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Usefulness of circulating microRNAs for the prediction of early preeclampsia at first-trimester of pregnancy

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To assess the usefulness of circulating microRNAs (miRNAs) as non-invasive molecular biomarkers for early prediction of preeclampsia, a differential miRNA profiling analysis was performed in first-trimester pooled sera from 31 early preeclampsia patients, requiring delivery before 34 weeks of gestation, and 44 uncomplicated pregnancies using microfluidic arrays. Among a total of 754 miRNAs analyzed, the presence of 63 miRNAs (8%) was consistently documented in the sera from preeclampsia and control samples. Nevertheless, only 15 amplified miRNAs (2%) seemed to be differentially, although modestly, represented (fold change range: 0.4–1.4). After stem loop RT-qPCR from individual samples, the statistical analysis confirmed that none of the most consistent and differentially represented miRNAs (3 overrepresented and 4 underrepresented) were differentially abundant in serum from preeclamptic pregnancies compared with serum from normal pregnancies. Therefore, maternal serum miRNA assessment at first-trimester of pregnancy does not appear to have any predictive value for early preeclampsia.

Preeclampsia, a pregnancy-related syndrome that complicates approximately 2–5% of pregnancies, is a major cause of maternal and perinatal morbidity and mortality[1]. Nowadays, preeclampsia still lacks a safe and effective therapy, as well as reliable, early means of diagnosis and prediction. An accurate prediction of preeclampsia is a major challenge in contemporary obstetrics, and resources are now focalized in the first trimester of pregnancy, where prophylactic strategies may help to reduce the incidence of this disorder[2].

Preeclampsia has been named the "disease of theories"[3] since its precise origin remains elusive. Several hypotheses (e.g., immunological, placental ischemia, inflammatory and genetic) have been described to explain its pathogenesis, but still remains poorly understood.

MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs (19–25 nucleotides) that have proved important post-transcription regulators of gene expression[4]. Through sequence-specific base pairing on the 3′ untranslated regions of the target mRNAs and inhibition of protein translation or stimulation of transcript degradation, miRNAs have the capacity to influence both physiological and pathological processes involving cell differentiation, proliferation/growth, apoptosis, angiogenesis and inflammation[4–6]. Recent studies have also proposed a role for miRNAs in cell-to-cell communication[7]. Indeed, miRNAs have been detected in most extracellular fluids, particularly in plasma/serum[8,9]. Moreover, these circulating miRNAs appear resistant to endogenous ribonuclease activity and, therefore, very stable in these environments[9]. This is likely due to both their intrinsic structural features and their presentation, either particulate when associated to membranous vesicles such as apoptotic bodies or exosomes[9,10], or soluble when complexed to RNA-binding proteins such as Ago2, high-density lipoproteins, or nucleophosmin[11–13]. Consequently, circulating miRNAs have been recently postulated as useful biomarkers for a variety of conditions such as cancer, cardiovascular disorders, and immune-inflammatory diseases[14].

Since placental and fetal development and vascular homeostasis are disturbed in preeclampsia, important regulatory events such as epigenetic factors and miRNAs could in turn become deregulated in preeclampsia pathogenesis. Several studies have evaluated the expression of miRNAs in placental tissues with both conventional and microarray approaches, and they identified genes differentially expressed in the transcriptome of human placentas from women affected by preeclampsia[15–19]. Additionally, a few recent reports have identified

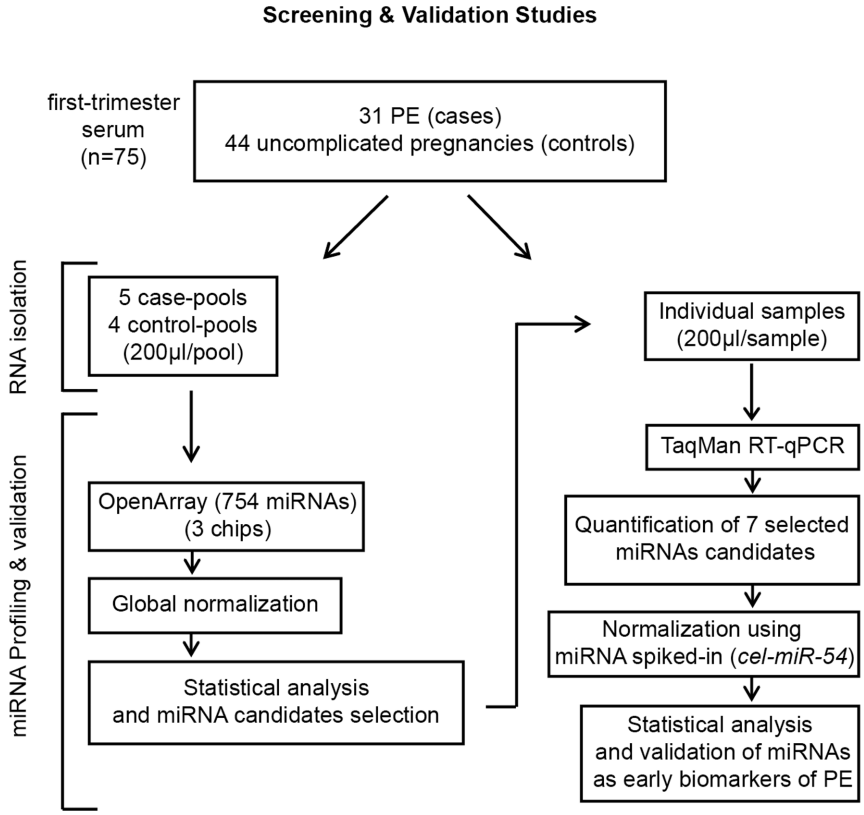

Figure 1 | Schematic diagram of the workflow for differential first-trimester circulating miRNA quantification of preeclampsia versus uncomplicated pregnancies. 754 miRNAs were screened (OpenArray), and 7 selected miRNAs were further validated (TaqMan RT-qPCR) in first-trimester sera from 31 preeclampsia (PE) cases and 44 controls.

altered circulating miRNAs later in pregnancy that are associated with preeclamptic risk using RT-qPCR[20–22] or high-throughput technologies such as microarrays[23,24] and next-generation sequencing (NGS)[25,26]. For example, using microarray analysis Wu et al. evaluated the expression of circulating miRNA from third-trimester preeclamptic women and reported 13 miRNA up-regulated, and 2 down-regulated, as compared to normal pregnancies[23]. Furthermore, application of NGS technology to profile circulating miRNAs in the serum of preeclampsia versus normal pregnant women has also found 22 miRNAs dys-regulated. Among these, 3 of them had been previously reported to be dys-regulated in the placentas of preeclampsia pregnancies[25]. Nevertheless, none of the reported differentially abundant miRNAs overlapped in both studies.

To the best of our knowledge, no study has evaluated the presence of circulating miRNAs in the maternal serum at first-trimester of pregnancy. In the present study, our aim was to explore the potential of miRNAs as an early predictive, minimally invasive biomarker of preeclampsia.

Results

Differential circulating miRNA profiling from first-trimester preeclampsia and healthy pregnancies. We designed a two-stage case-control study, including first-trimester sera from 31 cases of early preeclampsia and 44 uncomplicated pregnancies (controls), to determine whether maternal circulating miRNA profiling could predict preeclampsia at an early stage of development (Fig. 1). Cases and controls were similar regarding maternal age, nulliparity and smoking status. Conversely, they differed significantly for first-trimester MAP and UtA Doppler evaluation ($p < 0.001$) and for perinatal outcome data, such as gestational age at delivery ($p < 0.001$), mode of delivery ($p < 0.001$), and neonatal outcome (24 newborns in the preeclampsia group (77%) were SGA neonates, and 4 of them (13%) died during the perinatal period). The main features of the studied population, according to the study groups, are summarized in Table 1.

To estimate whether there was difference in the generalizable first-trimester serum miRNA signatures between preeclampsia and uncomplicated pregnancies, we employed three TaqMan OpenArray Human miRNA Panels and a study population of 31 early preeclampsia cases and 44 controls. These were interrogated using pools of the different serum samples as surrogate training samples. This pooling approach would only detect circulating miRNAs present in the majority of patients/subjects within the group, reducing variation between individuals and enriching for miRNAs most likely to change between cases and control groups. On the other hand, circulating miRNAs present at low levels or in only a few individuals would not be detected. Thus, the 31 cases of preeclampsia were randomly divided in 4 pools and the 44 controls in 5 pools (60 µl/individual serum sample) and the corresponding pre-amplified cDNAs were dispensed in each of 3 nanofluidic arrays (OA) according to the following distribution: OA1 interrogated 2 pools of cases (n = 6/each) and 1 pool of controls (n = 12), OA2 interrogated 1 pool of cases (n = 11) and 2 pools of controls (n = 6/each), and OA3 interrogated 1 pool of cases (n = 8) and 2 pools of controls (n = 10/each).

Among a total of 754 miRNAs analyzed, 63 could be correctly amplified ($Ct < 31$) in all sample pools and therefore were present in the sera from preeclampsia and control samples (Supplementary Fig. S1 online). We obtained a relatively good correlation of Ct values both between control pools and between sample pools, which reinforce the consistency and reliability of our pooling approach (Supplementary Fig. S2 online). Furthermore, using a rather conservative selection criteria to assess differential circulating miRNA abundance between preeclampsia and controls (fold change ≥ 1.3

Table 1 | Main clinical characteristics of the study groups

Characteristics	n	Preeclampsia	n	Controls	*p*-Value
Age (years)	31	32.6 ± 6.6	44	32.3 ± 5.6	0.822
Pre-pregnancy BMI (kg/m^2)	31	24.5 ± 4.4	44	22.8 ± 3.2	0.060
Non-Caucasian ethnicity	31	15 (48.4%)	44	8 (18.2%)	<**0.001**
Nulliparity	31	18 (58.1%)	44	24 (54.5%)	0.474
Smoking status	31	2 (6.5%)	44	5 (11.4%)	0.384
MAP (mmHg); median (IQR)*	31	90 (79–98)	37	71 (69–77)	<**0.001**
Mean UtA PI; median (IQR)*	31	2.4 (1.8–2.9)	37	1.6 (1.0–2.4)	<**0.001**
GA at delivery (weeks)	31	31.4 ± 2.5	44	37.5 ± 6.3	<**0.001**
Cesarean section	30	25 (80.6%)	42	7 (15.9%)	<**0.001**
Birthweight (g)	30	1374 ± 512	42	3232 ± 726	<**0.001**
Stillbirth or neonatal death	31	4 (12.9%)	44	0 (0.0%)	**0.026**
SGA newborns	31	24 (77.4%)	44	4 (9.1%)	<**0.001**

BMI: Body mass index; MAP: Mean arterial pressure; IQR: Interquartile range; UtA PI: Uterine artery Doppler pulsatility index; GA: gestational age; SGA: Small for gestational age; Data are reported as a mean ± SD, or n (%); bold font: statistically significant results.
*Data recorded at first-trimester screening (11 + 0-13 + 6 weeks' gestation).

or ≤ 0.7, and at least 3 of the 4 sample pools having the same tendency –towards higher abundance or lower abundance- respect to the control pools) we found 15 of them differentially represented (roughly one third more abundant and two thirds less abundant, compared to their corresponding control pool counterparts) (Fig. 2a). However, the overall differences in relative miRNA abundance between preeclampsia and controls were not statistically significant (except for miR-127; $p = 0.041$, although no miRNA passed the false discovery rate (FDR) correction) and fairly modest, as the fold changes ranked between 0.4 and 1.4.

Circulating miRNA abundance validation by real-time quantitative stem-loop RT-PCR analysis. According to the OpenArray profiling outcome, we proceeded to validate 7 of the most differentially abundant miRNAs: miR-192, -143 and -125b (overrepresented), and miR-127, -942, -126# and -221 (underrepresented) (Fig. 2b). Thus, these particular miRNAs were subsequently re-analyzed in each of the individual case and control samples (validation set) using stem-loop RT-qPCR[27]. The tendencies among the 7 miRNAs analyzed were conserved between the OpenArray platform and the RT-qPCR assays (Fig. 3 and Table 2). Moreover, their differential levels obtained by RT-qPCR were comparable, although somehow slighter than those found by Open Array analysis (as an example, see Supplementary Fig. S3 online). Consequently, no significant differences between preeclampsia and controls were found regarding the first-trimester levels of the above circulating miRNAs (Fig. 4). Furthermore, the only significant correlations between the levels of the 7 circulating miRNAs and the clinical parameters of the preeclampsia patients was a moderate negative correlation between miRNA-942 and the mean arterial pressure (MAP) ($r_s = -0.418$; $p = 0.019$), and rather weak correlations between miR-143 and the ethnicity ($r_{bs} = -0.401$; $p = 0.028$), the parity ($r_{bs} = 0.385$; $p = 0.036$) and the mean uterine artery Doppler pulsatility index (UtA PI) ($r_s = -0.373$; $p = 0.043$) (Supplementary Table S2 and Supplementary Fig. S4 online).

Discussion

The prediction of preeclampsia is one of the most important goals in maternal-fetal medicine due to the lack of a specific therapy for this severe disorder, which affects the mother and consequently the fetus, often requiring premature delivery. A screening test that could identify women early in pregnancy later developing preeclampsia would allow not only to increase surveillance of those women at risk, but also to identify those who would benefit from a prophylactic treatment[2]. Indeed, it has been recently proposed that the use of low-dose aspirin initiated at or before 16 weeks of gestation may reduce by 89% the incidence of preterm preeclampsia (delivery < 37 weeks)[28].

A growing body of evidence suggests that there are two forms of preeclampsia based on differences in placental insufficiency, clinical manifestations, and also fetal involvement[1,29–31]. The early-onset form, requiring delivery before 34 weeks of gestation, is the most studied and the one for whom first-trimester screening tests are achieving increased sensitivities[32–34].

Nevertheless, owing to its heterogeneity, elusive pathophysiology, and the lack of specific markers, preeclampsia is unlikely to be detected early by a single predictive parameter with sufficient accuracy to be clinically useful. Therefore, current efforts intend combining maternal characteristics and both biophysical and biochemical markers assessed in the first-trimester, into multivariate algorithms so that the risk of preeclampsia can be estimated with performance levels that could reach clinical utility[32–34]. The importance of biophysical parameters, such as maternal blood pressure and uterine artery Doppler, has already been demonstrated in several reports[29,34]. Moreover, a huge amount of different biochemical markers have been evaluated: products of fetal and placental origin, markers of renal or endothelial damage, markers of oxidative stress, angiogenic and anti-angiogenic factors among others[35]. However the evidence is not yet consistent, and studies assessing new biomarkers are highly encouraged to better clarify the complex pathogenesis of the disorder and, consequently, the best way to predict it.

The sera contain stable cell-free miRNAs, small non-coding RNAs with important regulatory roles in proliferation, apoptosis and cell-cell communication, which can be identified and quantified, making them the ideal biomarker candidates for non-invasive diagnostic/prognostic purposes[14]. Moreover, aberrant miRNA expression has been recently linked to pregnancy complications, such as preeclampsia[36]. Thus, we aimed to assess the potential of circulating miRNAs as sensitive and specific biomarkers for the early diagnosis of preeclampsia, either by themselves or in predictive models when combined with other biometrical variables, such as sonographic markers, into multivariate algorithms[35,37]. Nevertheless, this study suggests that circulating miRNAs in maternal serum are not useful for the early prediction of women at risk of preeclampsia. In fact, circulating miRNA levels did not differ significantly in women who developed early preeclampsia as compared with uncomplicated pregnancies when assessed at first-trimester of pregnancy using the OpenArray platform, a reliable high-throughput technology allowing profiling all known miRNAs with power similar to microarray and precision/specificity of qPCR. The OpenArray technology is based on high-density array of nanoliter PCR assays, making possible to do up to 3,072 real-time PCRs at a single experiment[38]. In contrast, three high-

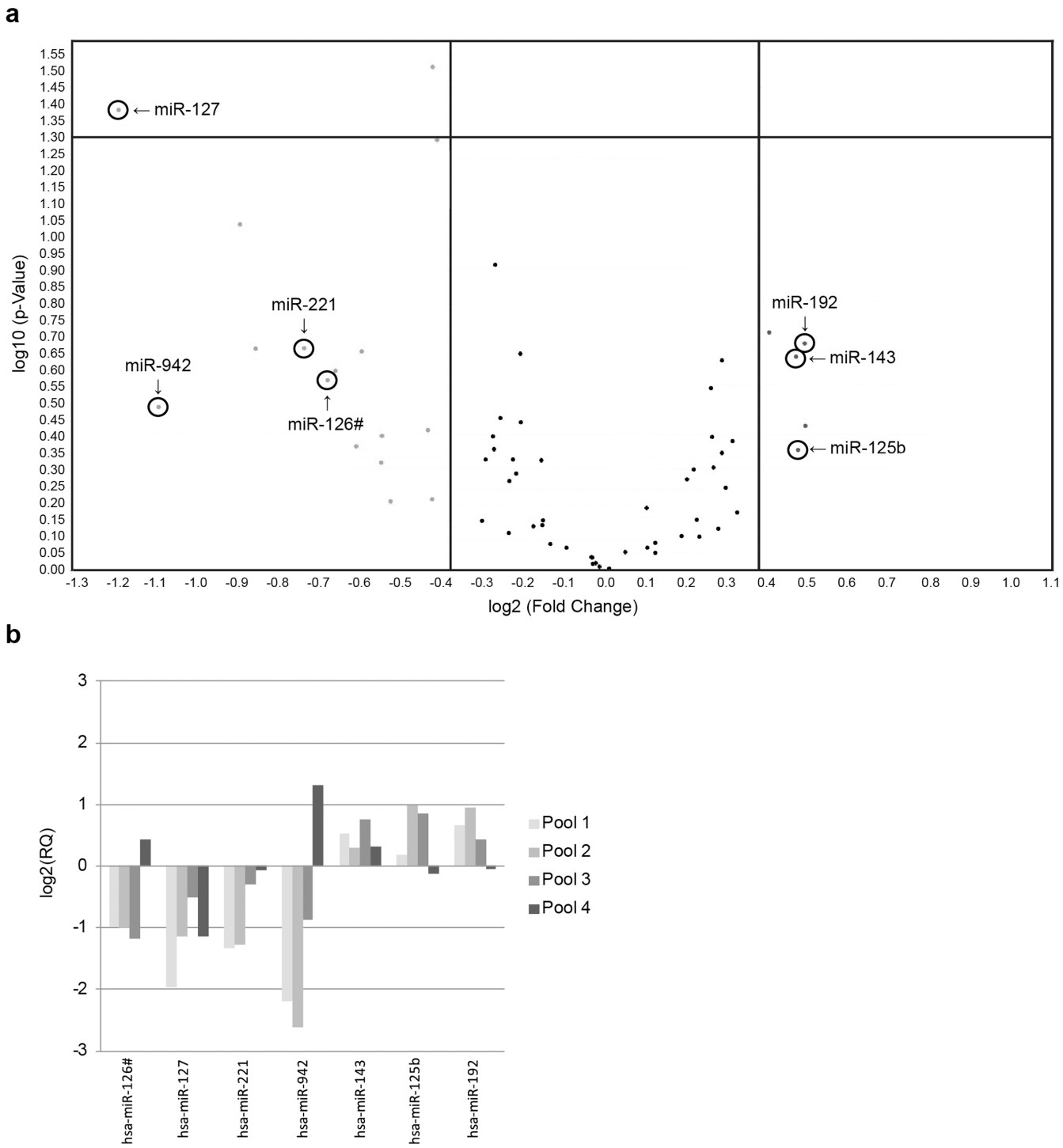

Figure 2 | Differential abundance profile of first-trimester serum miRNAs in preeclampsia and uncomplicated pregnancies by the OpenArray platform. (a) volcano plot of nanofluidics data shows fold changes in miRNA abundance between preeclampsia patients and control pregnancies. The 7 miRNAs selected for further validation (3 overrepresented and 4 underrepresented) are highlighted. (b) Levels of the above-selected circulating miRNAs in preeclampsia compared with the mean circulating miRNAs in uncomplicated pregnancies. Columns: preeclampsia serum sample pools. Baseline: mean of 4 serum sample pools from normal control pregnancies.

throughput studies have also recently assessed the expression profiles of circulating miRNAs in serum samples of pregnant women with and without preeclampsia, and reported several miRNAs to be dysregulated in the serum/plasma of preeclampsia pregnancies[23,25,26]. However, these reports were conducted using serum/plasma samples from a more advanced stage of gestation (third-trimester). Moreover, although Yang *et al.*[25] and Li *et al.*[26], belonging to the same research group, established a proof-of-concept regarding the feasibility, comprehensiveness and sensitivity of the next generation sequencing technology, the small number of samples analyzed in both studies (study 1: control group, n = 1; mild preeclampsia group, n = 2; severe preeclampsia group, n = 2; study 2: control group, n = 4; mild preeclampsia group, n = 4; severe preeclampsia group, n = 4) casts doubts about the overall reliability of their data for preeclampsia diagnosis/prognosis. Alternatively, using a hybridization-based microarray approach, Wu *et al.*[23] reported that among 821 human

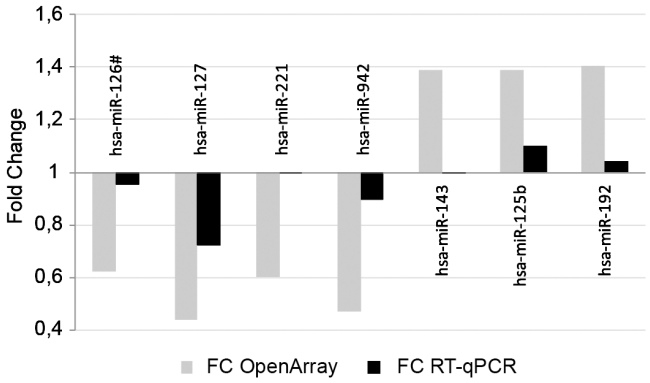

Figure 3 | Comparison of first-trimester serum miRNA abundance between the OpenArray and the TaqMan RT-qPCR platforms. The miRNAs analyzed are listed on the X-axis. The mean fold changes between preeclampsia cases and healthy pregnancies are displayed in the Y-axis. Gray bars represent OpenArray measurement results while solid bars represent TaqMan measurements from the same miRNA (see Table 2).

Table 2 | Differential circulating miRNA levels in preeclampsia compared to uncomplicated first-trimester pregnancies at both the screening (OpenArray) and the validation (RT-qPCR) stages

miRNA	FC (OpenArray)	*p*-Value	FC (RT-qPCR)	*p*-Value
hsa-miR-126#	0.624	0.267	0.952	0.918
hsa-miR-127	0.437	0.041	0.720	0.404
hsa-miR-221	0.599	0.214	0.999	0.846
hsa-miR-942	0.467	0.322	0.896	0.530
hsa-miR-143	1.387	0.228	0.993	0.797
hsa-miR-125b	1.391	0.433	1.101	0.472
hsa-miR-192	1.407	0.208	1.043	0.749

FC, fold change.

miRNAs detected by microarray, only 15 differentially expressed miRNAs of twofold changes or more (and merely two of them with fold changes higher than 4) were identified in plasma of women with severe preeclampsia. Therefore, these outcomes further support the notion that in our study the lack of miRNA discriminatory power during the early, preclinical phase of preeclampsia is not due to a limited sensitivity of the miRNA analysis technologies employed, but would appear an outcome of the own etiology of the disease. In fact, the overt clinical symptoms of preeclampsia do not develop until week 20 of gestation onward. Accordingly, our study suggests that circulating miRNAs may be a relatively late event in preeclampsia development. Although aberrant uterine vascular remodeling is a hallmark of preeclampsia pathogenesis, leading to reduced placental perfusion during early pregnancy, perhaps the degree of stress/damage inflicted into trophoblasts and endothelial cells by an enhanced inflammatory response due to environmental/maternal characteristics, genetic predisposition and/or immunological maladaptation is still scarce to induce abnormal levels of circulating miRNAs during the first-trimester of pregnancy. Thus, increased placental dysfunction may stimulate the gradual release of placental mediators (including miRNAs) into the maternal circulation, leading to further widespread maternal vascular injury and enhanced differential circulating miRNA profiles only at more advanced stages of gestation.

Interestingly, although the differential abundance of 7 circulating miRNAs uncovered by OpenArray analysis and chosen by further validation was fairly small between preeclampsia and uncomplicated pregnancies, most of them are multipurpose miRNAs that have been clearly implicated in angiogenesis (miR-125b, miR-143, miR-942)[39–41], inflammation (miR-126#, miR-127, miR-192, miR-221)[42–45], hypoxia/ischemia (miR-127)[46], and cell migration/remodelling (miR-125b, miR-143, miR-127)[47–49], and some of them have been found altered in placentas from pre-eclamptic pregnancies (miR-126#, miR-192)[36]. Two of these circulating miRNAs (miR-221 and miR-125b) have been found differentially abundant in late-screening preeclampsia samples through high-throughput sequencing by oligo ligation detection (SOLiD)[25,26] and microarray[23] profiling studies. Moreover, we have found in preeclampsia patients a negative correlation between miR-942 levels and the maternal arterial pressure, and between miR-143 levels and the uterine artery Doppler pulsatility index, in agreement to their recently reported roles in the regulation of blood pressure and vascular function[41,50], which might be suggestive of usefulness as prognostic markers. Indeed, altered angiogenic balance, systemic inflammation and placental hypoxia and ischemia, among others, contribute to the pathogenesis of preeclampsia[51].

However, we could not further confirm the differential abundance of these 7 miRNAs in individual samples by TaqMan stem-loop RT-qPCR, a prominent assay designed to detect and quantify mature miRNAs in a fast, specific, accurate and reliable manner[52].

For miRNA measurement, there are conflicting reports regarding the reproducibility of different high-throughput profiling technologies[53–55]. Nevertheless, the results obtained in our study suggest correlation, rather than lack of correlation, between both the OpenArray and the TaqMan RT-qPCR techniques. Indeed, the tendency (toward higher or lower abundancy) of the 7 miRNAs is the same comparing the OpenArray system (performed with "pooled" samples) and the TaqMan RT-pPCR system (performed with individual samples). After all, the slight differences in differential fold changes between both platforms could be due to minor variations in the cDNA pre-amplification step from both technologies to enhance sensitivity, or could be ascribed simply to the different normalization methods employed ("global mean normalization" in the OpenArray platform, versus "*spiked-in cel-miR-54* normalization" in the Taq-Man RT-qPCR platform). In fact, a recent report analyzing maternal plasma miRNA expression profiles in preeclamptic pregnancies compared with normal pregnancies through sequencing by oligo ligation detection (SOLiD), identified 51 miRNAs differentially expressed, and 4 of them were further validated by TaqMan RT-qPCR[26]. Thus, a comparison of the two platforms employed in the referred study (SOLiD sequencing versus TaqMan RT-qPCR) revealed that the differential abundance of these 4 validated miRNAs was significantly attenuated in the TaqMan RT-qPCR platform respect to that obtained in the SOLiD sequencing platform, analogously to that observed in our study between the OpenArray and the TaqMan RT-qPCR platforms.

Among the limitations of the current analysis we acknowledge that this was a nested case-control study including small numbers of patients with preeclampsia. However, we believe it unlikely that increasing the sample size would have resulted in remarkable differences in the results. In addition, we conducted the study in a low risk Mediterranean population. We cannot yet exclude that studies in other populations might yield different outcomes. Finally, in contrast to the NGS technologies, the fixed number of miRNAs represented in array-based platforms such as the high-throughput OpenArray system might overlook non-represented (e.g., isomirs) or inconsistently hybridized/amplified circulating miRNAs, although genuine differentially abundant, influencing the outcome of the present study.

In summary, the development of a non-invasive, efficient screening procedure to identify women at risk of preeclampsia would be beneficial for early-targeted preventive/prophylactic interventions. Using miRNA microarray and real-time stem-loop RT-qPCR analyses, our study demonstrates that the maternally circulating miRNA abundance profile in serum samples from early preeclampsia pregnancies is not significantly different in early gestation compared with that from normal pregnancies. This suggests a minor predictive and

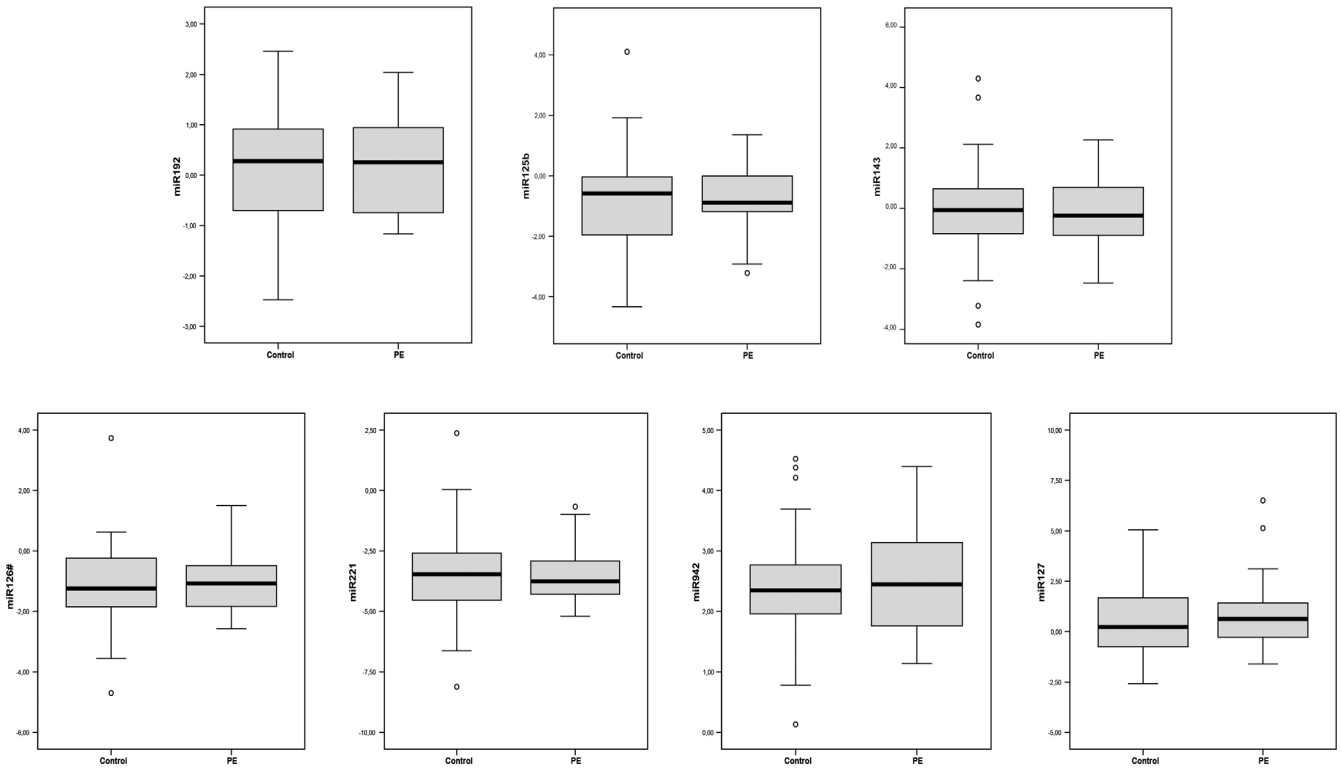

Figure 4 | First-trimester circulating miRNAs in patients with preeclampsia versus uncomplicated pregnancies. Circulating levels of miR-192, miR-125b, miR-143, miR-126#, miR-221, miR-942 and miR-127, selected according the OpenArray results outcome, obtained from first-trimester-analyzed preeclampsia patients (PE; n = 31) and healthy pregnancies (control; n = 44) and measured by real-time quantitative stem-loop RT-PCR. Serum miRNA levels were calculated by the $2^{-\Delta\Delta CT}$ method. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. Upper and lower horizontal bars denote the 90th and 10th percentiles. The values are normalized to cel-miR-54 and shown as relative expression at y-axis. The first-trimester serum levels of all miRNAs analyzed were not significantly different between preeclampsia and control groups.

functional pathophysiological relevance of circulating miRNAs during the premature phase of the disease.

Methods

Study population. This nested case-control study was performed at the Department of Maternal-Fetal Medicine (Hospital Clínic de Barcelona) and at the Molecular Genetics Lab (IDIBELL). Maternal serum samples were obtained from a prospective cohort composed of 5,759 unselected singleton pregnancies referring for routine first-trimester screening for aneuploidies (11 + 0-13 + 6 weeks of gestation) between May 2009 and October 2011. Cases corresponded to women subsequently developing preeclampsia and requiring delivery before 34 weeks of gestation (defined as early preeclampsia). Controls were women referring in the same study period who had normotensive pregnancies uncomplicated by proteinuria. The Clinical Research Ethics Committee of the Hospital Clínic de Barcelona (CEIC Hospital Clínic) approved the study protocol and each patient provided written informed consent.

Preeclampsia was defined as systolic blood pressure (BP) $\geq$ 140 mmHg and/or diastolic BP $\geq$ 90 mmHg on at least two occasions 4 hours apart, developing after 20 weeks of gestation in a previously normotensive women, and proteinuria $>$ 300 mg in a 24-hour urine specimen[56].

Exclusion criteria for both study groups were pregnancies with aneuploidies, major fetal abnormalities, and those ending in termination, miscarriage or fetal death before 24 weeks of gestation. Gestational age in all pregnancies was calculated based on the crown-rump length at first-trimester ultrasound[57]. Medical records were used to obtain information on risk factors, pregnancy history, and perinatal outcome. Data on maternal mean arterial pressure (MAP) and mean uterine artery (UtA) Doppler pulsatility index (PI) measured at first-trimester screening were also recorded. Small for gestational age (SGA) newborns were defined with birth weight below the 10th centile according to local standards[58]. All experiments were carried out in accordance with the approved guidelines and regulations, in line with the tenets of the Declaration of Helsinki.

Sample collection and miRNA extraction. Thirty-one cases of early preeclampsia and 44 controls were used in the study. The serum fractions were obtained according to standard protocols. Briefly, the blood samples were collected in EDTA-containing tubes, processed within one hour by centrifugation at 2,000 g for 10 min at 4°C, and the supernatants were quickly removed, aliquoted, and stored immediately at −80°C. For analysis, serum samples were thawed on ice and centrifuged at 3,000 g for 5 min, avoiding the presence of traces of red blood cells and other cellular debris susceptible to affect the microRNA profile. RNA was isolated from 200 µl of serum using the miRNeasy Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's protocol, and eluted with 50 µl of nuclease-free water.

miRNA Profiling on high-throughput OpenArray™ system. The TaqMan® OpenArray® Human MicroRNA Panel (Applied Biosystems, Foster City, CA, USA) is a complete set of assays represented in Megaplex pools A and B. A total of 758 unique nanoliter PCR assays per sample, for 754 well-characterized human miRNAs (Sanger miRBase v14) and 4 controls, are arranged on the OpenArray plate such that 3 samples can be interrogated on a single nanoplate. The assays are arranged so that those from different pools are located in different subarrays with one replicate of each control assay present in every subarray[59].

Megaplex™ RT Primers, Human Pool A v2.1 and Human Pool B v3.0 (Applied Biosystems) were used both for reverse transcription and for pre-amplification, according to the manufacturer's miRNA profiling workflow optimized for low sample input. Briefly, reverse transcription (RT) (3 µl RNA/sample) was performed using the TaqMan MicroRNA RT kit. The cDNA samples underwent unbiased PCR pre-amplification using the TaqMan PreAmp Master Mix, and finally were PCR amplified by incubation with the TaqMan OpenArray Real-Time PCR Master Mix onto the through-hole nanofluidic OpenArray plate. The plates were processed in the UPF Genomics Core Facility from Pompeu Fabra University (UPF, Barcelona).

OpenArray data analysis. OpenArray Real-Time qPCR Analysis Software (Applied Biosystems) was used to analyze and review the amplification plots and threshold cycle (Ct) values obtained. In some cases, manual adjustments to the baseline parameters were made to individual assays. miRNA data analysis was performed with DataAssist™ Software (Applied Biosystems) through global mean normalization of all miRNAs that had Ct values lower than 31. The Benjamini-Hochberg procedure for multiple testing correction was applied to calculate the False Discovery Rate (FDR) for each of the *p*-values obtained.

Validation of OpenArray results. A fixed volume of 5 µl RNA per individual sample was reverse transcribed using the TaqMan MicroRNA RT kit and 2 µl of miRNA-specific stem-loop primers (Applied BioSystems) in a small-scale RT reaction [comprised of 1.10 µl of nuclease-free water, 1 µl of 10× of RT Buffer, 0.13 µl of

RNase-Inhibitor (20 units/ml), 0.10 μl of 100 mM dNTPs and 0.67 μl of Multiscribe Reverse Transcriptase], using a 9700 PCR thermocycler system (Applied BioSystems). To enhance sensitivity, a 2.5 μl aliquot of undiluted RT product was combined with 7.5 μl of pre-amplification solution [5 μl of TaqMan PreAmp Master Mix and 2.5 μl of TaqMan miRNA Assay] and amplified in the 9700 PCR thermocycler system (Applied Biosystems) by heating to 95°C for 10 min, followed by 10 cycles of 95°C for 15 s and 60°C for 4 min.

Finally, 2.5 μl of diluted (1 : 5) pre-amplified product was combined with 5 μl of TaqMan Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems), 0.5 μl of TaqMan microRNA Assay 20× and 2 μl of nuclease-free water in a 10 μl PCR reaction. Real-time PCR was carried out on an Applied BioSystems 7900 HT thermocycler (50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Data were analyzed with SDS RQ Manager 1.2.1 and DataAssist™ Software (Applied BioSystems). The amplification curves were individually inspected and miRNAs with abnormal amplification patterns were removed from analysis. Normalization of sample-to-sample variation in RNA isolation was approached including the synthetic *C. elegans* miRNA *cel-miR-54* (Integrated DNA Technologies, Coralville, IA, USA) to each denatured serum sample (after the addition of QIAzol Lysis Reagent in the initial RNA extraction procedure) to avoid its degradation by endogenous serum RNases. The spiked-in *cel-miR-54* (5 fmol) was measured concomitantly to the miRNAs of interest. All reactions were run in triplicate. Data normalization across samples was performed using a median normalization procedure. The Ct values were determined using the fixed threshold settings. For each sample, the relative abundance of target miRNAs were determined by the equation $2^{-\Delta Ct}$, in which $\Delta Ct = Ct_{sample} - Ct_{cel\text{-}miR\text{-}54}$. ΔΔCt was then calculated by subtracting the ΔCt of controls from the ΔCt of cases. The fold change in miRNA abundance was calculated with the equation $2^{-\Delta\Delta Ct}$. Detailed information regarding the miRNAs employed in this study is provided in Supplementary Table S1 online.

Statistical analysis. Comparisons between the clinico-pathological parameters of preeclampsia and control groups were carried out using one-way ANOVA for numerical data and Chi-Square for categorical data. Comparisons between cases versus controls in the validation step were analyzed using the Mann-Whitney U-test for unpaired data. The relationships of the miRNAs with the preeclampsia patient clinical characteristics were analyzed by Spearman's rank correlation or by Point-Biserial correlation, where appropriate. All data analyses were performed using the SPSS software version 15.0 (SPSS 15.0, SPSS Inc., Chicago, IL, USA). All tests were two-tailed and $p < 0.05$ was considered statistically significant.

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Author contributions

A.L. and A.F. performed experimental work; Fr.C., Fa.C. and F.F. performed clinical work; E.G. and J.M.A. designed and co-directed research. All authors reviewed the manuscript.

Additional information

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