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Pre-eclampsia predictive ability of maternal miR-125b: a clinical and experimental study.

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Running head: miR-125b and pre-eclampsia onset

Abbreviations:

PE: pre-eclampsia;

Trop-2: Trophoblast cell surface antigen-2;

miR-125b: microRNA-125b.

Abstract

Pre-eclampsia (PE) is a systemic maternal syndrome affecting 2-8% of pregnancies worldwide and involving poor placental perfusion and impaired blood supply to the foetus. It manifests after the 20th week of pregnancy as new-onset hypertension and substantial proteinuria and is responsible for severe maternal and newborn morbidity and mortality. Identifying biomarkers that predict PE onset prior to its establishment would critically help treatment and attenuate outcome severity. MicroRNAs are ubiquitous gene expression modulators found in blood and tissues. Trophoblast cell surface antigen (Trop)-2 promotes cell growth and is involved in several cancers. We assessed the PE predictive ability of maternal miR-125b in the first trimester of pregnancy by measuring its plasma levels in women with normal pregnancies and with pregnancies complicated by PE on the 12th week of gestation. To gain insight into PE pathogenesis we investigated whether Trop-2 is targeted by miR-125b in placental tissue. Data analysis demonstrated a significant association between plasma miR-125b levels and PE, which together with maternal body mass index before pregnancy provided a predictive model with an area under the curve of 0.85 (95% confidence interval, 0.70-1.00). We also found that Trop-2 is a target of miR-125b in placental cells; its localization in the basal part of the syncytiotrophoblast plasma membrane suggests a role for it in the early onset of PE. Altogether, maternal miR-125b proved a promising early biomarker of PE, suggesting that it may be involved in placental development through its action on Trop-2 well before the clinical manifestations of PE.

Keywords: miR-125b, trophoblast, pre-eclampsia, hypoxia, epigenetic changes, biomarker, placenta, maternal plasma.

Introduction

PE is a maternal pregnancy disorder arising after the 20th week of gestation and characterized by new-onset hypertension and substantial proteinuria. It involves multiple organs including kidney, liver and brain (1-4) and affects 2–8% of pregnancies worldwide, causing severe maternal and newborn morbidity and mortality (5,6). According to the "two-stage model" of PE, inadequate trophoblast invasion of the placental bed in early pregnancy results in insufficient remodelling of uteroplacental spiral arteries (first stage of PE) (7). Poor placental perfusion may become clinically relevant at different times of gestation by affecting the blood supply to the foetal-placental unit; oxidative stress then induces the release into the maternal circulation of factors like Flt-1 and s-Endoglin, which induce the clinical features of PE, where hypertension (blood pressure ≥ 140/90 mm Hg) and proteinuria (≥ 300 mg in 24 h) combine with clotting and liver dysfunction (second stage of PE) (8-11). Since a normal syncytiotrophoblast is critical for the establishment and maintenance of a healthy pregnancy, its impaired function directly affects foetal development and maternal adaptation to pregnancy. Syncytiotrophoblast necrosis, an extreme condition, involves a significantly higher release of syncytiotrophoblast extracellular vesicles into the maternal circulation, particularly in early-onset PE (12,15). However, the key mechanisms underlying placental pathophysiology remain to be elucidated. Investigation of the molecules regulating placental adaptation processes is therefore critical to gain a greater understanding of PE pathogenesis.

MicroRNAs (miRNAs), small non-coding RNAs that regulate the translation and degradation of specific mRNA targets, play a critical role in cellular functions such as proliferation, apoptosis, death, stress response, differentiation and development (16,18). Dysregulation of tissue and/or circulating miRNAs has been described in a variety of disorders (16-18). At least 500 miRNAs are known to be expressed in placental trophoblasts (19,20). After the early study by Pineles et al. in 2007 (21), mounting evidence has been suggesting a potential association between some miRNAs and PE pathogenesis (21-26). Although several miRNAs have been tested for their ability to predict PE onset (27-31), results have been inconclusive.

Among the miRNAs implicated in pregnancy disorders (32), miR-125b is especially interesting because it is involved in angiogenesis (33) and cell migration/remodelling (34) and is highly conserved in several species (35).

Trophoblast cell surface antigen (Trop)-2, also known as tumour-associated calcium signal transducer (TACSTD2), is a 35-49 kDa transmembrane glycoprotein (36) involved in the regulation of cell-cell adhesion, cell proliferation and maintenance of basement membrane integrity (37-39); it can function as a cell surface receptor for specific ligands to increase intracellular calcium levels (39) and plays a role in cell growth (38,40). Trop-2 was first identified by Lipinski et al. (41) as a marker of trophoblast and choriocarcinoma cell lines and has since been described as a major tumorigenic factor in several cancers (42-47). Interestingly, Trop-2 mRNA has been identified as a miR-125b target in breast, head and neck, and urothelial cancer (48-50), whereas no data are available for placental tissue.

Based on these considerations, a clinical, biomolecular and morphological study was devised i) to evaluate the ability of maternal miR-125b plasma levels measured at 12 weeks of pregnancy to predict PE onset; ii) to investigate whether Trop-2 protein is a miR-125b target in placental tissue; and iii) to elucidate the functional role of miR-125b in modulating Trop-2 in human trophoblasts.

Materials and methods

Study design

The participants who provided the blood samples were prospectively recruited from women attending a routine antenatal care visit at Obstetrics and Gynaecology outpatient clinic of Hospital 'S. Maria della Misericordia' (University of Perugia, Italy) from 2014 to 2016 and followed until delivery (Table 1).

This is a case-control study nested in a cohort study where 158 plasma samples from women with healthy pregnancies (n=144) or with pregnancy complicated by PE (n=14) had been analysed. The present case-control study involved 31 samples, 18 from women with healthy pregnancies and 13 from gestational age-matched women whose pregnancy was complicated by PE (the 14th sample was no longer available because it had been used for other tests).

Placenta samples were collected from 30 women, 20 with a healthy pregnancy and 10 whose pregnancy was complicated by PE. The former 20 samples were collected from women who had had voluntary terminations in the 1st trimester of gestation (n=10, Gynaecology and Obstetrics Unit, Department of Clinical Sciences, Università Politecnica delle Marche, Ancona, Italy) or who had had an uneventful term delivery (n=10, Department of Obstetrics and Gynaecology, Catholic University of the Sacred Heart, Roma, Italy). The 10 samples from pregnancies complicated by PE were collected at delivery at the Department of Obstetrics and Gynaecology, Catholic University of the Sacred Heart (Table 1).

The eligibility criteria were those described by Gesuita et al. (51). Briefly, women aged 18 to 45 years with a singleton pregnancy and no genetic diseases (*e.g.* aneuploidy) who gave their written informed consent to participate were considered eligible. Exclusion criteria were multiparity, multiple gestation, a history of hypertension, renal or cardiac disease, diabetes mellitus, thyroid and immune diseases and congenital or acquired thrombophilia.

Baseline demographics and information on the mother's medical (including obstetric) history and current and earlier lifestyle factors (smoking, diet and physical activity) was collected through an interview. The body mass index (BMI) was calculated based on the data obtained during the interview. The gestational age was calculated from the last menstrual period and was confirmed by ultrasound crown-rump-length measurement.

Newborn data included gender, health and birth weight. Placenta samples were not collected from stillbirths or from live births where the newborn suffered from chromosomal or other foetal abnormalities.

The study was approved by the institutional Ethics Board of the University of Perugia.

Healthy pregnancies were normotensive pregnancies with normal uterine and umbilical Doppler flow velocimetry during gestation and where the foetus was appropriate for the gestational age (newborns $\geq 10^{th} \leq 90^{th}$ percentile for gender and gestational age according to Italian charts [52]).

PE was diagnosed after the 20th week based on two blood pressure readings ≥ 140/90 mmHg taken at least 4 h apart and on proteinuria ≥ 300 mg in 24 h (or 2 dipstick readings of at least +2 of midstream or catheter urine specimens if 24-hour urine collection was not available) in previously normotensive patients (1).

Sample collection

Blood samples for the clinical study

Venous blood was collected on the 12th week of pregnancy in EDTA-containing VACUETTE® tubes and centrifuged at 1500 g for 15 min at 4 °C. Plasma was aliquoted in 500 µl tubes and stored at -80 °C until processing within 2 years of collection. To avoid bias in miRNA dosage, samples were thawed only once.

Placenta samples for the biomolecular and morphological study

Six tissue blocks were collected from each placental sample. Three blocks were immediately frozen at -80 °C for use in Western blot analysis; the other 3 blocks were immediately fixed in 4% neutral buffered formalin for 8-12 h at 4 °C and paraffin-embedded at a temperature not exceeding 56 °C (44), to obtain sections for immunohistochemistry (2 µm), immunofluorescence and RT-PCR analysis of miR-125b (10 µm).

Analysis of tissue and plasma miR-125b for the clinical study

MiRNA-125b was measured in placental samples and plasma using Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada). Synthetic *Caenorhabditis elegans* cel-miR-39 (Spike-In Kit, Norgen Biotek Corp.) was spiked into 100 µl of plasma before RNA extraction. Total RNA was stored at -80 °C until use. Reverse transcription was performed using TaqMan microRNA Reverse Transcription kit a miR-125b-specific probe (cat. # 4427975 Assay ID 000449, both from Applied Biosystem, Life Technologies, Monza, Italy). Normalization of miR-125b values in placenta and plasma was also performed by reverse transcription with primers for RNU48 (cat. # 4427975 Assay ID 001006), a constituent siRNA, and for cel-miR-39 (cat. # 4427975 Assay ID 000200, both from Applied Biosystem, Life Technologies), respectively. The conditions for reverse transcription were as follows: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. The temperature was then reduced to 4 °C.

MiRNA expression was quantified using TaqMan Fast Universal PCR Master Mix and probes for miR-125b, RNU48, and cel-miR-39 (all from Applied Biosystem, Life Technologies). The RT-PCR reaction conditions involved one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The threshold cycle (Ct) value was defined as the cycle number at which the signal exceeded a predetermined

threshold. The relative amount of miR-125b to RNU48 and to cel-miR-39 was calculated by the equation $2^{-\Delta Ct}$, where ΔCt = Ct miR – 125b – Ct RNU48 (placenta) and ΔCt = Ct miR-125b – Ct cel-miR-39 (plasma) (28).

Statistical analysis for the clinical study

MiR-125b expression was analysed after log transformation. We used a non-parametric approach, because the Shapiro test demonstrated that the quantitative variables had a non-normal distribution. The quantitative variables were summarized using median and interquartile range (IQR, 1^{st}-3^{rd} quartiles), respectively, as a measure of centrality and variability; the qualitative variables were expressed as absolute and percent frequency. Between-group comparisons were performed using Wilcoxon's rank sum test and Fisher's exact test for quantitative and qualitative variables, respectively.

Multiple logistic regression was applied to estimate the independent effect of women' characteristics and log miR-125b on the probability of developing PE, and 95% confidence intervals (95% CI) were calculated. The most parsimonious model was obtained by including the variables that were significant on the likelihood ratio test. The model's goodness of fit was assessed with the Hosmer-Lemeshow test. The accuracy of the model in predicting PE was analysed using a ROC plot, the Area Under the Curve (AUC) and 95% CIs. The R statistical program was used for the analyses; a probability of 0.05 was set as the threshold for statistical significance.

In vitro *cultures to establish whether placental Trop-2 protein is a miR-125b target*

To investigate Trop-2 localization, the first-trimester cytotrophoblast cell line HTR-8/SVneo (kindly provided by C. H. Graham, Queen's University, Kingston, ON, Canada) and the BeWo human placental cell line derived from choriocarcinoma (kindly provided by S. Alberti, Laboratory of Cancer Pathology, CeSI-MeT, University 'G. d'Annunzio', Chieti, Italy) were used as *in vitro* cytotrophoblast and syncytiotrophoblast models, respectively (53).

HTR-8/SVneo cells were cultured in RPMI-1640 medium (Euroclone S.p.A., Pero, Italy) with 10% foetal bovine serum (FBS; Gibco, Life Technologies, Waltham, MA, USA), 1% penicillin, 1% streptomycin, 1% L-glutamine at 37 °C, 20% O_2 and 5% CO_2. BeWo cells were cultured in Dulbecco's Modified Eagle

Medium (DMEM) and Ham's F12 (both from Euroclone S.p.A.) 1:1 supplemented with 10% FBS, 1% penicillin, 1% streptomycin at 37 °C, 20% O_2 and 5% CO_2.

The BeWo cell line was used as a model to study Trop-2 expression in hypoxic conditions, to mimic PE. To mimic hypoxia, BeWo cells were incubated at 37 °C with 3% O_2, 92% N_2 and 5% CO_2 for 48 h. To demonstrate whether Trop-2 is a miR-125b target, BeWo cells ($2.5x10^5$) incubated at 37 °C with 20% O_2 and 5% CO_2 were seeded in 6-well plates and transfected with a complex containing X-tremeGene 9 DNA Transfection Reagent (Roche Applied Science, Penzberg, Germany) and mirVana miRNA mimic (cat. # MC10148 MIMAT0000423) or mirVana miRNA inhibitor (cat. # MH10148 MIMAT0000423, both from Ambion, Life Technologies, Monza, Italy). The ratio of transfecting agent to mimic or inhibitor was 6:1; the final oligonucleotide concentration was 30 nM. Cells were incubated for 48 h. The experiments were performed 3 times with two biological replicates each time. MG63 cells and placental tissue from women in the 3rd trimester of gestation were used as positive controls for Trop-2 (54).

Western blot analysis of Trop-2 in HTR-8/SVneo and BeWo cells

For protein extraction, cell pellets were collected and incubated with lysis buffer in ice for 30 min and centrifuged at 14,000 g for 20 min at 4 °C. Supernatants were aliquoted and stored at -80 °C until use. Protein concentration was determined by the Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Milano, Italy). Protein samples (50 µg each) were fractionated in 15% SDS-PAGE gel and electrophoretically transferred to Trans-Blot Turbo Mini Nitrocellulose membranes with Trans-Blot Turbo Transfer System (all from Bio-Rad Laboratories).

Membranes were incubated with 5% milk in distilled water to block non-specific sites and then with monoclonal mouse Trop-2 antibody (Table 2) in TBS with 0.05% Tween 20 (TBS-T) at 4 °C overnight. After washing with TBS-T, membranes were incubated with the secondary anti-mouse antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) at 1:1500 dilution. Antibody binding was detected with Clarity Western ECL Substrate and images were acquired with Chemidoc (both from Bio-Rad Laboratories). Densitometric analysis was performed with ImageJ software (https://imagej.nih.gov/ij/download.html). The housekeeping genes *GAPDH* and β-actin were used to

normalize cell and tissue values, respectively (see antibodies in Table 2). HTR-8/SVneo cells were negative for Trop-2.

Trop-2 immunohistochemistry and immunofluorescence in placental tissue

Trop-2 localization in normal and PE samples from 1st- and 3rd-trimester placenta was investigated by immunohistochemistry.

Paraffin sections were deparaffinized and rehydrated with xylene and a graded ethyl alcohol series. To inhibit endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide in methanol and treated with 0.3% Tween 20 in phosphate-buffered saline (PBS) 1X for 20 min at room temperature for antigen retrieval. To block non-specific background, sections were incubated with normal horse serum (Vector Laboratories, Burlingame, CA, USA) diluted 1:75 in PBS. Sections were incubated with mouse monoclonal Trop-2 antibody (Table 2) overnight at 4 °C. After washing in PBS, sections were incubated with horse anti-mouse biotinylated antibody diluted 1:200 (Vector Laboratories) for 30 min. The avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories) was performed using 3',3'-diaminobenzidine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) as the chromogen. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted with Eukitt solution (Kindler GmbH and Co., Freiburg, Germany). Negative controls involved omitting the primary antibody; normal human skin was the positive control (55,56).

Trop-2 protein localization was investigated by immunofluorescence and confocal microscopy. Two double reactions were performed using Bcl-2 as a syncytiotrophoblast (57) and Trop-2 marker and RKIP as a cytotrophoblast (58) and Trop-2 marker, to establish whether Trop-2 is localized in one or the other cell type.

Tissue samples were deparaffinized and hydrated with xylene and a graded alcohol series. To reduce autofluorescence, samples were incubated with 0.1% Sudan Black B (Sigma-Aldrich) in 70% ethanol for 30 min then washed with PBS 1X with 0.03% Tween 20. In double-staining reactions, sections for antigen retrieval were incubated i) with Tris-EDTA buffer pH 9.0 at 95 °C for 30 min (Trop-2/Bcl-2) or ii) with 0.3% Tween 20 in PBS for 20 min at room temperature (Trop-2/RKIP). Sections were then washed twice

with PBS 1X. Non-specific sites were blocked with 3% bovine serum albumin in PBS 1X for 30 min, then sections were incubated with mouse monoclonal Trop-2 antibody (Table 2) overnight at 4 °C. Sections were washed and incubated with anti-mouse secondary antibody (Alexa Fluor® 594, 1:400 dilution; Invitrogen, Carlsbad, CA, USA). After washing, they were incubated with mouse monoclonal Bcl-2 antibody as a syncytiotrophoblast marker (57) or with RKIP as a cytotrophoblast marker (58), both overnight at 4 °C (Table 2). Samples were washed and incubated with donkey anti-mouse (Bcl-2) or donkey anti-rabbit (RKIP) Alexa Fluor® 488 secondary antibody (1:400 dilution, Invitrogen) for 30 min. Slides were then incubated with TO-PRO-3® Iodide (1:3000 dilution, Invitrogen) for 10 min for nuclear staining, washed and mounted onto glass slides using Vectashield mounting medium (Vector Laboratories). Negative controls were performed by omitting the primary antibody.

Sections were analysed with a motorized Leica DM6000 microscope (Leica Microsystems srl, Milano, Italy) at different magnifications. Fluorescence was detected with a Leica TCS-SL spectral confocal microscope equipped with an Argon and a He/Ne mixed gas laser. Fluorophores were excited with the 488, 543 and 649 nm lines and imaged separately. Images (1024 × 1024 pixels) were obtained sequentially from two channels using a confocal pinhole of 1.1200 and stored as TIFF files.

Western blot analysis of placental tissue sections

For protein extraction, 1st- and 3rd-trimester placenta samples from healthy pregnancies and pregnancies complicated by PE were homogenized using an Ultra-Turrax T8 apparatus (IKA-WERKE, Lille, France) in lysis buffer containing PBS 1X, 0.1% SDS, 1% NONIDET-P40, 1 mM orthovanadate sodium, 12 mM deoxycholate sodium, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1.7 µg/ml aprotinin and centrifuged at 16,000 g for 20 min at 4 °C. Pellets were discarded and supernatants were collected and stored at -80 °C until use. Western blot analysis was performed as described above.

Analysis of tissue miR-125b for the biomolecular and morphological study

Total RNA was extracted from formalin-fixed paraffin-embedded (FFPE) samples of 1st- and 3rd-trimester placenta from healthy and PE pregnancies using FFPE RNA/DNA Purification Kit (Norgen Biotek Corp.) according to the manufacturer's protocol. RT-PCR and miR-125b quantification were as described above.

Statistical analysis for the biomolecular and morphological study

Trop-2 levels were reported as mean ± standard deviation (SD) and represented as histograms. MiR-125b levels were analysed after log transformation. Median and IQR were used respectively as a measure of centrality and variability and graphically represented as boxplots. The Mann-Whitney test was applied to between-group comparisons and the Kruskal-Wallis test to comparisons among three groups.

GraphPad Prism 7 (https://www.graphpad.com/scientific-software/prism/) was used for the analyses; a probability of 0.05 was set as the threshold for statistical significance.

Results

Clinical study: PE prediction model based on miR-125b levels

The demographics, lifestyle factors, BMI values and log miRNA-125b plasma levels of women with a normal pregnancy and of those whose pregnancy was complicated by PE are reported in Table 3. Whereas healthy women and PE cases were similar in terms of age, smoking and dietary habits, physical activity, gestational age at delivery and newborn birth weight, PE cases had a significantly higher BMI before pregnancy and significantly higher log miR-125b levels at 12 weeks. The multiple logistic regression analysis (Table 4) showed that PE was significantly associated with log miR-125b (relative expression), maternal age and BMI before pregnancy. In particular, the likelihood of developing PE more than doubled with every additional log miR-125b unit and it increased by about 24% with every additional BMI unit.

The ROC plot showed that the model displayed a good accuracy in predicting PE, as demonstrated by an AUC of 0.85 (95%CI: 0.70-1.00) (Figure 1).

Trop-2 is a miR-125b target, as demonstrated by an in vitro *placental model*

An *in vitro* approach was devised to document whether Trop-2 is targeted by miR-125b. HTR8/SV-neo cells, used as a cytotrophoblast model, were negative for Trop-2 (Figure 2A), indicating that the protein is not found in villous cytotrophoblasts. Forskolin-induced syncytialization (58) demonstrated that BeWo cells could be used as an *in vitro* syncytiotrophoblast model and, as expected, they were positive for Trop-2 (Figure 2B). Syncytialized BeWo cells cultured in hypoxic conditions (3% O_2) to mimic the PE environment demonstrated significant (p=0.03) Trop-2 downregulation compared with control cells cultured

in normal atmospheric conditions (20% O_2) (Figure 2C; quantification in Figure 2D). Their transfection with the miR-125b mimic also induced significant (p=0.0005) Trop-2 downregulation, as demonstrated by the Western blots (Figure 2D; quantification in Figure 2F), thus confirming that Trop-2 is targeted by miR-125b.

Trop-2 protein, a miR-12b target, is expressed in normal and PE placenta, as demonstrated by biomolecular and morphological analyses

Immunohistochemical analysis demonstrated Trop-2 protein (brown staining in Figure 3,A-F) in the syncytiotrophoblast basal plasma membrane in 1st-trimester (Figure 3A,B) and 3rd-trimester (Figure 3C,D) normal and PE placenta (Figure 3E,F). Trop-2 localization in the syncytiotrophoblast was demonstrated by double immunofluorescence in first trimester placenta (Figure 4A e 4B). In fact, yellow staining (merged image, yellow arrows) showed that Trop-2 (red) co-localized with the syncytiotrophoblast marker Bcl-2 (green) (Figure 4A), whereas the absence of yellow staining demonstrated that Trop-2 (red) did not co-localize with the cytotrophoblast marker RKIP (green) (Figure 4B).

Western blot analysis demonstrated a significant increase in Trop-2 from the 1st to the 3rd trimester in placenta from normal pregnancies (Figure 5A) and a significantly decreased in gestational age-matched PE placenta (Figure 5B).

MiR-125b is expressed in normal and PE placental tissue, as demonstrated by biomolecular analysis

From the 1st to the 3rd trimester, miR-125b levels in normal placenta (Figure 6A) showed a significantly greater increase compared to gestational age-matched PE placenta (Figure 6B).

Discussion

This study describes how an early PE prediction model based on plasma miR-125b levels, maternal age and BMI before pregnancy was developed and tested. Since the experimental work also established that Trop-2 is a miR-125b target, we also found that miR-125b may be involved in PE development through modulation of Trop-2 expression in the syncytiotrophoblast. Our findings therefore have clear clinical implications.

Although the key pathological changes related to PE arise well before the 20th week of gestation, its clinical symptoms do not manifest before this time (1). From a clinical point of view, pregnant women who are at high risk of developing PE seem to benefit from prophylactic treatment; for instance, low-dose aspirin administered before the 16th week has been seen to reduce the risk and attenuate the severity of the maternal and foetal outcomes, not to mention the social, economic and healthcare impacts of PE (60,61).

Early detection would of course be more effective. However, although several promising predictors have been identified – *e.g.* uterine artery ultrasonography, maternal serum/urinary levels of human chorionic gonadotropin, inhibin A and activin A, pregnancy-associated plasma protein A, sex hormone-binding globulin, placental growth factor, soluble fms-like tyrosine kinase 1 and serum placental protein 13 (62-65) – PE screening in early pregnancy is still poorly effective. Several stable miRNAs with important regulatory roles in proliferation, apoptosis and cell-cell communication have been identified and quantified during pregnancy (27-29,66-79). Some are expressed in the 3rd trimester whereas for some miRNAs identified in the 1st trimester a target protein has not yet been identified. MiR-125b overexpression in the 1st trimester has been reported in women who went on to develop PE, as also documented in our study. The results of randomized controlled trials of a number of biomarkers, tested to identify high-risk women, have also been disappointing, possibly because the sensitivity of most of them is highest in the 2nd trimester, long after PE has become clinically manifest. A recent study of the ability of a miRNA panel to predict PE (27) has found that none showed a good performance (*i.e.* greater than 0.70). Some studies have denied a PE predictive value for miRNAs in 1st trimester maternal serum/plasma. However, the population investigated by Luque et al (80) included only early PE and no late PE cases; other studies evaluated miR-125b only in the 3rd trimester (81) or compared only two cases to two controls (82,83). However, in 2013 Gu et al. (20)

described miR-125b localization in trophoblasts during gestation (20), whereas two interesting papers have described an association between miR-125b and PE, suggesting a possible role for this miRNA in PE onset (66,67). The present study found significant plasma miR-125b overexpression in 1st-trimester plasma from women who went on to develop PE. Since according to multiple regression analysis BMI before pregnancy and maternal age are associated with PE development, these three factors were entered in a model for PE prediction. Testing of the model demonstrated that it showed a good performance, with an AUC equal to 0.85.

As regards the biomolecular and morphological study, data analysis also demonstrated that miR-125b was underexpressed and Trop-2 was downregulated in PE placenta compared with control samples. These data suggest that maternal miR-125b is involved in Trop-2 modulation in placental tissue. Combined with the *in vitro* demonstration that Trop-2 is targeted by miR-125b, which reduces its expression, our findings suggest that maternal miR-125b overexpression at 12 weeks of gestation could be involved in the early onset of PE through an action on Trop-2 concentrations from the earliest phases of placental development.

We feel that this paper has a number of strengths: i) a complex and rigorous design, involving the prospective collection of 1st-trimester plasma samples, the collection of healthy placenta at two different time points and the collection of gestational-age-matched normal and PE placenta; ii) biomolecular and morphological analyses to establish that placental Trop-2 is targeted by miR-125b and to identify its site of action, which has implications for diagnostic and treatment strategies; and iii) the development of an accurate PE prediction model based on easily measurable parameters, *i.e.* BMI before pregnancy and maternal age.

Its limitations include the facts that we did not investigate other miRNAs that may be involved in Trop-2 modulation; that the overexpression of plasma miR-125b at 12 weeks of gestation in women who will eventually develop PE could be the effect, rather than the cause of PE, with Trop-2 downregulation being the downstream effect; and the limited sample size used to evaluate the prognostic role of miR-125b. However, PE is a rare condition, and the size of the sample was determined by the number of women who developed PE in the cohort study (51) in which this case-control study was nested. Moreover, our analysis

demonstrated a significant and accurate predictive role for miR-125b. Further research is clearly needed to confirm its role and to elucidate the contributions of other differentially expressed miRNAs in the placenta of PE patients; a more powerful, large-scale, multicentre study is required to validate the model.

In conclusion, the practical, non-invasive and efficient screening method to identify women at risk of developing PE described herein can contribute to its prevention and early treatment, whereas the identification of a protein target of miR-125b in placental tissue and its downregulation in PE placenta advances our mechanistic understanding of the complications of PE and provide insights for PE prevention strategies.

Brief Commentary

This is a multidisciplinary study on the role of miR-125b on the development of preeclampsia. This study was undertaken to assess the preeclampsia predictive ability of maternal plasma miR-125b at 12 weeks of gestation and to establish whether miR-125b targets Trophoblast cell surface antigen-2 protein. We propose that maternal plasma miR-125b overexpression at 12 weeks of gestation inhibits placental Trophoblast cell surface antigen-2 protein and can directly favour preeclampsia onset by causing placental maldevelopment.

Translational Significance: These data advance our mechanistic understanding of preeclampsia complications and provide insights to develop new prevention strategies.

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Table 1- Characteristic of placental tissue from healthy pregnancies and pregnancies complicated by pre-eclampsia				
	1st trimester (n=10)	3rd trimester (n=10)	Pre-eclampsia (n=10)	p value
Maternal age (years) (mean±SD)	30.2 ± 7.59	32.6 ± 4.05	36.9 ± 5.25	0.07
Gestational age (weeks) (mean±SD)	8.43 ± 1.95	37.25 ± 3.48	32.71 ± 3.85	0.0001

Table 2- Primary antibodies used

Antibody	Specificity	Catalogue #	Application	Concentration	Source
Mouse mAb	Human Trop-2	sc-376746	IHC, IF, WB	1:50 (IHC, IF) 1:250 (WB)	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Rabbit pAb	Human CD138	PA5-16918	IF	1:100	Thermo Fisher Scientific, Rockford, IL, USA
Mouse mAb	Human Bcl-2	M0887	IF	1:50	Dako, Glostrup, Denmark
Rabbit pAb	Human RKIP	07-137	IF	1:400	Upstate Biotechnology, Lake Placid, NY, USA
Goat pAb	Human β-actin	sc-1616	WB	1:200	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Rabbit pAb	Human GAPDH	A300-641A-T	WB	1:1000	Bethyl Laboratories, Montgomery, TX, USA

mAb: monoclonal antibody; pAb: polyclonal antibody; IHC: immunohistochemistry; IF: immunofluorescence; WB: western blotting.

Table 3. Demographics, lifestyle factors, clinical characteristics and log miR-125b levels in relation to participant health status

Variables	**Healthy women** n=18	**Women with pre-eclampsia** n=13	P
Maternal age (years)[#]	30 (28;35)	33 (31;34)	0.199
Gestational age[#]	39 (38;40)	39 (38;39)	0.367
Smoking (yes/quit)[§]	3 (16.7)	1 (7.7)	0.621
Poor diet[§]	5 (27.8)	4 (30.8)	0.999
Physical activity (yes)[§]	13 (72.2)	7 (53.8)	0.114
BMI before pregnancy[#]	20 (19;22)	23 (21;28)	0.039
log miR-125b[#]	-8.14 (-9.04;-7.88)	-7.16 (-8.27;-6.1)	0.040
Birth weight (gg)[#]	3.175 (2.95;3.5)	3.1 (2.95;3.3)	0.410

BMI: body mass index; # median and interquartile range (1^{st}-3^{rd} quartiles); § absolute and percent frequencies
P values: # Wilcoxon rank sum test; § Fisher's exact test

Table 4. Effect estimate of the factors associated with pre-eclampsia. Results of multiple logistic regression analysis

	Odd ratio	95% confidence interval
log miR-125b	2.17	1.11; 5.35
Maternal age (years)	1.15	0.94; 1.48
BMI before pregnancy (kg/m^2)	1.24	1.02; 1.68

Hosmer and Lemeshow goodness of fit test: χ^2 test with 8 df, χ^2=8.11, p= 0.423 Likelihood ratio test: χ^2 test with 3 df, χ^2=13.01, p=0.004

Figure legends

Figure 1 ROC curve for the predictiveness of pre-eclampsia (black curve=raw data; red curve=smoothed ROC curve). The ROC curve was estimated by entering in the model log miR-125b, BMI before pregnancy and gestational age at delivery (variables with $p < 0.10$). SE: Sensitivity; SP: Specificity; AUC: Area Under Curve; 95%CI: 95% Confidence Interval.

Figure 2 *In vitro* model of Trop-2 protein, a miR-125b target. A) HTR-8/SVneo cells were negative for Trop-2 whereas BeWo cells (B, shown in triplicate) with forskolin-induced syncytialization were positive for Trop-2. The MG63 cell line was used as a positive control. C) Representative Western blot of BeWo cells with forskolin-induced syncytialization cultured at 3% O_2 and 20% O_2 and histogram representing band quantification (D). Representative blot (E) and histogram (F) of BeWo cells treated with forskolin and transfected with miR-125b mimic and negative mimic (*: $p = 0.03$; ***: $p = 0.0005$). CTR: control placental tissue in the 3rd trimester of gestation.

Figure 3 Trop-2 expression in normal placenta and placenta from pregnancies complicated by PE by immunohistochemistry.

Trop-2 expression in 1st-trimester placenta (A, B), 3rd-trimester placenta (C, D) and in placental samples from pregnancies complicated by PE (E,F). The brown Trop-2 staining is localized in the basal part of the syncytiotrophoblast in all samples. B) Note the brown immunostaining of the syncytiotrophoblast between two villous cytotrophoblast cells (*).

Figure 4A Double staining detected by confocal microscopy in a 1st-trimester placental villus showing Trop-2 and Bcl-2 colocalization in the syncytiotrophoblast. Trop-2 stained red whereas Bcl-2, a syncytiotrophoblast marker, stained green. The nuclei are blue. Merge (yellow) shows that Trop-2 and Bcl-2 are colocalized in the basal part of the syncytiotrophoblast (yellow arrows). 100X magnification.

Figure 4B Double staining detected by confocal microscopy in a 1st-trimester placental villus showing no Trop-2/RKIP colocalization. Trop-2 stained red; RKIP, a villous cytotrophoblast marker, stained green (green arrows). Trop-2 and RKIP are not colocalized in the villous cytotrophoblast. Blue arrow points at syncytiotrophoblast nuclei. No yellow staining is detected in Merge. 40X magnification.

Figure 5 Western blot analysis of Trop-2 in placental tissue. A) Trop-2 increased significantly from the 1st to the 3rd trimester; B) Trop-2 protein decreased significantly in PE placenta (*: $p \leq 0.05$; **: $p \leq 0.01$; ***; $p \leq 0.001$). CTR: normal gestational age-matched placenta. PE=pre-eclampsia.

Figure 6 Relative miR-125b expression in 1st- and 3rd-trimester normal placenta and in placenta from pregnancies complicated by PE. A) Boxplot of relative miR-125b expression (RT-PCR) in 1st- and 3rd-trimester placenta showing miR-125b overexpression in placental tissue at term; B) Boxplot of relative miR-125b expression (RT-PCR) in PE placenta showing a significant reduction of miR-125b (**: $p \leq 0.01$; ***: $p \leq 0.001$). CRT: normal gestational age-matched placenta. PE=pre-eclampsia.