



Preeclamptic plasma stimulates the expression of miRNAs, leading to a decrease in endothelin-1 production in endothelial cells



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ABSTRACT

Preeclampsia is a major cause of maternal and fetal morbidity and mortality worldwide. It is a multisystem pregnancy syndrome characterized by general endothelial dysfunction caused mainly by plasma factors and debris in endothelial cells. It is widely accepted that endothelin-1 (ET-1) is involved in the pathophysiology of preeclampsia, and so it is of interest to ascertain whether the ET-1 gene (*EDN1*) can be targeted with tools such as miRNAs. Therefore, we investigated the relationship between the expression of miRNAs that putatively target *EDN1* (and so affect ET-1 levels) in HUVECs incubated with plasma from preeclamptic women. *EDN1* expression and ET-1 levels in HUVECs incubated with plasma from women with preeclampsia were similar to those in plasma from healthy pregnant women. Expression of miRNAs let-7a, -7b, and -7c, and to a lesser degree 125a and 125b, was increased in preeclampsia. Expression of miRNAs of the let-7 family was significantly negatively correlated with ET-1 levels in preeclampsia. Transfection of the preeclampsia cultures with mimic miRNA let-7 decreased ET-1 levels. Our findings show that preeclamptic plasma stimulates the expression of miRNAs in HUVECs, leading to a decrease in ET-1 levels, which suggests that therapeutic miRNAs may aid in the management of preeclampsia.

1. Introduction

Preeclampsia is a multisystem syndrome diagnosed by hypertension after 20 weeks of gestation associated with proteinuria and/or edema [1,2]. It affects approximately 2–8% of all pregnancies and is a major cause of maternal and fetal morbidity and mortality worldwide [3,4]. In preeclampsia, the ischemic placental tissue produces and releases factors and debris into the maternal circulation, leading to the general endothelial dysfunction characteristic of the syndrome, which is responsible for its clinical manifestations [5,6].

Through the incubation of endothelial cells with plasma/serum from preeclamptic women, an *in vitro* model of preeclampsia, this

pathophysiological mechanism can be observed [7–16]. Plasma/serum taken from pregnant women who subsequently developed preeclampsia was shown to impair endothelial function in myometrial vessels [7] and to dysregulate *c-FOS* expression in endothelial cells [8]. In addition, as shown by our group and others, plasma/serum taken from women with clinically verified symptoms of preeclampsia induced alterations in endothelial cells [8–12], including changes in endothelin-1 (ET-1) expression [10,11,14–16], a potent vasoconstrictor peptide which has been extensively studied in preeclampsia [17–21].

Recently, it was demonstrated that microRNAs (miRNAs) are involved in the post-transcriptional regulation of the ET-1 gene (*EDN1*) [22–26]. miRNAs are small (18–24 nucleotides) endogenous noncoding

Abbreviations: BMI, body mass index; c-FOS, FOS proto-oncogene; DBP, diastolic blood pressure; EDN1, endothelin-1 gene; ET-1, endothelin-1; GA, gestational age; HEK293A, human embryonic kidney cell; HP, healthy pregnant; HUVEC, human umbilical vein endothelial cell; miRNA, microRNA; PE, preeclampsia; qPCR, quantitative PCR; RT, reverse transcription; SBP, systolic blood pressure

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single-stranded RNAs that post-transcriptionally regulate gene expression by repressing protein expression [27]. Mounting evidence for alterations in miRNA expression has been found in placental tissue and plasma/serum from preeclamptic women, suggesting a role of miRNAs in preeclampsia [28–32]. However, to our knowledge no study has examined the relationship between miRNA expression and ET-1 in endothelial cells incubated with plasma/serum from preeclamptic women.

In the current study, we investigated the relationship between expression of miRNAs that putatively target *EDN1* and ET-1 levels in endothelial cells incubated with plasma taken from pregnant women who subsequently developed clinically verified preeclampsia. We also investigated circulating ET-1 levels to verify whether the *in vitro* results are found systemically.

2. Materials and methods

2.1. Study population

We recruited two groups of women. One group was derived from a broader observational study in two Brazilian cities, Ribeirão Preto (in the state of São Paulo) and São Luís (in the state of Maranhão), although the women analyzed here came from Ribeirão Preto only. The study was approved by the Institutional Review Board at Ribeirão Preto Medical School, Brazil (reference 4116/2008, approval date November, 11 2008). A total of 1417 pregnant were evaluated at the Hospital das Clínicas Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (HCFMRP-USP) at 20–25 weeks of gestation. Of these women, 17 did not return and another 460 gave birth in other hospitals. From the 940 pregnant women remaining, 30 developed preeclampsia and 45 healthy pregnant women were randomly selected as controls. For this study, we included only 29 samples from women who developed preeclampsia, because one sample was too small (in terms of volume) to perform all the experiments.

The other group of women was recruited from the ambulatory clinic at HCFMRP-USP. That study was also approved by the Institutional Review Board at Ribeirão Preto Medical School, Brazil (reference 4682/2006, approved date June 20, 2006). This group likewise included both preeclamptic (cases) and healthy women (controls).

‘Cases’ are those women analyzed before the onset of clinical symptoms, with a corresponding group of ‘controls’, and the ‘PE’ group are those women analyzed after the onset of clinical symptoms, with a corresponding ‘HP’ group of women with a healthy pregnancy throughout (see Fig. S1).

Both studies were performed following the principles of the Declaration of Helsinki. All subjects gave written informed consent. Exclusion criteria included twin pregnancy, hemostatic abnormalities, chronic hypertension, diabetes mellitus, fetal abnormalities, cancer, and cardiovascular, autoimmune, renal, and hepatic diseases. Preeclampsia was characterized as systolic blood pressure (SBP) ≥ 140 mmHg and diastolic blood pressure (DBP) ≥ 90 mmHg on two occasions more than 4 h apart, occurring after 20 weeks of gestation in a previously normotensive woman, in association with proteinuria (≥ 300 mg/24-h urine) [2].

All maternal venous blood samples were collected in tubes containing heparin. The tubes were rapidly centrifuged (1000g for 3 min) and plasma samples were stored at -80°C .

3. Endothelial cell culture and plasma incubation

The human umbilical vein endothelial cell (HUVEC) line (CRL 2873) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). HUVECs were cultured in DMEM medium (Gibco, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco), 50 $\mu\text{g}/\text{ml}$ penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin and 0.5 $\mu\text{g}/\text{ml}$ amphotericin B (Gibco) at 37°C in an incubator with a 5% CO_2 atmosphere. Cells

were grown in 75-cm^3 tissue culture flasks (Corning, Costar, Netherlands) and after reaching 80% confluence, they were detached from the flask surface by trypsin/EDTA (0.5/0.2 mg/ml in phosphate-buffered saline, PBS) and split at a 1:3 ratio into 25-cm^3 flasks. At passage 3, HUVECs were resuspended in DMEM medium and re-plated in 48-well tissue culture plates (Corning), where they were grown to 80% confluence for plasma incubation. After reaching confluence, the medium was removed and the cells were washed twice in PBS. The cells were then incubated in medium with 20% (v/v) plasma from controls and cases, and from HP and PE women, for 24 h at 37°C , 5% CO_2 . Cells were used for these experiments until passage 8.

4. RNA isolation

Total RNA from HUVECs incubated with plasma from controls, cases, the HP group and the PE group was isolated using the miRNeasy Mini Kit (Qiagen, Leusden, Netherlands) according to the manufacturer’s protocol. Quantification of isolated RNA was performed using a NanoDrop Spectrophotometer (Thermo Scientific, MA, USA), and it was consistently found to be pure.

5. PCR array analysis for mRNA expression

RT² Profiler™ PCR Array Human Endothelial Cell Biology (Qiagen®-PAHS-015ZC) was used to verify the expression of 84 genes related to endothelial cell biology (Table S1), as well as five housekeeping genes. Before gene expression analysis using RT² Profiler PCR Arrays, the RT² RNA QC PCR Array (Qiagen®) was used to assess the quality of isolated RNA, according to the manufacturer’s protocol. The array contains controls for RNA integrity, presence of inhibitors of reverse transcription and PCR amplification, and presence of genomic or other DNA contamination. All RNA samples were consistently found to be intact and pure.

For the PCR array, total RNA was converted into cDNA using the RT² First Strand Kit (Qiagen®), according to the manufacturer’s protocol. qPCR was performed using the RT² SYBR Green qPCR Mastermix (Qiagen®), which contained 1350 μL of SYBR Green Master Mix, 1248 μL of RNase-free water and 102 μL of cDNA (1.5 ng/ μL) from each sample in a final volume of 2700 μL per plate. Thermal cycling was performed under the following conditions: 10 min at 95°C , 40 two-step cycles of 15 s at 95°C , and 60 s at 60°C , and a final step for the dissociation curve. Normalization was performed using the five housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, *RPL0*) in the array. Relative quantification was calculated using the comparative $2(-\Delta\Delta\text{C}_t)$ method [33].

6. Messenger RNA expression by qPCR

To validate the differential findings regarding the expression of messenger RNA (mRNA) in the PCR array in women after the onset of symptoms of preeclampsia, qPCR was performed using the KiCqStart Universal SYBR Green qPCR (Sigma-Aldrich, Poole, UK). For qPCR, the same isolated RNAs used in the PCR array were used as templates using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, ON, Canada), according to the manufacturer’s protocol. Each reaction contained 10 μL KiCqStart SYBR Green qPCR Ready Mix, 300 nM of each primer (forward and reverse), a variable amount of nuclease-free water and 5 μL of cDNA (1.5 ng/ μL), to give a final volume of 20 μL .

The following primers were synthesized by Sigma-Aldrich: *EDN1*, forward 5’CAAGCAGGAAAAGAACTCAG-3’ and reverse 5’CTGGTTTGTCTTAGGTGTTCC-3’; *HPRT1*, forward 5’ATAAGCCAGACTTTGTTGG-3’ and reverse 5’ATAGGACTCCAGATGTTTCC-3’. The *HPRT1* gene was chosen as an endogenous control as it was the gene most stable in the samples from the panel of housekeeping genes in the RT² RNA QC PCR Array (Qiagen®). Thermal cycling was performed under the following conditions: 10 min at 95°C , 40 two-step cycles of 15 s at 95°C and 60 s

at 60 °C, and a final step for the dissociation curve for both primers. Relative quantification was calculated using the comparative 2(-Delta Delta C(T)) method [33]. All PCR reactions were performed in duplicate for each sample.

7. miRNA selection

We initially selected miRNAs to target the mRNA of the *EDN1* gene (Table S2) based on bioinformatics analysis using two databases (Mi-crona.org [34] and miRWalk 2.0 [35]). This selection was refined using two sub-criteria:

1%2 miRNAs expressed in the human umbilical vein endothelial cell line (HUVEC) [36].

2%2 miRNAs associated with preeclampsia [29,37,38].

Based on these criteria, the following miRNAs were selected: let-7a, let-7b, let-7c, miR-1, miR-125a, miR-125b, miR-135a, miR-144 and miR-199.

8. miRNA expression by qPCR

Reverse transcription (RT) was performed using the miScript II RT Kit (Qiagen®), according to the manufacturer's protocol. The reaction was performed from total isolated RNA using miScript HiSpec Buffer, which selectively converts mature miRNAs and certain small nucleolar RNAs and small nuclear RNAs into cDNA. For qPCR, the miScript SYBR® Green PCR Kit (Qiagen®) was used and each reaction contained 10 µL of QuantiTect SYBR Green PCR Master Mix, 2 µL of universal primer, 2 µL of each primer of miRNA, 4 µL of nuclease-free water and 2 µL of cDNA (1.5 ng/µL), in a final volume of 20 µL. The primers of the selected miRNAs were obtained from Qiagen®. Thermal cycling was performed under the following conditions: 15 min at 95 °C, 40 three-step cycles of 15 s at 94 °C, 30 s at 55 °C and 30 s at 70 °C, and a final step for the dissociation curve. Relative quantification was calculated using the comparative 2(-Delta Delta C(T)) [33] and normalization was performed to U6 snRNA. In all PCR reactions were performed in duplicate for each sample.

9. Transfection with miRNA mimics

HUVECs were seeded at a density of 2×10^5 cells per well in a 6-well plate and, after reaching 80% confluence, transfected with miRNA mimic let-7b or miRNA mimic negative control #1 (Ambion, CA, USA) (30 pmol) for 12 h, using Lipofectamine®2000 Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The mimic negative control was a random miRNA sequence with no known homology to the human transcriptome. After transfection, cells were allowed to incubate for 12 h at 37 °C in an incubator with a 5% CO₂ atmosphere, followed by RNA isolation and measurement of ET-1 in the supernatants. Cells were used for these experiments until passage 8.

10. Measurement of ET-1 concentrations

The ET-1 concentrations in the HUVECs culture supernatants and plasma samples were measured using enzyme-linked immunosorbent assay kit Quantikine® ELISA Endothelin-1 (R&D Systems, MN, USA), according to the manufacturer's protocol.

10.1. Statistical analysis

Clinical characteristics and ET-1 levels were compared between preeclampsia groups and their respective controls using t-tests. Pearson's correlation coefficients were used in the correlation analyses. These statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA). Gene and miRNA expression data were

Table 1

Clinical characteristics of patients used to perform *in vitro* studies.

Parameters	Before		After	
	Controls	Cases	HP	PE
N	6	6	10	14
GA at sampling (weeks)	23 ± 2	23 ± 2	36 ± 2	35 ± 3
Age (years)	28 ± 5	28 ± 4	27 ± 5	26 ± 5
BMI (kg/m ²)	28 ± 4	30 ± 6	30 ± 4	31 ± 6
SBP at sampling (mmHg)	104 ± 6	118 ± 1	113 ± 7	133 ± 1*
DBP at sampling (mmHg)	64 ± 4	77 ± 15	73 ± 6	85 ± 8*
GA at delivery (weeks)	39 ± 3	36 ± 2*	39 ± 2	36 ± 3*

Values are the means ± S.D. or percentage. HP, healthy pregnant; PE, preeclampsia; GA, gestational age; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. * P < 0.05 vs controls.

analyzed using the GeneGlobe Data Analysis Center (Qiagen®) online platform. For all tests, a P value < 0.05 (two-tailed) was considered significant.

11. Results

11.1. Effects of plasma incubation on the expression of genes involved in endothelial cell biology in HUVECs

The study workflow is shown in Fig. S1. We compared the plasma samples from pregnant women who subsequently developed preeclampsia (cases) with those from women who remained healthy throughout gestation (controls), and from pregnant women with preeclampsia symptoms established (the PE group) with those from healthy pregnant women (the HP group). First, we compared the effects of plasma incubation on the expression of 84 genes (determined using the PCR array) in HUVECs (Table S1). The clinical characteristics of the women whose plasma samples were collected and used to perform the *in vitro* studies are shown in Table 1. As expected, we observed significantly increased systolic blood pressure (SBP) and diastolic blood pressure (DBP) in the PE group compared with the HP group. The gestational age (GA) at delivery was significantly lower for both cases and the PE group compared with their respective controls.

Out of the 84 genes evaluated (Table S1), *EDN1* expression was the one that differed most significantly between cases and controls (Table S1; Fig. 1A, p < 0.05 and fold change > 2.0). In HUVECs incubated with plasma from the PE group compared with HP group, however, there was no significant difference in *EDN1* expression between them (Table S1; Fig. 1B, p < 0.05 and fold change > 2.0). To confirm the PCR array data, we increased the number of samples from the PE and HP groups (n = 14 and n = 10, respectively), but *EDN1* expression remained not significantly different between the groups (1.19 ± 0.22 vs. 1.02 ± 0.22 fold change, respectively) (Fig. S2).

11.2. Effects of plasma incubation on ET-1 levels in HUVECs

We examined ET-1 levels in supernatants from the HUVECs incubated with plasma from case and control groups, and from PE and HP groups. ET-1 levels were significantly increased (p = 0.027) in the cases (1.24 ± 0.14 pg/mL) compared with controls (0.79 ± 0.09 pg/mL) (Fig. 1C). ET-1 levels were similar, though, in the PE and HP groups (1.68 ± 0.37 vs. 1.55 ± 0.33 pg/mL, respectively, p = 0.794) (Fig. 1D).

11.3. Expression of miRNAs that target *EDN1* in HUVECs

We examined whether miRNAs that target *EDN1* could modulate ET-1 levels. From the miRNAs selected, four were not expressed under our conditions (miR-1, miR-135a, miR-144 and miR-199). In HUVECs incubated with plasma from the case and control groups, there were no

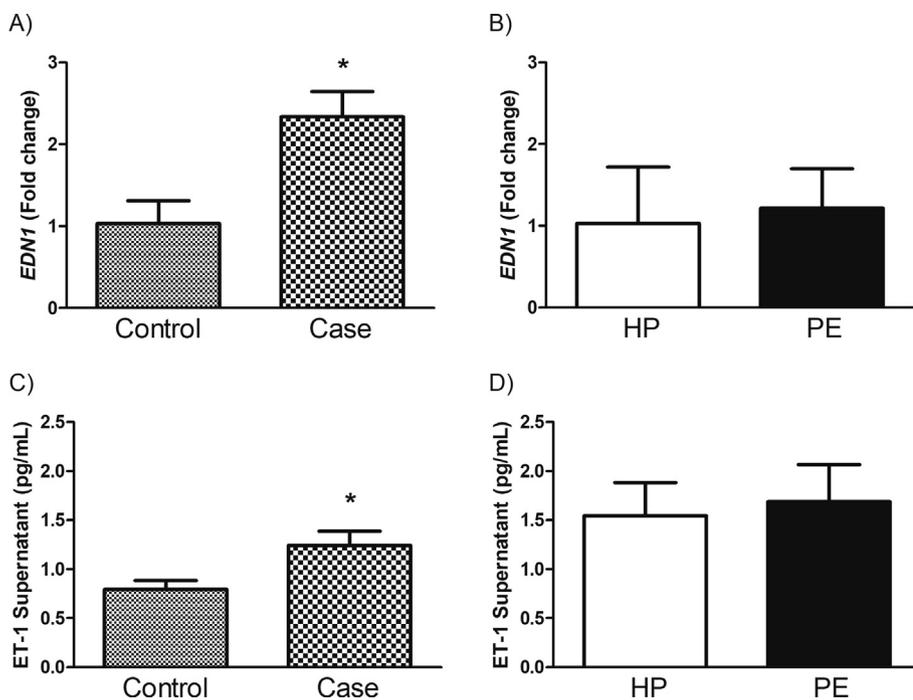


Fig. 1. *EDN1* expression and ET-1 levels in HUVECs incubated with plasma from patients before and after clinical symptoms of preeclampsia. HUVECs cells were incubated with 20% (v/v) plasma samples from pregnant women who subsequently developed preeclampsia (case) and who remained healthy during gestation (control), and from pregnant women with PE symptoms established (PE) and healthy pregnant women (HP) for 24 h. *EDN1* expression by PCR array before (A) and after (B) clinical symptoms of preeclampsia (n = 6 per group); ET-1 levels in supernatant by *ELISA* before (C) and after (D) clinical symptoms of preeclampsia (control and case, n = 6; HP, n = 10; PE, n = 14) (B). Values are fold change ± S.E.M. relative to respective control (control or HP) (A and B); and means ± S.E.M. (C and D), * P < 0.05 vs. respective control.

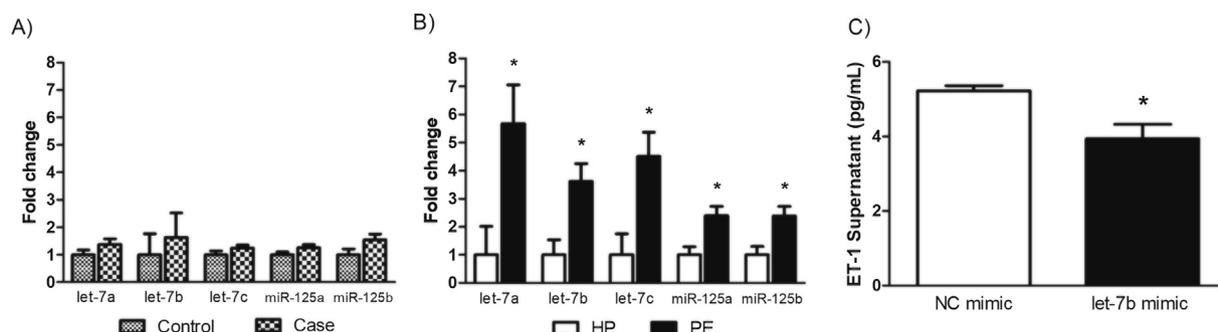


Fig. 2. Expression of miRNAs and effect of miRNA mimic transfection on ET-1 levels in HUVECs. HUVECs cells were incubated with 20% (v/v) plasma samples from pregnant women who subsequently developed preeclampsia (case) and who remained healthy during gestation (control), and from pregnant women with PE symptoms established (PE) and healthy pregnant women (HP) for 24 h. miRNAs expression by qPCR before (A) and after (B) clinical symptoms of preeclampsia (control and case, n = 6; HP, n = 10; PE, n = 14). HUVECs cells were transfected with miRNA let-7b mimic or negative control (30 pmol) for 12 h. ET-1 levels in supernatant by *ELISA* (n = 6) (C). Values are fold change ± S.E.M. relative to respective control (control or HP), * P < 0.05 vs. respective control (A and B) and means ± S.E.M., * P < 0.05 vs. negative control (C).

significant differences in miRNA expression between groups (Fig. 2A, p > 0.05 and fold change < 2.0). However, in HUVECS incubated with plasma from PE women, miRNAs let-7a, let-7b, let-7c, 125a and 125b were significantly upregulated compared with the HP group, and more so for the miRNAs from the let-7 family (Fig. 2B, p < 0.05 and fold change > 2.0).

Next, we performed correlation analyses on miRNA expression and supernatant ET-1 levels in the PE and HP groups (Table 2).

Table 2

Correlations between Delta C_T of miRNAs and ET-1 levels (plasma collected after the onset of clinical symptoms of preeclampsia).

miRNA	HP	PE
miR-125a	-0.15 (P = 0.607)	-0.47 (P = 0.090)
miR-125b	-0.41 (P = 0.248)	-0.02 (P = 0.910)
let-7a	-0.03 (P = 0.933)	-0.74 (P = 0.003)*
let-7b	-0.01 (P = 0.932)	-0.76 (P = 0.002)*
let-7c	-0.06 (P = 0.838)	-0.78 (P = 0.001)*

Values are Pearson Correlation Coefficient (P-value). HP, healthy pregnant; PE, preeclampsia. * P < 0.05.

Interestingly, we found significant inverse correlations between expression of the miRNAs in the let-7 family (a, b and c) and ET-1 levels in the PE group, but not in the HP group.

11.4. Effect of miRNA mimic let-7b transfection on ET-1 levels in HUVECs

In order to validate the interaction between miRNAs of the let-7 family and ET-1 levels, suggested by the significant inverse correlations, we evaluated the effect of miRNA mimic let-7b transfection on ET-1 levels in the HUVEC supernatants. Mimic let-7b transfection significantly decreased ET-1 levels by approximately 25% compared with the negative control (3.94 ± 0.38 vs. 5.22 ± 0.14 pg/mL, respectively, p = 0.010) (Fig. 2C). Transfection with the miRNA mimic was confirmed using real-time PCR (qPCR) (Fig. S3).

11.5. ET-1 levels in plasma from patients before and after the onset of preeclampsia symptoms and from controls who had healthy pregnancies

Finally, we increased the sample size and quantified plasma ET-1 levels in all the study groups. The clinical characteristics of each group

Table 3
Clinical characteristics of patients used to quantify circulating ET-1 levels.

Parameters	Before		After	
	Control	Case	HP	PE
N	45	29	26	37
GA at sampling (weeks)	24 ± 2	23 ± 1	37 ± 2	35 ± 3
Age (years)	27 ± 6	28 ± 6	24 ± 5	28 ± 7*
BMI (kg/m ²)	29 ± 4	31 ± 6	31 ± 6	31 ± 7
SBP at sampling (mmHg)	110 ± 10	117 ± 12*	113 ± 9	140 ± 13*
DBP at sampling (mmHg)	66 ± 6	74 ± 9*	71 ± 7	86 ± 10*
Nulliparous (%)	36	38	58	46
GA at delivery (weeks)	39 ± 2	36 ± 4*	40 ± 1	37 ± 3*
Newborn weight (g)	3368 ± 467	2793 ± 987*	3302 ± 385	2854 ± 882*

Values are the means ± S.D. or percentage. HP, healthy pregnant; PE, preeclampsia; GA, gestational age; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. * P < 0.05 vs. controls.

are shown in Table 3. As expected, both cases and the PE group had significantly higher SBP and DBP than the controls. Moreover, both preeclampsia groups (cases and PE) had a lower GA at delivery and lower newborn weight compared with the respective control groups.

We found higher ET-1 levels in the cases compared with controls (0.75 ± 0.05 vs. 0.61 ± 0.03 pg/mL, respectively, p = 0.023) (Fig. 3A) and in the PE group compared with the HP group (1.53 ± 0.17 vs. 1.11 ± 0.08 pg/mL, respectively, p = 0.040) (Fig. 3B).

12. Discussion

In this study, we found increased expression of miRNAs that putatively target *EDN1* in HUVECs incubated with plasma from the PE group, compared with plasma from the HP group. The same was not found before the clinical onset of preeclampsia. In addition, we observed significant inverse correlations between miRNA expression of the let-7 family and ET-1 levels in the PE group, but not in the HP group. Therefore, as *EDN1* expression and ET-1 levels in the supernatant was increased in cases compared with controls and were not different in the PE group compared with the HP group, we suggest that miRNAs may modulate ET-1 levels in HUVECs in the presence of preeclamptic plasma.

It is widely accepted that ET-1 is involved in the pathophysiology of preeclampsia [17–21], and so it is interest to ascertain whether the ET-1 gene can be targeted with tools such as miRNAs. The seed sequence predicted in which miRNAs let-7 a, b and c bind to *EDN1* 3'UTR is CUACCUC (positions 572–579). In order to verify the interaction between these miRNAs and the *EDN1* gene, we transfected HUVECs with miRNA mimic let-7 and found a significant decrease in ET-1 levels

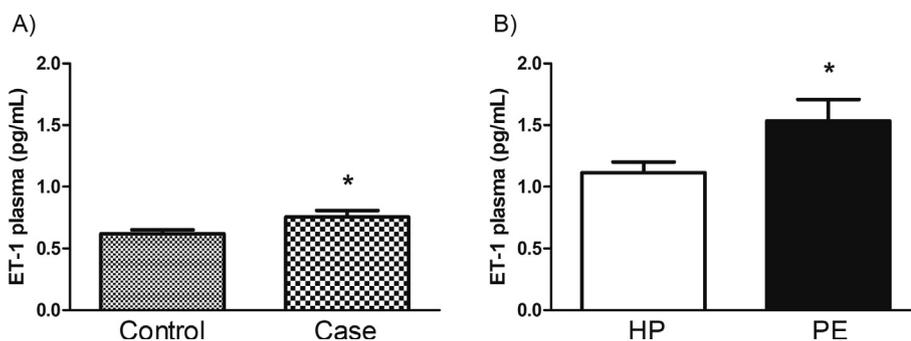


Fig. 3. ET-1 levels in plasma from patients before and after clinical symptoms of preeclampsia. Plasma samples were collected from pregnant women who subsequently developed preeclampsia (case) and who remained healthy during gestation (control), and from pregnant women with PE symptoms established (PE) and healthy pregnant women (HP). ET-1 levels in plasma by ELISA before (A) and after (B) clinical symptoms of preeclampsia (control, n = 45; case, n = 29; HP, n = 26; PE, n = 37). Values are means ± S.E.M., * P < 0.05 vs. respective control.

compared with the miRNA negative control condition. Furthermore, the interaction between miRNAs and *EDN1* was confirmed by luciferase reporter assays [25,26]. The let-7c inhibited luciferase activity by > 50%, and let-7b also slightly suppressed the luciferase activity in gastric cancer cells [25]. Moreover, the interaction between miR-125a, miR-125b and *EDN1* has also been reported in human embryonic kidney cells (HEK293A) [26]. These functional studies validated the interaction between these miRNAs and *EDN1*, further supporting our hypothesis that these miRNAs modulate ET-1 levels.

The mechanism by which plasma from preeclamptic women stimulates miRNA expression in endothelial cells needs further investigation. It could be through alterations at the transcriptional level or at the post-transcriptional level. At the transcriptional level, it could be changes in the expression of host genes where the miRNA is encoded (for intragenic miRNAs), in transcription factors (for intergenic miRNAs) or in methylation of the promoter in both intragenic and intergenic miRNAs [39]. At the post-transcriptional level, changes in miRNA expression could be due to alterations in miRNA biogenesis, for example in the activity of miRNA biogenesis enzymes such as Dicer and Drosha [39].

Discordant findings have been reported regarding the effects of plasma (collected after the onset of preeclampsia) from preeclamptic women on ET-1 levels in endothelial cells [10,11,14–16]. Increased production of ET-1 was reported in endothelial cells incubated for 24 h with serum (20%, v/v) from preeclamptic women compared with serum from women with a normal pregnancy [10,11]. Conversely, other studies showed decreased ET-1 levels [14,16] or similar levels [15], which is in agreement with our findings. However, it is important to note that these controversial findings may have resulted from differences in experimental protocols, such as: the plasma/serum concentration during incubation; the time of incubation and proliferation; and the viability and type of HUVEC used (primary or immortalized cell line). Moreover, according to our findings, miRNAs that target *EDN1*, mainly the let-7b family, may decrease ET-1 levels in HUVECs incubated with plasma from preeclamptic patients.

Many studies have examined the therapeutic potential of miRNAs for pathologies such as cancer, diabetes and cardiovascular diseases, but few miRNAs are in the pre-clinical or clinical stage of development as therapeutic agents [40,41]. We found that transfection with miRNA mimic let-7 decreased ET-1 levels in HUVECs, which suggests that miRNA mimics of let-7 might be useful candidates for therapeutic intervention in the management of preeclampsia. However, more studies should be performed to explore this possibility.

To our knowledge, the only study regarding ET-1 levels in plasma samples from pregnant women collected before the onset of symptoms of preeclampsia found higher ET-1 levels in samples from women who subsequently developed mild to severe preeclampsia and eclampsia [42]. This result is in agreement with our findings and, taken together with our *in vitro* findings, suggests that ET-1 may be a useful biomarker to help identify women at risk of preeclampsia. Furthermore, ET-1 has been validated to predict cardiovascular risk [43]. In pregnant women who had already developed preeclampsia, plasma ET-1 levels were also

higher compared with those of healthy pregnant women, which has been extensively observed in previous studies of preeclampsia [17,18,21]. This shows that the suppression of ET-1 levels by miRNAs in our *in vitro* findings occurs locally, not systemically, in preeclamptic women. Moreover, as a limitation of the study, we used an immortalized cell line of HUVECs, which is a healthy cell line; however, it is well established that the systemic endothelial dysfunction characteristic of preeclampsia occurs because the maternal endothelium is already impaired [44].

13. Conclusions

In conclusion, we have shown that plasma from preeclamptic women stimulates the expression of miRNAs, mainly miRNAs of the let-7 family, leading to a decrease in ET-1 production in HUVECs. This suggests that miRNAs may be a therapeutic tool in the management of preeclampsia.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.preghy.2018.03.001>.

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