscarriages	1.4 ± 1.4	2.9 ± 1.6	2.1 ± 1.6	2.1 ± 1.3	2.0 ± 2.0
No. of previous IVF failures	1.3 ± 1.3	1.8 ± 2.4	1.0 ± 1.1	1.0 ± 1.0	1.0 ± 1.4
Gestational age of sample (no. of days from LMP)	50.9 ± 15.9	50.3 ± 21.1	65.8 ± 17.8	60.6 ± 12.6	73.0 ± 22.9
No. of 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
% of patients using IVIg therapy	32% (6/19)	25% (2/8)	33% (4/12)	29% (2/7)	40% (2/5)
Delivery outcomes					
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 (%)	42% (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)

LMP last menstrual period.

For Trizol™ sample preservation, PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from 24-h-old heparinized blood that had been maintained at room temperature. PBMC cell populations ($<1 \times 10^7$ viable cells) were then collected in 1 ml of TRIzol (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C until use. Total RNA was isolated according to the TRIzol protocol as described in the manufacturer's instructions (Invitrogen, 2013).

PBMCs were preserved in Trizol™ (Invitrogen, Carlsbad, CA, USA) and maintained at −20 °C.

2.4. MicroRNA associations with immunological assays

To assess how well immunological tests corresponded with the differential expression of microRNAs, patient immunological results were separated into those with "high" (above the clinical cut-off) and "low" (below the clinical cut-off) groups for each of the immunological assays being assessed: TNFα/Il-10 ratio (high > 30; low < 24), the IFNγ/Il-10 ratio (high > 11; low < 6) (Ng et al., 2002), CD56+16+% of peripheral blood lymphocytes (high >12%; low <5%), NK cytotoxicity (high >18%; low <5%) (Beer and Kwak, 1997; Kwak et al., 1998a, 1998b) and T regulatory cell percentage (high >1.1%; low <0.7%) (Winger and Reed, 2011). All selected samples had experienced little or no intravenous immunoglobulin (IVIg; patients either had no IVIg or had IVIg ≥35 days from the blood draw date). All available stored samples that fulfilled these criteria were included in the analysis (35 blood samples from 29 pregnant patients). The mean microRNA Ct levels for specimens within the expected ranges and outside the expected ranges were calculated. When the difference between the means divided by the average of the standard deviations exceeded 1.0, the microRNA was hypothesized to be more clinically useful and was selected to be a candidate for the microRNA scoring system.

2.5. Developing a clinical scoring system using seven microRNAs

Previously, we had shown that low microRNA Ct values for clinically relevant microRNAs are more often associated with poor pregnancy outcome (Winger et al., 2014). To generate scores, results for each microRNA were arranged from lowest to highest. A "cutoff" value was defined as the Ct value segregating the lowest 20% of Ct values from the remaining values. For each patient sample a point was scored for each microRNA Ct falling below the cutoff. Each patient who received a Ct value below the cutoff for each microRNA level received a score of "1." Points for each of the seven microRNAs were summed, creating a total risk factor score for each patient (maximum possible score is 7).

2.6. Statistical analysis

Clinical assays must differentiate individual patient samples into affected and non-affected groups. The statistical parameter, the "*p* value," is useful for quantifying the probability that two groups of patients may be distinguished by a given parameter. However, in the clinic a test must be able to segregate a single patient sample into either the affected or the non-affected group. Here, ROC curve analysis is utilized to confirm this capability.

2.6.1. ROC curve analysis

The ROC curve is a commonly preferred parameter for assessing the diagnostic power of a clinical assay. A continuous curve is generated tracing the ratios of true-positive to false-positive results as the detection threshold is raised. With a robust test, false-positive results are excluded at relatively low threshold values and few true-positive individuals are lost. In strong assays, the ROC curve appears highly arched. However, with a weak test, the ROC curve appears shallow. The "area under the curve" or "AUC" quantifies the arching of the curve and is the commonly used measure of the usefulness of a test. The AUC of a "perfect" assay approaches 1.0 (100%); when the AUC of a weak assay approaches 0.5 (50%) the assay has no discriminatory power. In our study, we performed ROC curve analysis using MedCalc™ Statistical Software version 13.0.6 (MedCalc™ Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014).

2.6.2. MicroRNA cluster association with metabolic pathways

MicroRNA clusters associated with specific pregnancy outcomes and treatment effects were then analyzed using the online microRNA analysis program (Vlachos et al., 2012).

2.7. 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 to the 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 before analysis. Patient-identifying information was maintained in accordance with HIPAA (US Department of Health and Human Services) requirements. Patient confidentiality was strictly maintained. The study was approved by the Western Institutional Review Board (WIRB) Study Number 1151255, Pro. Number 20142368, waiver of authorization under 45 CFR 164.512 and waiver of consent under 45 CFR 46.116(d).

2.8. Immunological testing

2.8.1. Th1:Th2 cytokine assay

The ratio of Th1/Th2 cytokine-expressing CD3+CD4+ lymphocytes was determined by flow cytometry. PBMCs were stimulated with phorbol myristic acid (PMA) and ionomycin in the presence of an inhibitor of Golgi

transport (Golgiplug™ [brefeldin A]) for 18 h. Subsequently, mononuclear cells were permeabilized and then reacted with phycoerythrin-conjugated anti-cytokine antibodies to TNF-α, or IFN-γ and IL-10. Multi-color flow cytometry was used to identify the percentages of intracellular cytokine-expressing CD3+CD4+ lymphocytes. CD4+ is down-regulated following stimulation. Therefore, CD3+CD4+ cells were identified as CD3+CD8− cells using a combination of ECD-conjugated anti-CD3 and FITC-conjugated anti-CD8. The percentages of intracellular cytokine-containing CD3+CD8− cells were determined for each of the three cytokines TNF-α, IFN-γ and IL-10. From this, the TNF-α:IL-10 and IFN-γ/IL-10 ratios were calculated. A TNF-α:IL-10 ratio above 30.6 and/or the IFNγ:IL-10 ratio above 20.5 was considered abnormally elevated (Ng et al., 2002).

2.8.2. NK cytotoxicity

Natural killer cytotoxicity was assessed by flow-cytometric quantification of the relative proportion of killed-K562 target cells assessed by propidium-iodide uptake following co-incubation with isolated patient mononuclear cells. These patient mononuclear cells were incubated for 2 h (37 °C with 5%CO_2), with target K562 cells pre-stained with PKH-67, a lipophilic dye that binds to the cell membrane permitting their identification in a mixture comprising unstained effector cells (patient PBMC's). K562 cells killed by NK cells become permeabilized permitting DNA staining by propidium-iodide. The percentage of killed target cells is quantified by flow cytometry. NK cytotoxicity was tested at an effector to target ratio of 50:1. Cytotoxicity was regarded as increased when target cell killing exceeded 15 percent (Beer and Kwak, 1997; Kwak et al., 1998a, 1998b).

2.8.3. Immunophenotyping

Flow cytometry was performed on peripheral blood mononuclear cells to assess the percentage of NK cells (CD56+CD3−). A percentage of CD56+CD3− lymphocytes greater than 12% was defined as elevated (Coulam et al., 1995).

2.8.4. T regulatory cell assay

The T regulatory cell percentage (CD4+CD25+Foxp3) was performed by membrane staining for CD25 and CD4 followed by membrane permeabilization and intracellular staining for the FoxP3 transcription factor. Low T regulatory cell % (<0.7) in the first trimester is correlated with an increased risk of miscarriage (Winger and Reed, 2011).

2.9. Immunological treatment

Intravenous immunoglobulin (IVIg) was administered at 400 mg/kg body weight for a history of immunological pregnancy failure and/or infertility with one or more of the following test abnormalities present: elevated Th1:Th2 (>30), elevated % $CD56^+$ cells (>12%) and/or elevated % NK cytotoxicity (>15%) (Carp, 2007; Clark et al., 2006; Winger and Reed, 2011). 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. MicroRNA results in no way influenced patient treatment.

2.10. Pregnancy outcome criteria

2.10.1. Healthy delivery

A "healthy" delivery was defined as the delivery of a singleton normal karyotype baby with the following pregnancy criteria: (1) delivered at 37–40 weeks' gestation, (2) birth-weight of ≥6 lb(2.721 g), (3) normal maternal blood pressure throughout pregnancy, or (4) twin delivery with gestational age ≥35 weeks and birth weights of ≥5.1 lb (2.313 g), and (4) no other pregnancy or delivery complications present (Alexander et al., 1998).

2.10.2. Preeclampsia

"Preeclampsia" was defined as a pregnancy condition in which the pregnancy of a singleton/twin/triplet normal karyotype baby expressed the following characteristics: (1) IUGR defined as less than 90% normal weight for gestational age; (2) blood pressure >140 systolic and/or >90 diastolic (two separate readings taken at least 6 h apart) (3) ≥300 mg of protein in a 24-h urine sample (Longo and Dan, 2012). "Early-onset preeclampsia" was defined as onset at <34 weeks' gestation. "Late-onset" preeclampsia" was defined as onset at ≥34 weeks' gestation (Raymond and Peterson, 2011).

2.10.3. Miscarriage

A "miscarriage" was defined as a failed pregnancy at <24 weeks' gestation that had reached a minimum β-HCG level of 25 mIU/ml and/or demonstrated a visible uterine pregnancy sac via ultrasound (RCOG guidelines, 2014). All known karyotypically abnormal miscarriages were excluded from this study.

2.10.4. Estimated date of implantation

When only the date of the last menstrual period was known for a pregnancy, the estimated date of implantation was the number of days past the last menstrual period minus 21 days.

3. Results

3.1. Selection of microRNAs for the assessment of pregnancy risk and treatment response

3.1.1. Differences in preconception microRNA levels between healthy and preeclampsia patients

Sixteen patients were assessed using a single preconception blood draw. These patients included 5 with preeclampsia and 11 healthy patients (mean day of blood draw 60.7 ± 53.6 days before the conception cycle LMP [last menstrual period] day). All patients who had microRNA samples available preconception were included. None of

Table 2
MicroRNA levels in different pregnancy outcome groups. Column 1: preconception microRNA levels of patients who developed preeclampsia and those who experienced a healthy delivery were compared. *Column 2:* first-trimester microRNA levels of healthy and preeclampsia patients were compared. *Column 3:* first-trimester microRNAs of healthy and miscarriage patients were compared. *Column 4:* sequential samples, differences in the mean change in the first-trimester microRNA levels between the preeclampsia group and the healthy group (Δ preeclampsia, Δhealthy). For columns 1, 2, and 3, ↑signifies higher microRNA levels in the healthy pregnancy group; ↓signifies lower microRNA levels in the healthy pregnancy group. For column 4, ↑signifies a higher increase in the microRNA level in the healthy group compared with the preeclampsia group); ↓signifies a lower increase in microRNA levels in the healthy group compared with the preeclampsia group (no IVIg was used between these sequential samples).

Experiment	1	2	3	4
	[**]Preconception microRNA levels: healthy compared with preeclampsia (↑higher in healthy; ↓lower in healthy)	First trimester microRNA levels: healthy compared with preeclampsia (↑higher in healthy; ↓lower in healthy)	First trimester microRNA levels: healthy compared with miscarriage (↑higher in healthy; ↓lower in healthy)	First trimester change (delta Δ) in microRNA level (Δpreeclampsia – Δhealthy; ↑greater increase in healthy; ↓smaller increase in healthy) no IVIg used
Patient groups	11 healthy; 5 preeclampsia	9 healthy; 5 preeclampsia	9 healthy; 7 miscarriage	13 healthy; 8 preeclampsia
Timing of blood draw(s)	Mean 60.7 ± 53.6 days before the conception cycle LMP day	Mean 44.0 ± 13.9 days post-implantation	Mean 41.6 ± 19.7 days post-implantation	No. 1 Mean 29.2 ± 20.1 days post-implantation; No. 2 Mean 51.0 ± 21.7 days post implantation
MicroRNA levels	↓hsa-miR-144-3p, ↓hsa-miR-148a-3p, ↓hsa-miR-16, ↓hsa-miR-223, ↓hsa-miR-582-5p, ↓hsa-miR-7-5p	↑hsa-miR-1229, ↑hsa-miR-1267, ↑hsa-miR-148a-3p, ↑hsa-miR-16, ↑hsa-miR-199b-5p, ↑hsa-miR-223, ↑hsa-miR-30e-3p, ↑hsa-miR-301a-3p, ↑hsa-miR-340-5p, ↑hsa-miR-424-5p	↑hsa-miR-1267, ↑hsa-miR-146a, ↑hsa-miR-155, ↑hsa-miR-16, ↑hsa-miR-210	↑hsa-miR-132, ↑hsa-miR-133b, ↑hsa-miR-146a, ↑hsa-miR-210, ↑hsa-miR-1244, ↓hsa-miR-1229p

[**] Preconception samples used only two microRNAs to calculate pregnancy risk: hsa-miR-223, and 148a-3p.

these patients used IVIg at the time of the blood draw, though all used IVIg later in their subsequent pregnancy. From this analysis, it was found that the preconception expression ratio for hsa-miR-223, 7-5p, 148a-3p, 144-3p, 7-5p, 16, and 582-5p for preeclampsia exceeded 1.0 (Table 2, column 1).

3.1.2. Differences in microRNA levels between preeclampsia and healthy patients in the first trimester

Differences in microRNA levels in the first trimester of pregnancy between healthy and preeclampsia patients without IVIg were assessed. Nine healthy pregnancies and 5 patients with preeclampsia met these inclusion criteria (44.0 ± 13.9 days post-implantation). The expression ratio in the first trimester for preeclampsia for hsa-miR-223, 16, 30e, 424, 1267, 148a-3p, 1229, 301a-3p, 340-5p, and 199b-5p exceeded 1.0 (Table 2, column 2).

3.1.3. Differences in microRNA levels between miscarriage and healthy patients in the first trimester

Differences in microRNA levels in the first trimester of pregnancy between healthy and miscarriage patients without IVIg were assessed. Nine healthy pregnancies and 7 miscarriages met these inclusion criteria (41.6 ± 19.7 days post-implantation). The expression ratio for hsa-miR-210, 1267, 146a, and 16 for miscarriage exceeded 1.0 (Table 2, column 3).

3.1.4. Differences in sequential microRNA levels in early pregnancy between preeclampsia and healthy pregnancies

Sequential blood draws were studied for patterns of microRNA change in response to pregnancy (no IVIg was used before or between the blood draws, although IVIg may have been added later in the pregnancy). Two sequential microRNA levels were tracked approximately 22 days apart. Eight preeclampsia and 13 healthy pregnancies met the inclusion criteria. Ct values for the second sample were subtracted from the first. The mean day of the first blood draw was 29.2 ± 20.1 days post-implantation. The mean day of the second blood draw was 51.0 ± 21.7 days post-implantation. The microRNA differences were sorted from highest to lowest Ct differences (Δ preeclampsia – Δ healthy). MicroRNAs with an expression ratio >1.5 were selected as most differentially expressed between preeclampsia and healthy pregnancy (to keep the microRNA number manageable for this particular population, we chose a higher ratio than the other groups). MicroRNAs meeting this criterion were hsa-miR-133b, 132, 1244, 210, 146a, and 1229 (Table 2, column 4).

3.1.5. Differences in microRNA response to IVIg therapy between preeclampsia and healthy pregnancies

Differences in microRNA response to IVIg therapy between outcome groups were examined. Mean IVIg

Table 3

Differences in the microRNA response to IVIg therapy between preeclampsia and healthy pregnancies. MicroRNA was quantified using real-time polymerase chain reaction (PCR). The term "cycle threshold" ("Ct") represented the amplification cycle at which a reliable signal generated by the amplified product is first detected. Each cycle of amplification represented a doubling of the target polynucleotide. A higher Ct value corresponds with a lower microRNA quantification. Differences in microRNA response to IVIg therapy between outcome groups were examined. Mean IVIg response in preeclampsia (7 patients) was compared with the mean IVIg response in healthy pregnancy (7 patients). IVIg response was calculated using the mean microRNA sequential changes of healthy patients before and after IVIg (mean day of first blood draw 41.4 ± 18.8 days post-implantation; mean day of second blood draw 65.7 ± 21.4 days post-implantation) and compared with similar patients not using IVIg (3 healthy pregnancies). A similar IVIg comparison was performed for the preeclampsia pregnancies (mean day of first blood draw 48.0 ± 40.3 days post-implantation; mean day of second blood draw 76.4 ± 40.2 days post-implantation) and compared with similar patients not using IVIg (2 preeclampsia pregnancies). Results (with and without IVIg) for each outcome group were sorted from largest difference to smallest difference. This demonstrated the "IVIg response" by specific microRNAs associated with different pregnancy outcomes. The "IVIg responses" between the preeclampsia and the healthy pregnancies were converse (highlighted cells).

MicroRNA	Difference: No IVIg-Yes IVIg (Ct values)	** Healthy pregnancy miR Ct levels decreased w/ IVIg (increased activity)	MicroRNA	Difference : No IVIG-Yes IVIg (Ct values)	***Preeclampsia miR Ct levels decreased w/ IVIg (decreased activity)
hsa-miR513a-5p	-9.58	**	hsa-miR575	-15.59	***
hsa-miR219-5p	-1.07		hsa-miR219-5p	-14.87	
hsa-miR132	-0.97		hsa-miR33a-5p	-7.70	***
hsa-miR193a-3p	-0.56	**	hsa-miR199a-5p	-6.26	***
hsa-miR133b	0.43	***	hsa-miR1	-3.66	***
hsa-miR181a	0.46		hsa-miR144-3p	-3.60	***
hsa-miR155	0.77		hsa-miR221-5p	-3.06	***
hsa-miR223	0.78		hsa-miR133b	-2.80	***
hsa-miR671-3p	0.88		hsa-miR671-3p	-2.02	
hsa-miR146a	1.23		hsa-miR7-5p	-1.84	***
hsa-miR1267	1.24		hsa-miR30e-3p	-1.83	***
hsa-miR210	1.40		hsa-miR199b-5p	-1.41	
hsa-miR30e-3p	1.52	***	hsa-miR1229	-1.27	***
hsa-miR1229	1.61	***	hsa-miR181a	-1.22	
hsa-miR221-5p	1.63	***	hsa-miR148a-3p	-1.10	***
hsa-miR148a-3p	1.91	***	hsa-miR16	-0.52	***
hsa-miR144-3p	2.02	***	hsa-miR340-5p	-0.36	***
hsa-miR16	2.02	***	hsa-miR132	-0.24	
hsa-miR301a-3p	2.06		hsa-miR301a-3p	-0.21	
hsa-miR582-5p	2.17		hsa-miR513a-5p	0.00	**
hsa-miR340-5p	2.27	***	hsa-miR223	0.14	
hsa-miR424-5p	2.33		hsa-miR582-5p	0.53	
hsa-miR7-5p	2.44	***	hsa-miR146a	0.53	
hsa-miR575	4.46	***	hsa-miR155	0.59	
hsa-miR1	5.12	***	hsa-miR210	0.74	
hsa-miR199a-5p	5.83	***	hsa-miR1267	1.07	
hsa-miR33a-5p	17.60	***	hsa-miR424-5p	1.13	
hsa-miR-199b-5p	NA		hsa-miR193-3p	3.82	**

NA = failed sample.

*** Increased microRNA activity in healthy pregnancy.

** Decreased microRNA activity in healthy pregnancy.

Table 4a

MicroRNA correlations with immune test results: TNFα/IL-10 and IFNγ/Il-10. The mean microRNA Ct levels for specimens within the expected ranges and for those exceeding the expected ranges were calculated. Highlighted microRNAs demonstrate a mean microRNA Ct difference between high- and low-risk testing of immunological groups that were greater than the mean of the standard deviations of the two compared groups: (TNF/IL10 ratio: high risk >30; low risk <24; IFN/IL10 ratio: high risk >11; low risk <6.) When the difference between the means divided by the average of the standard deviations exceeded 1.0, the immune test correlation with the microRNA was selected as being most potentially clinically useful (yellow highlighted cells).

MicroRNA	Difference between high and low risk TNF/IL10 sample groups (microRNA Ct's)[c]	Mean SD of the high and low risk groups		MicroRNA	Difference between high and low risk IFN/IL10 sample groups (microRNA Ct's)	Mean SD of the high and low risk groups
hsa-miR1	3.61	2.36		hsa-miR144-3p	3.81	2.91
hsa-miR199a-	3.09	1.49		hsa-miR1	3.38	2.30
hsa-miR133b	3.02	2.38		hsa-miR199a-5p	3.21	2.45
hsa-miR144-3p	2.68	3.53		hsa-miR1229	2.07	4.15
hsa-miR575	2.48	3.99		hsa-miR181a	1.43	1.65
hsa-miR199b-	1.66	1.35		hsa-miR671-3p	1.37	1.06
hsa-miR7-5p	1.46	1.58		hsa-miR148a-3p	1.33	1.49
hsa-miR146a	1.43	1.24		hsa-miR7-5p	1.32	1.45
miR16	1.39	0.87		hsa-miR1244	1.18	1.96
hsa-miR340-5p	1.38	1.47		hsa-miR340-5p	1.10	1.87
hsa-miR132	1.35	1.64		hsa-miR30e-3p	1.07	1.68
hsa-miR301a-	1.29	1.67		hsa-miR301a-3p	0.84	1.42
hsa-miR223	1.22	1.08		hsa-miR133b	0.78	2.65
hsa-miR1267	1.17	1.24		hsa-miR575	0.41	4.39
hsa-miR155	1.00	1.18		hsa-miR199b-5p	0.30	1.58
hsa-miR1229	0.98	4.40		hsa-miR16	0.06	0.99
hsa-miR30e-3p	0.93	1.80		hsa-miR223	-0.23	1.18
hsa-miR1244	0.81	2.02		hsa-miR582-5p	-0.24	1.50
hsa-miR210	0.78	2.18		hsa-miR210	-0.48	2.28
hsa-miR148a-	0.65	1.80		hsa-miR132	-0.63	1.17
hsa-miR582-5p	0.38	1.48		hsa-miR1267	-0.81	1.14
hsa-miR193a-	0.34	1.83		hsa-miR146a	-0.87	1.30
hsa-miR181a	0.33	1.87		hsa-miR155	-1.03	0.94
hsa-miR671-3p	0.25	1.09		hsa-miR193a-3p	-2.30	1.57

[a] MicroRNA was quantified using real-time polymerase chain reaction. The term "cycle threshold" ("Ct") represented the amplification cycle at which a reliable signal generated by the amplified product is first detected. Each cycle of amplification represented a doubling of the target polynucleotide. A *higher* Ct value corresponds with *lower* microRNA quantification.

response in preeclampsia (7 patients) was compared with the mean IVIg response in healthy pregnancy (7 patients). IVIg response was calculated using the mean microRNA sequential changes of healthy patients before and after IVIg (mean day of first blood draw 41.4 ± 18.8 days post-implantation; mean day of second blood draw 65.7 ± 21.4 days post-implantation) and compared with sequential changes of a similar group not using IVIg (2 patients). A similar IVIg comparison was performed for the preeclampsia pregnancies (first blood draw, mean 48.0 ± 40.3 days post-implantation; second blood draw, mean 76.4 ± 40.2 days post-implantation) and those preeclampsia cases not using IVIg (3 patients). Results (before and after IVIg and those using no IVIg) for each outcome group were sorted from largest difference to smallest difference. This demonstrated the "IVIg response" by specific microRNAs associated with different pregnancy outcomes. The "IVIg responses" between the preeclampsia and the healthy pregnancies were converse for hsa-miR-513a-5p, 193a-3p, 1, 133b, 1229, 148a-3p, 7-5p, 575, 221-5p, and hsa-miR-199a-5p, among others (Table 3). Interestingly, we demonstrated that many of the microRNAs that corresponded with IVIg response also corresponded with pregnancy outcome (Table 2, column 4). It would appear from these data that patients with many of the "dysregulated" microRNAs associated with pregnancy disease also have the potential for treatment with IVIg.

3.1.6. MicroRNA associations with immunological test results

Immunological testing was performed on the same blood samples used for the microRNA analysis and immunological associations with specific microRNAs were found. The most differentially expressed microRNAs between "high" and "low" TNFα/IL-10 patient groups

Table 4b
MicroRNA associations with immune test results: percentage of T regulatory cells (CD4+CD25+Foxp3+) and percentage of peripheral blood natural killer cells (CD56+16+). Highlighted microRNAs demonstrate a mean microRNA Ct difference between high- and low-risk testing of immunological groups that was greater than the mean of the standard deviations of the two compared groups: (T regulatory cell percentage: high risk <0.7%, low risk >1.1%; CD56+16+% of peripheral blood lymphocytes: high risk >12%; low risk <5%). When the difference between the means divided by the average of the standard deviations exceeded 1.0, the immune test correlation with the microRNA was selected as being more potentially clinically useful (yellow highlighted cells).

MicroRNA	Difference between high and low risk Treg sample group (microRNA Ct levels)	Mean SD of the high and low risk groups		MicroRNA	Difference between high and low risk CD56+16+% group (microRNA Ct levels)	Mean SD of the high and low risk groups
hsa-miR1229	2.43	1.32		hsa-miR199b-	0.68	1.48
hsa-miR1267	1.18	1.06		hsa-miR199a-	0.68	2.49
hsa-miR1244	1.1	1.17		hsa-miR340-5p	0.40	1.55
hsa-miR30e-3p	0.99	0.82		hsa-miR193a-	0.34	2.07
hsa-miR199b-5p	0.92	1.44		hsa-miR1267	0.04	1.34
hsa-miR155	0.86	1.23		hsa-miR210	-0.01	2.10
hsa-miR146a	0.77	1.23		hsa-miR582-5p	-0.13	0.99
hsa-miR210	0.77	1.57		hsa-miR146a	-0.16	1.23
hsa-miR671-3p	0.64	0.66		hsa-miR155	-0.22	1.27
hsa-miR7-5p	0.56	1.17		hsa-miR132	-0.23	1.74
hsa-miR582-5p	0.4	0.72		hsa-miR223	-0.23	1.04
hsa-miR148a-3p	0.37	0.92		hsa-miR671-3p	-0.30	1.21
hsa-miR16	0.35	1.06		hsa-miR7-5p	-0.32	1.51
hsa-miR133b	0.32	2.64		hsa-miR1244	-0.40	1.67
hsa-miR193a-3p	0.28	1.22		hsa-miR16	-0.55	1.07
hsa-miR223	0.21	0.78		hsa-miR181a	-0.72	1.31
hsa-miR301a-3p	0.01	1.59		hsa-miR133b	-0.97	2.65
hsa-miR575	-0.01	1.66		hsa-miR301a-	-1.26	1.34
hsa-miR199a-5p	-0.24	1.46		hsa-miR148a-	-1.28	1.39
hsa-miR132	-0.24	1.67		hsa-miR1	-1.34	2.07
hsa-miR181a	-0.26	1.32		hsa-miR30e-3p	-1.42	1.58
hsa-miR1	-0.34	2.26		hsa-miR144-3p	-1.62	2.52
hsa-miR340-5p	-0.69	1.38		hsa-miR575	-1.67	3.76
hsa-miR144-3p	-2.08	2.48		hsa-miR1229	-3.11	3.54

were hsa-miR-1, 199a-5p, 133b, 146a, 223, 16, and 199b-5p (Table 4a). This microRNA cluster was found to be associated with the PI3K-Akt signaling pathway using DIANA miRPath online software (Vlachos et al., 2012). The cluster associated with the IFNγ/Il-10 ratio assay consisted of hsa-miR-144-3p, 1, and 199a-3p (Table 4b). This cluster was also found to be associated with the PI3K-Akt signaling pathway. Two microRNAs, hsa-mir-1267 and 1229, demonstrated the greatest differential expression between high and low T regulatory cell levels (Table 4b). These two microRNAs were found to regulate the TGF-beta signaling pathway. Hsa-miR-1229 Ct was only weakly differentially expressed with a differential CD56 + percentage (Table 4b). Interestingly, none of the 30 microRNAs included in our analysis showed differential expression with differential NK 50:1 cytotoxicity (data not shown).

3.1.7. MicroRNA clusters are associated with metabolic pathways

Using the DIANA miRPath online software (Vlachos et al., 2012), microRNA clusters that demonstrated both IVIg response in addition to pregnancy risk were together associated with the PI3K-Akt signaling pathway. This existence of a common pathway between pregnancy risk and IVIg response suggests that this microRNA cluster might hold both diagnostic and treatment monitoring potential (Tables 3 and 5).

3.2. Venn diagram for microRNA selection

A Venn diagram was constructed that contained four clinically relevant microRNA groups. These groups represented microRNAs demonstrating the greatest differential expression among:(1) Th1/Th2 response, (2) T regulatory cell percentage, (3) prediction of pregnancy risk, and (4) prediction of treatment response (Fig. 1). MicroRNAs that were contained in both the Th1/Th2 and T regulatory cell groups (Tables 4a and 4b), which also overlapped within the pregnancy outcome and therapy response groups, were selected for scoring (as they appeared to demonstrate maximal clinical relevance). Five optimal microRNAs were identified using this process: hsa-miR-1, 133b, 1267, 1229, and 199a-5p. Two additional microRNAs were selected that would also predict pregnancy outcome preconception: hsa-miR-223 and 148a-3p.

Table 5
Clinical features of microRNAs used in our seven-microRNA scoring system.

MicroRNA	Elevated level associated with *high* pregnancy risk at implantation	Elevated level associated with strongly with low TH1/Th2	Elevated level associated with strongly with low Treg	Elevated level associated with weakly with elevated CD56	Elevated level associated with *low* pregnancy risk preconceptio n	MicroRNA distinguishes between miscarriage and preeclampsia risk	Associated Metabolic pathways [d]
Hsa-miR-1	x	x					PI3-Akt signaling pathway; cell cycle; apoptosis
Hsa-miR-133b	x	x					PI3-Akt signaling pathway; cell cycle; apoptosis
Hsa-miR-199a-5p	x	x					PI3-Akt signaling pathway; cell cycle; apoptosis
Hsa-miR-1267	x		x				TGF-beta signaling pathway; Angiogenesis, Apoptosis, Immunosuppression
Hsa-miR-1229	x		x	x		x	TGF-beta signaling pathway; Angiogenesis, Apoptosis, Immunosuppresion
Hsa-miR-223	x	x			x	x	PI3-Akt signaling pathway; cell cycle; apoptosis
Hsa-miR-148a-3p	x			x	x		ECM-receptor interactions; Focal adhesion

[a] DIANA miRPath v.2.0.

3.3. Clinical application of the seven-microRNA testing panel

The seven-microRNA scoring system was then applied to a population of 39 patients (19 healthy deliveries, 12 preeclampsia [7 late-onset and 5 early-onset] and 8 miscarriages) with samples drawn at a mean of 34.9 ± 19.2 days post-implantation for the first blood draw. In addition, the scoring system was also applied to 20 patients who had samples drawn 2–14 weeks preconception (mean of 59.2 ± 56.8 days before upcoming conception LMP, mean cycle day 17.6 ± 9.5 post-last LMP.) See Table 1b for patient details.

3.4. ROC curve analysis results

The seven-microRNA panel was used to predict pregnancy risk in samples from 39 patients quantified early during the first trimester (Table 6). A ROC curve with an AUC of 0.92 was calculated for miscarriage (mean 30.9 ± 18.6 days post0implantation) and an ROC curve with an AUC 0.90 was calculated for preeclampsia (mean 36.3 ± 18.7 days post-implantation; Fig. 2a and b). When the preeclampsia group was divided into late and early preeclampsia subgroups, the AUC for the early preeclampsia group was 0.86 (mean 35.9 ± 20.5 days post-implantation) and the AUC for the late preeclampsia group was 0.92 (mean 33.3 ± 16.4 days post-implantation; Fig. 2c and d. ROC curve analysis for 20 preconception samples (mean 59.2 ± 56.8 days before the conception cycle LMP) demonstrated an AUC of 0.81 (Fig. 2e.) These AUCs confirmed that the smaller seven-microRNA panel maintained essentially the same level of sensitivity and specificity in early pregnancy comparable to that of the full 30-microRNA panel in our previous study. Based on these AUC results, the seven-microRNA panel would seem to make an effective clinical tool for diagnosing and monitoring immune-based pregnancy conditions, both preconception and early in the first trimester (Table 5).

4. Discussion

We report the quantification of maternal cellular microRNA in women during the preconception period and early during the first trimester of pregnancy. We show that patterns of microRNA expression differ between patients who develop pregnancy disorders and those who have healthy deliveries. Previously, we reported a scoring system using a panel of 30 microRNAs derived from differences in responses to therapeutic intervention among clinical groups. ROC curve analysis demonstrated the superior power of peripheral blood cell microRNA over conventionally used immune tests to predict pregnancy outcome.

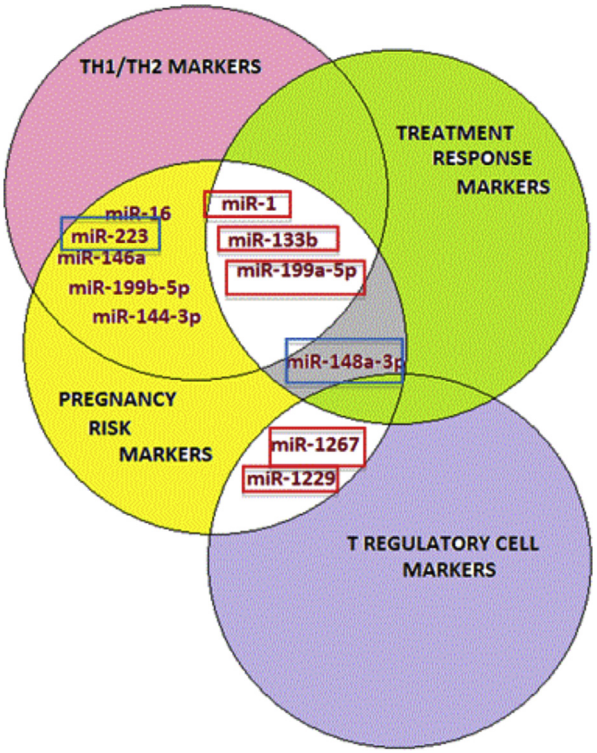

Fig. 1. *A Venn diagram showing the relationship between immunological testing, pregnancy outcome and treatment response.* The seven highlighted microRNAs are included in our microRNA scoring system (blue outlined microRNAs are preconception markers).

In our current study, we reduced the 30-microRNA panel to a seven-member panel without sacrificing either sensitivity or specificity. In an assay performed on a sample from a single time-point, we report the successful prediction of miscarriage (AUC 0.92) and preeclampsia (AUC 0.90) and for each of the endpoints in patient samples collected during the first trimester (Fig. 2a and b). The traditional cell-based immune tests have been found to achieve AUCs only between 0.60 and 0.70 (Lee et al., 2013; Thum et al., 2004). When the preeclampsia group was divided into late and early preeclampsia subgroups, the AUC calculated for the early preeclampsia group was 0.86 while the AUC for the late preeclampsia group was 0.92 (Fig. 2c and d). Moreover, different microRNA clusters revealed different clinical associations with immune parameters. For example, we found that microRNAs associated with negative pregnancy outcome were associated with low T regulatory cell levels and low TNF-Il-10 levels (Tables 4a and 4b). These parameters have been associated with poor outcome in the literature (Chaouat et al., 2007; Winger and Reed, 2011). Also, microRNA clusters associated with adverse pregnancy outcome corresponded with clusters associated with PI3K-Akt signaling (Table 5). As the PI3K-Akt pathway regulates cell cycle progression and focal adhesion, its disruption during trophoblast invasion may be a causal factor in both implantation and preeclampsia risk (Chakraborty et al., 2002).

Table 6
MicroRNA pregnancy risk score using seven microRNAs in the first trimester of pregnancy for 39 patients with various pregnancy outcomes.

Patient no.	Outcome	No of days post-implantation	Seven-microRNA score
1	H	36	1
2	H	32	0
3	H	48	0
4	H	47	0
5	H	60	0
6	H	36	1
7	H	62	0
8	H	33	0
9	H	36	0
10	H	29	0
11	H	14	0
12	H	14	0
13	H	18	0
14	H	6	0
15	H	27	0
16	H	21	0
17	H	62	0
18	H	18	0
19	H	2	0
20	M	13	0
21	M	20	3
22	M	71	3
23	M	21	4
24	M	32	1
25	M	16	4
26	M	11	1
27	M	50	2
28	PE	59	1
29	PL	31	2
30	PE	70	1
31	PL	27	3
32	PL	62	7
33	PE	42	1
34	PL	36	5
35	PE	72	3
36	PL	29	2
37	PL	36	0
38	PL	44	6
39	PE	17	0

Key: PL = late-onset preeclampsia, PE = early-onset preeclampsia, M = miscarriage, H = healthy delivery.

In addition to microRNA cluster associations with immune tests, we also examined microRNA cluster associations with clinical outcomes. It was found that a cluster of maternal PBMC microRNAs that demonstrated increased activity with preeclampsia also demonstrated decreased activity with healthy pregnancy (Table 3). In addition, many microRNAs that appeared to respond to IVIg were also associated with microRNAs predictive for pregnancy outcome (hsa-miR-513a-5p, 193a-3p, 7-5p, 575, 221-5p, 133b, 1 and 199a-5p; Table 2, column 4). Of particular note, the group of microRNAs associated with both IVIg response and pregnancy outcomes also demonstrates correlations with the TNFα/1l-10 ratio (hsa-miR-1, hsa-miR-133b, and hsa-miR-199a-5p; Table 4a). Given these correlations, the potential value of microRNA testing becomes more compelling.

To our knowledge, the seven-member microRNA panel is the first practical diagnostic test to successfully predict preeclampsia risk early in the first trimester. Interestingly,

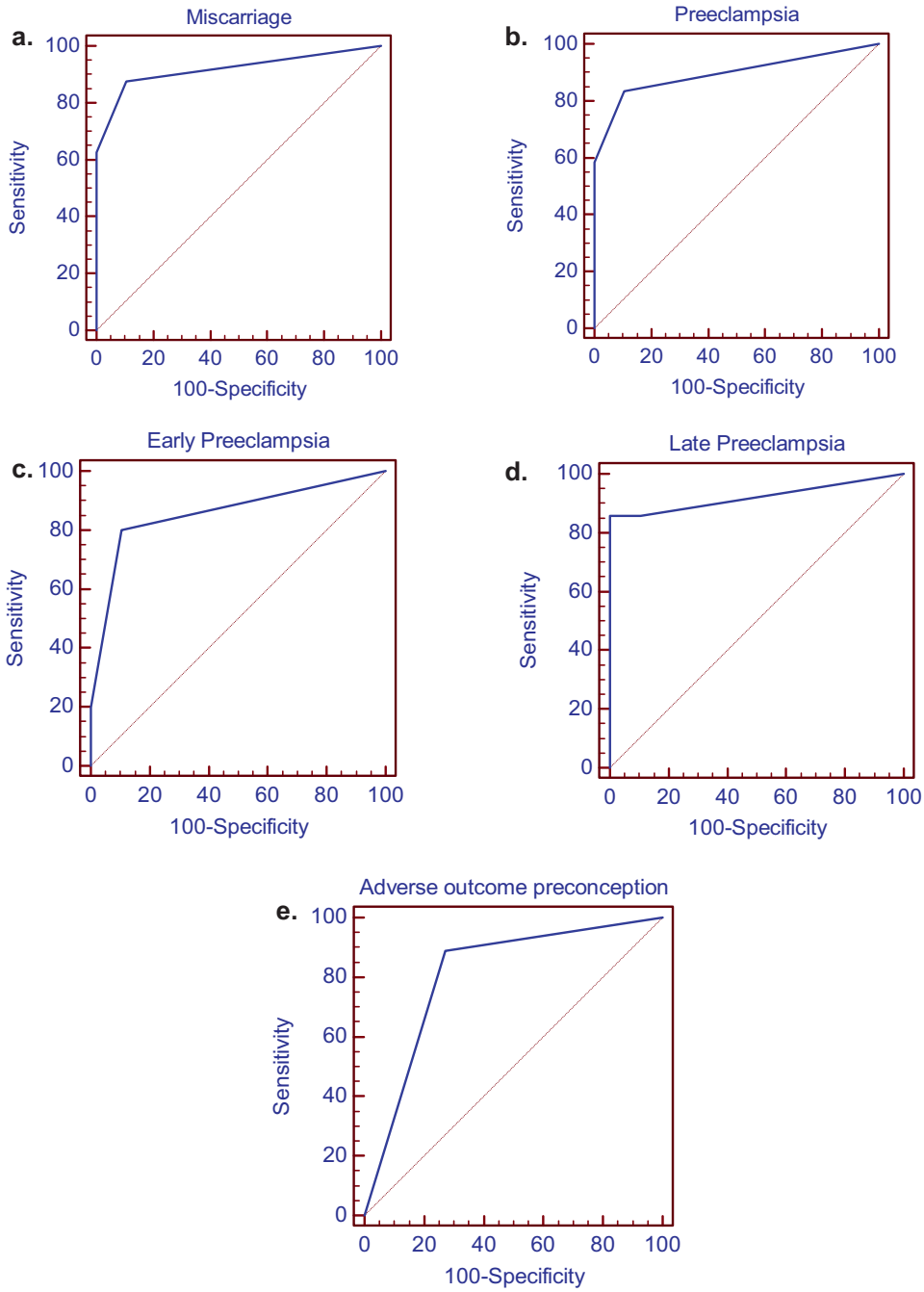

Fig. 2. *Receiver operational characteristic (ROC) curves.* ROC curves of the PBMC microRNA scoring system. Miscarriage and preeclampsia samples utilized seven microRNAs for the scoring system: hsa-miR-1, 133b, 1267, 1229 and 199a-5p, hsa-miR-223, and 148a-3p, and preconception samples used two microRNAs for the scoring system: hsa-miR-223 and 148a-3p. MicroRNAs were selected for scoring based on the Venn diagram shown in Fig. 1. (a) An AUC of 0.92 was calculated for miscarriage, (b) an AUC 0.90 was calculated for preeclampsia, (c) an AUC of 0.86 was calculated for the early preeclampsia, (d) an AUC of 0.92 was calculated for late preeclampsia, (e) an AUC of 0.81 was calculated for "compromised pregnancy" (miscarriage and preeclampsia combined) in the preconception time period. Additional ROC curve details are listed in Table 7.

of the two subtypes of preeclampsia known, early and late, microRNA predicted late preeclampsia with the higher ROC curve AUC (AUC of 0.92; mean 33.3 ± 16.4 days post-implantation). No pregnancy marker has yet been published that predicts late preeclampsia on samples collected during the first trimester. Not only does this finding offer hope for an earlier approach to preeclampsia treatment, but it also suggests that maternal factors as identified in PBMC microRNA might play a more central role in preeclampsia development than has been previously recognized.

Table 7
ROC calculations for "affected" versus "healthy" pregnancy for various outcomes using a seven-microRNA scoring system. Miscarriage and preeclampsia samples utilized seven microRNAs for the scoring system: hsa-miR-1, 133b, 1267, 1229 and 199a-5p, hsa-miR-223, and 148a-3p, and preconception samples used 2 microRNAs for the scoring system: hsa-miR-223 and 148a-3p. MicroRNAs were selected for scoring based on the Venn diagram shown in Fig. 1.

Outcome	"Compromised" outcome (miscarriage and preeclampsia combined) for preconception samples only[a]	Miscarriage	Preeclampsia (all)	Late preeclampsia	Early preeclampsia
AUC	0.81	0.92	0.90	0.92	0.86
Criteria (microRNA score for diagnosis)	>0	>0	>0	>0	>0
Specificity	72.7	89.5	89.5	89.5	89.5
Sensitivity	88.9	87.5	83.3	85.7	80.0
PPV	0.73	0.78	0.83	0.75	0.67
NPV	0.89	0.94	0.89	0.94	0.94
Diagnosis 1 (No. of affected)	11	8	12	7	5
Diagnosis 2 (No. of healthy)	9	19	19	19	19
No. of total samples	20	27	31	26	24
Day of blood draw (mean ± SD)	59.2 ± 56.8 days before the conception cycle LMP (mean menstrual cycle day 17.6 ± 9.5)	30.9 ± 18.6 days post-implantation	36.3 ± 18.7 days post-implantation	33.3 ± 16.4 days post-implantation	35.9 ± 20.5 days post-implantation
p Value	0.0006	<0.0001	<0.0001	<0.0001	0.0008

[a] Preconception samples used only two microRNAs to calculate pregnancy risk: hsa-miR-223 and 148a-3p.

In summary, microRNA may offer a logical and compelling alternative to conventional immune testing. Heretofore, clinicians have been obliged to employ multiple and complex cell-based immune tests to identify patients who might benefit from therapy. MicroRNA testing, on the other hand, allows for greater precision, simpler interpretation, and greater predictive value in the clinic. Although our research is preliminary, we hope that future studies will build upon our investigations and enhance the power of maternal cell microRNA to predict pregnancy risk in the clinic.

Acknowledgements

We thank Holden Maecker, PhD, Director, Human Immune Monitoring Center, Stanford University, for critical reading and editing.

We thank Professor Sudesh K. Srivastav, Ph.D., School of Public Health and Tropical Medicine, Department of Biostatistics and Bioinformatics, Tulane School of Medicine, for his kind discussion and suggestions.

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