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Placental effects and transfer of sildenafil in healthy and preeclamptic conditions

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ABSTRACT

Background: The phosphodiesterase-5 inhibitor (PDE5) sildenafil has emerged as a promising treatment for preeclampsia (PE). However, a sildenafil trial was recently halted due to lack of effect and increased neonatal morbidity.

Methods: *Ex vivo* dual-sided perfusion of an isolated cotyledon and wire-myography on chorionic plate arteries were performed to study the effects of sildenafil and the non-selective PDE inhibitor vinpocetine on the response to the NO donor sodium nitroprus-side (SNP) under healthy and PE conditions. *Ex vivo* perfusion was also used to study placental transfer of sildenafil in 6 healthy and 2 PE placentas. Furthermore, placental mRNA and protein levels of eNOS, iNOS, PDE5 and PDE1 were quantified.

Findings: Sildenafil and vinpocetine significantly enhanced SNP responses in chorionic plate arteries of healthy, but not PE placentas. Only sildenafil acutely decreased baseline tension in arteries of both healthy and PE placentas. At steady state, the foetal-to-mater-nal transfer ratio of sildenafil was 0.37±0.03 in healthy placentas versus 0.66 and 0.47 in the 2 PE placentas. mRNA and protein levels of PDE5, eNOS and iNOS were comparable in both groups, while PDE1 levels were lower in PE.

Interpretation: The absence of sildenafil-induced NO potentiation in arteries of PE pla-centas, combined with the non-PDE-mediated effects of sildenafil and the lack of PDE5 upregulation in PE, argue against sildenafil as the preferred drug of use in PE. Moreover, increased placental transfer of sildenafil in PE might underlie the neonatal morbidity in the STRIDER trial.

INTRODUCTION

Suboptimal development of the placenta results in serious pregnancy complications such as preeclampsia (PE) and foetal growth restriction (FGR), that contribute significantly to perinatal and maternal morbidity and mortality.[1,2] Besides increasing the risk of adverse events during pregnancy, placenta-related diseases have lifelong consequences for the health of both mother and child. For example, PE increases the maternal risk of developing cardiovascular disease[3,4] and can cause persistent vascular dysfunction in the systemic and pulmonary circulation of the offspring,[5] whereas preterm birth and low birthweight greatly increase the risk of cardiopulmonary morbidity and neurodevelopmental impairment later in life.[6,7] Currently, PE treatment is aimed at symptom relief and prevention of further complications in an attempt to prolong pregnancy until term. Many antihypertensive agents have been studied over the years, but they at most temporarily stabilise the clinical manifestations of PE, without directly targeting placental hypoperfusion.[8,9] The only cure is termination of pregnancy to deliver the placenta, which often leads to preterm birth of the foetus. Therefore, developing new treatment options to treat PE and safely prolong pregnancy is of great importance.

Although the aetiology of PE remains largely unknown, it finds its origin in early pregnancy with suboptimal placentation, characterised by increased placental vascular resistance and hypoperfusion, leading to systemic endothelial dysfunction.[10-12] One of the key features of this endothelial dysfunction is a decreased activity of the nitric oxide (NO) pathway.[13-15] NO acts as an important vasodilator, synthesised by a family of nitric oxide synthases (NOS), predominantly endothelial NOS (eNOS) and inducible NOS (iNOS). NOS enzymes are present in various cell types including endothelial cells and foetal trophoblasts, and stimulation of these enzymes (e.g., by endothelial shear stress or oestrogen) results in the production of NO through catalysis of L-arginine. By activating soluble guanylate cyclase (sGC), NO induces an increase in production of cyclic guanosine 3′,5′-monophosphate (cGMP), leading to vasodilation through closure of Ca^{2+} channels.[13,14]

Besides regulation of vascular tone, NO also plays an important role in cytotrophoblast invasion of the receptive endometrium and subsequent spiral artery remodelling during early pregnancy.[16] Throughout normal pregnancy there is an increase in maternal plasma levels of NO,[17] as well as NO-stimulating factors such as vascular endothelial growth factor and placental growth factor.[18,19] However, in women with PE these plasma levels are significantly lower.[17,20,21] Sildenafil, as a phosphodiesterase-5 (PDE5) inhibitor, enhances vasodilation mediated by the NO pathway, by inhibiting degradation of active cGMP into inactive GMP by PDE5. Sildenafil is currently approved for the treatment of erectile dysfunction and pulmonary hypertension.[22] In PE animal models, sildenafil

improved foetal outcome and diminished maternal symptoms by increasing blood flow to the uterus.[23,24]

Because of its potential to improve placental hypoperfusion by increasing systemic vasodilation, sildenafil has been considered for the treatment of PE and FGR over the last years. However, an international consortium of large multi-centre randomised controlled trials (the STRIDER study), investigating the effect of sildenafil compared to placebo on pregnancy outcome in extreme FGR due to placental insufficiency,[25,26] was recently halted due to lack of beneficial effects in the first two cohorts[27,28] and an increase in neonatal morbidity and mortality in the treatment group of one cohort.[28,29] These results emphasise the importance of taking into account the possible effects that maternal medication use can have on the foetus. When considering drugs for treatment in human pregnancy, it is essential to know the trans-placental transfer and effects on the placental vasculature. Hence, besides transfer, study of the placenta can give insight into vascular effects of sildenafil in the foetus. Dual *ex vivo* perfusion of a single placental cotyledon is the only reliable experimental method to study drug transfer across the human placental barrier to date.[30] With this model Russo *et al.* recently showed that sildenafil crosses the placenta of healthy term pregnancies,[31] however to our knowledge this has never been performed in PE placentas. The aim of our study is to evaluate the effects of sildenafil on NO-mediated vasodilation in the foetoplacental vasculature, to evaluate placental transfer in healthy and PE placentas, and to study placental expression levels of components of the NO pathway under healthy and PE conditions. This could help to provide a possible explanation for the negative findings obtained with sildenafil in the above-mentioned clinical trials. Understanding these negative findings will be helpful for the development of future therapies.

METHODS

Patients and setting

The study received exemption for approval from the local institutional Medical Ethics Committee according to the Dutch medical Research with Human Subjects Law (MEC-2016-418 and MEC-2017-418), and all patients gave written consent prior to donating their placenta. Randomly selected placentas of uncomplicated singleton pregnancies and of patients with early onset PE (diagnosis before 34+0 weeks of gestation) were collected immediately after delivery at Erasmus University Medical Center, Rotterdam, The Netherlands. Because it is generally believed that late onset PE (from 34+0 weeks of gestation onwards) has a different pathophysiological mechanism than early onset PE, being more a maternal rather than a placental syndrome,[32] and also showing clear histopathological differences in the placenta,[33] late onset PE was excluded from the current

study. Further exclusion criteria were retained placenta, viral infections (HIV, hepatitis B, Zika), the presence of foetal congenital abnormalities on ultrasound, participation in the STRIDER study and for the healthy controls any form of diabetes. Baseline characteristics such as maternal age, medical history, obstetrical history, use of medication, blood pressure, mode of delivery, gestational age at delivery, foetal sex, foetal weight, placental weight and pregnancy complications were obtained from the digital medical files.

Wire-myography experiments

Second order branches of chorionic plate arteries were identified, carefully dissected and stored overnight in cold, oxygenated Krebs-Henseleit buffer. The next morning the vessels were cut into segments of 2 mm and mounted in 6-mL organ baths (Danish Myograph Technology, Aarhus, Denmark), filled with Krebs-Henseleit buffer at 37°C and gassed with 95% O_2 – 5% CO_2. Tension was normalised to 90% of the estimated diameter at 100 mmHg effective transmural pressure. Maximum contractile responses were determined using 100 mmol/L potassium chloride (KCl). After washout of the KCl, precontraction was elicited using the thromboxane A2 agonist U46619 (10 nmol/L) resulting in 75-100% of the contraction induced by 100 mmol/L KCl. Subsequently, a concentration-response curve (CRC) was constructed for SNP (0·001 – 100 μmol/L). To study the effect of PDE inhibition on NO-mediated vasodilation, vessel segments were either pre-incubated for 30 minutes with sildenafil (1 μmol/L), the non-selective PDE inhibitor vinpocetine (10 μmol/L) or without additives as control.

Placental perfusion setup

The perfusion model used in our study has been adapted from the model previously described by Schalkwijk *et al.*[34] It exists of a perfusion chamber, two peristaltic roller pumps, heating devices and a water bath (37°C). The maternal and foetal perfusion media consisted of Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3), supplemented with heparin 5000 IU (0.5 mL/L) and aerated with 95% O_2 - 5% CO_2. After selecting an intact cotyledon, the foetal circulation was established by cannulating the chorionic artery and corresponding vein. Flow rate was carefully increased to 3 or 6 mL/min, depending on the experiment. When this was successful, the cotyledon was cut from the placenta and placed inside the perfusion chamber. Maternal circulation (flow rate 12 mL/min) was created by placing four blunt cannulas in the intervillous space through remnants of the spiral arterioles. Venous outflow was collected in a reservoir underneath the cotyledon and run back to the maternal reservoir. A placental washout period of approximately 45 minutes was performed before starting an experiment. Changes in pressure were continuously measured by pressure transducers and recorded throughout the experiment using acquisition software (Biopac, Goleta, CA, USA).

Placental vasoreactivity

To study the effect of sildenafil on the foetal vascular bed of the placenta, NO-mediated vasodilation was tested in the absence or presence of sildenafil. When a stable baseline pressure had been reached after the washout period, sildenafil (500 ng/mL) was added to the foetal medium in half of the placentas that were randomly selected. After approximately 15 minutes 1 μmol/L serotonin (5-HT) was administered to the foetal circulation to preconstrict the vasculature. Subsequently, the NO donor sodium nitroprusside (SNP) was used to induce vasodilation (1 μmol/L). Changes in placental vascular resistance were used to assess the magnitude of the vasodilator response.

Placental transfer of sildenafil

To study placental transfer, sildenafil was administered to the maternal circulation at start of the experiment in a concentration of 500 ng/mL, the maximal tolerated concentration in humans.[35] System adherence of sildenafil was tested by adding the drug to the perfusion system in the absence of a placenta. At t=0, antipyrine (100 mg/L) was added to the maternal buffer as a positive control of passive diffusion across the placental barrier and to prove adequate overlap between maternal and foetal circulations, and the macromolecule FITC-dextran (40 kDa, 36 mg/L) was added to the foetal buffer as a marker of integrity of the capillary bed. Samples of the maternal and foetal buffer were taken at seven set time points, and immediately stored at -80 °C.

Quality control

The placenta was excluded from further analysis when fluid leakage from the foetal circulation exceeded 3 mL/h, the foetal-to-maternal (F/M) ratio of antipyrine was <0.75 at t=180, or the maternal-to-foetal (M/F) ratio of FITC-dextran was >0.03 at t=180.[36]

Analysis of antipyrine and FITC-dextran

Antipyrine concentration was analysed by first deproteinising the samples with perchloric acid 6%. After this, a mixture of 0·2 mg/mL $NaNO_2$ and 0.6% H_2SO_4 was added in a 1:1 ratio to form nitroantipyrine. Using ultraviolet–visible spectroscopy (Shimadzu UV-1800), absorption at 350 nm was measured. For analysis of FITC-dextran, fluorescence was measured using a Multiwell Plate Reader (Victor X4 Perkin Elmer, excitation/emission 485/519 respectively).

LC-MS analysis of sildenafil

The concentration of sildenafil in placental perfusate was measured with a FDA validated liquid chromatography-mass spectrometry method (LC-MS), using Thermo TQS Vantage LC-MS/MS for the analysis. Column 2.1x100 mm Waters Acquity CSH C18 1.7 μm. The mobile phase A consisted of 2 mM ammonium acetate in 0.1% formic acid in water.

The mobile phase B consisted of 2 mM ammonium acetate in 0.1% formic acid in LC-MS methanol. Flow rate was 0.5 mL/min. The mobile phase composition changed linearly during analysis in a percentage mobile phase A (from 80% to 0) and B (from 2% to 100%). Total analysis time was four minutes. The injected volume was 10 μl. Internal standard was vardenafil. The method was validated according to FDA guidelines between 2-1000 μg/L for sildenafil and 2-500 μg/L for desmethylsildenafil.[37]

qPCR

Within 20 min after delivery of the placenta pieces of tissue were dissected from both the foetal and maternal side of the placenta and snap frozen in liquid nitrogen. For RNA extraction, small pieces of tissue were homogenised in RLT lysis buffer (Qiagen, Venlo, The Netherlands) with β-mercaptoethanol. After proteinase K treatment (Invitrogen, Breda, The Netherlands) for ten minutes at 55 °C, total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen). RNA was eluted in RNase free water, and concentration and purity were assessed on a NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific, Bleiswijk, The Netherlands). Complimentary DNA (cDNA) was synthesised from 0.5 μg RNA template with the SensiFast cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer's instructions. This cDNA was used for quantitative PCR (qPCR) using the SYBR Green qPCR Kit (Bioline, London, UK) and specific primer pairs on a CFX-96 light cycler (Bio-Rad, Hercules, CA, USA). qPCR was performed with the following conditions: initial denaturation at 95 °C for eight min and 30 s, followed by 40 cycles comprising 15 s at 95 °C, and 60 s at 60 °C. Target genes were normalised against the reference genes β-actin and Peptidylprolyl Isomerase A (PPIA) and relative gene expression was calculated by the ΔΔCt method. A melt curve was run for each gene to confirm amplification of a single PCR product. The specific primer pairs are listed in Table S1.

Western blot analysis

Snap frozen pieces of placental tissue were homogenised in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycolate, 0.1% SDS, 50 mM Tris PH 8.0) with protease and phosphatase inhibitors. After incubation on ice for 20 min, samples were centrifuged at 4 °C, 13000 rpm for three min. Supernatant was collected of each sample, and total protein concentration was determined with the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific). Per sample 50 μg of total protein was loaded onto a 4-15% mini-protean TGX gel (Bio-Rad). Samples were resolved at 80 V for 20 min followed by 110 V for 60 min (PDE5a) or 120 min (eNOS). Transfer of proteins to the membrane was done on ice for one h at 110 V. Subsequently, after blocking in TBS + 5% BSA for one h, the membranes were incubated with anti-PDE5A 1:1000 (Abcam Cat# ab64179, RRID:AB_1566572, 1 mg/ml), anti-eNOS 1:500 (BD Biosciences Cat# 610296, RRID:AB_397690, 250 μg/ml),

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or anti-iNOS 1:1000 (Abcam Cat# ab182640, 1 mg/ml), and anti-β-actin 1:1000 (Abcam Cat# ab8229, RRID:AB_306374) for 1.5 h at room temperature. Hereafter, the membranes were incubated with 1:15000 diluted fluorescently-labelled secondary antibody for one h. Bands were visualized by the Odyssey® Infrared Imaging System, and analysed in Image Studio Lite (LI-COR Biosciences). The density of each band was normalised to β-actin and displayed as arbitrary unit (AU) value.

Statistical analysis

A power analysis was performed based on our previous experience with wire-myography experiments.[38] Based on the same standard deviation, at an α level of 0.05 and with statistical power at 80%, a derived minimum sample size of 6 per group was determined. Because of skewed distributions, non-parametric tests were applied. Statistical analysis was performed with SPSS (version 21, SPSS Chicago, IL, USA) and GraphPad Prism (version 5, 2007, La Jolla, CA, USA) on Windows. Statistical analysis between groups was performed using either a Mann-Whitney U test or Wilcoxon matched pairs test for two groups, and a Kruskal-Wallis test or a Friedman test for repeated measures with a Dunn's post-hoc test for three groups where appropriate. Log10-transformed SNP values at which the half-maximal response occurred (pEC_{50}) were individually estimated with sigmoid curve fitting software (GraphPad Prism 5). Data are displayed as median (interquartile range) or mean±SEM unless stated otherwise. A p-value <0·05 was considered to be statistically significant.

RESULTS

Wire-myography experiments

Chorionic plate arteries of 12 healthy and six PE placentas were dissected and mounted into Mulvany wire-myographs. Clinical characteristics can be found in Table S2. For analysing the CRC of SNP, five healthy and one PE placentas were excluded because of severe spontaneous vasomotion, making it impossible to analyse SNP effects. Sildenafil acutely decreased baseline tension vs. control in vessel segments of both healthy (p=0.01) and PE placentas (p=0.05) during the incubation period (Figure 1a). Such effects were not seen for vinpocetine. SNP fully relaxed U46619-preconstricted vessels in all conditions, and both sildenafil and vinpocetine enhanced (p=0.02) the SNP response in healthy vessel segments (Figures 1b and 1c and Table 1). No such potentiation was seen in vessel segments obtained from PE placentas. The maximum effect of SNP was unaltered by PDE inhibition.
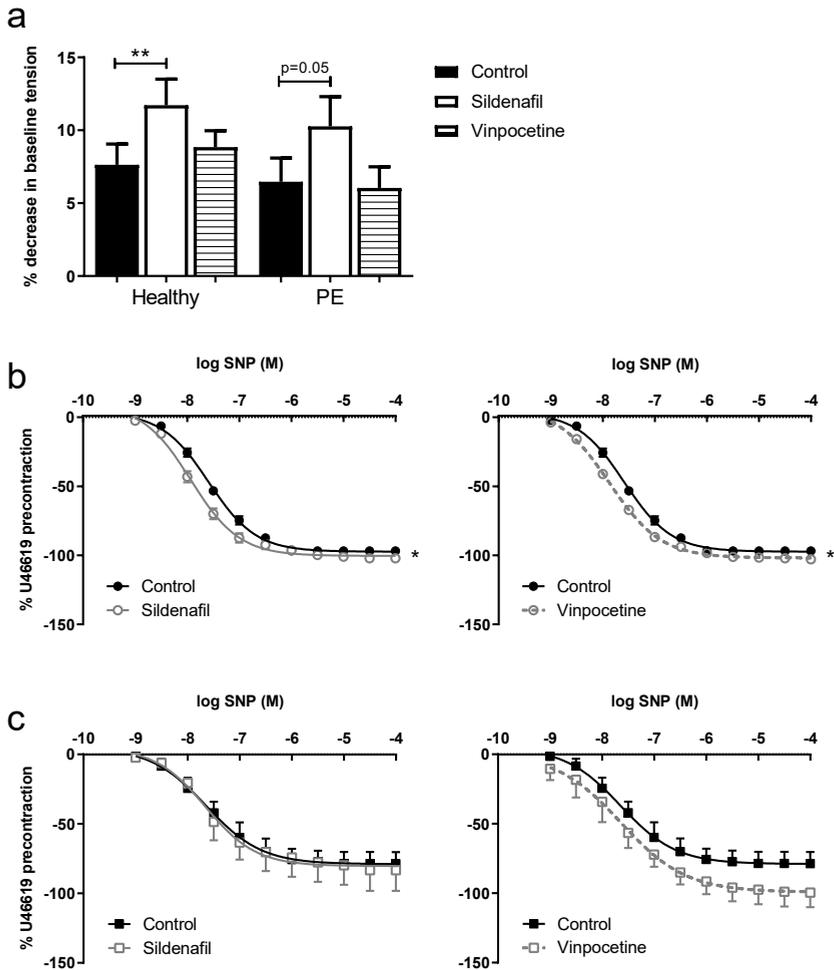
Figure 1. Wire-myography experiments. Panel a, decrease of baseline tension in response to PDE inhibitors. Panels b and c, effect of PDE inhibition with sildenafil or vinpocetine on SNP-mediated relaxation of chorionic plate vessels of seven healthy (b) and five PE (c) placentas. Data (mean±SEM) are expressed as % of U46619 precontraction. *p<0.05; **p<0.01 (Friedman test for repeated measures).

Table 1. Effects of sildenafil and vinpocetine on isolated chorionic plate arteries obtained from healthy and early onset preeclamptic (PE) placentas.

	pEC_{50}			E_{max}		
	control	**sildenafil**	**vinpocetine**	**control**	**sildenafil**	**vinpocetine**
Healthy	7.5 (7.9-7.3)	8.0 (8.3-7.6)*	8.0 (8.3-7.6)*	92 (86-110)	98 (87-112)	102 (97-111)
PE	7.6 (8.0-7.2)	7.6 (7.8-7.1)	7.5 (8.1-7.3)	85 (65-90)	97 (56-104)	90 (82-121)

Data are median (interquartile range). pEC_{50}: -$\log_{10}$-transformed SNP concentrations at which the half-maximal response occurred. E_{max}: maximal effect of SNP (% of U46619 precontraction). *p<0.05 vs. control (Friedman test for repeated measures).

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Placental vasoreactivity

A total of 26 (18 healthy and eight PE) placentas were included in the analysis. Clinical characteristics of these placentas can be found in Table S3. As expected, the gestational age and birth - and placental weight were lower in PE placentas, and they were associated with a higher maternal blood pressure. Baseline pressure at the start of the experiment was significantly lower in placentas from PE pregnancies compared to healthy placentas (p=0.03) (Table 2). Moreover, the 5-HT-induced pressure increase was lower in the PE group (p=0.07). SNP reversed the 5-HT-induced pressure increases, although significantly less in PE placentas (p=0.02) compared to controls. Under no condition did sildenafil significantly improve the SNP response (Table 2).

Placental transfer of sildenafil

Of the received placentas, a total of six out of 12 healthy and two out of 11 PE placentas met the quality control criteria, and were included in the analysis. For healthy term placentas the success rate of ~50% is higher than the average reported in literature.[36] To our knowledge there are no previous reports on success rate of perfusion experiments in preterm PE placentas.

Table 2. Placental vasoreactivity in healthy pregnancy and early onset preeclampsia (PE).

Parameter	Healthy (n=18)		PE (n=8)	
Baseline pressure (mm Hg)	34 (28-36)		23 (17-31)*	
5-HT-induced pressure increase (mm Hg)	60 (47-91)		47 (34-55)	
	control (n=9)	sildenafil (n=9)	control (n=4)	sildenafil (n=4)
Response to SNP (% 5-HT precontraction)	105 (97-112)	110 (103-120)	74 (55-96)*	85 (62-114)

Data are median (interquartile range). *p<0.05 compared to healthy (Mann-Whitney U test).

All included placentas showed good overlap of the maternal and foetal circulations with a F/M ratio for antipyrine of >0.75 (Figure S1). Table 3 shows the clinical characteristics of the included placentas. All women underwent elective caesarean section, because of previous caesarean section (four), previous shoulder dystocia (one) and intracranial hematoma that contra-indicated vaginal delivery (one). Both PE patients underwent caesarean section because of maternal illness and foetal distress. As expected, the placentas from PE pregnancies were born at an earlier gestational age (~32 weeks) and associated with a higher maternal blood pressure and a lower birth - and placental weight. The six healthy placentas were perfused at a foetal flow rate of 3 or 6 mL/min (n=3 for each). Since there were no significant differences in transfer ratios of antipyrine and sildenafil between the two flow rates (Figure S2), the results have been combined.

Figure 2a shows the placental transfer of sildenafil in healthy placentas. The transfer rate of sildenafil was highest in the first hour, and after ~90 min an equilibrium between

Table 3. Clinical characteristics of healthy and early onset preeclamptic (PE) placentas used for sildenafil transfer experiments.

Characteristic	Healthy						PE	
	1	2	3	4	5	6	1	2
Maternal age (y)	36	31	24	36	38	39	30	26
Parity	1	1	1	1	3	1	0	0
Caucasian ethnicity	yes	no	yes	no	no	yes	no	yes
Body mass index (kg/m^2)	24.6	27.3	27.8	20.8	29	24.7	28.5	23.1
Smoking	no	no	no	no	no	no	no	no
Highest DBP (mm Hg)	75	86	80	80	75	80	120	109
Gestational age (weeks)	39.0	38.0	38.5	38.6	39.1	39.0	32.0	31.4
Mode of delivery	CS	CS	CS	CS	CS	CS	CS	CS
Foetal Sex	M	M	M	M	F	F	M	M
Birth weight (g)	4325	3475	3420	4010	3815	2940	1150	1460
Birth weight (centile)	96	76	55	89	83	16	0	6
Placental weight (g)	784	550	581	659	825	618	348	339

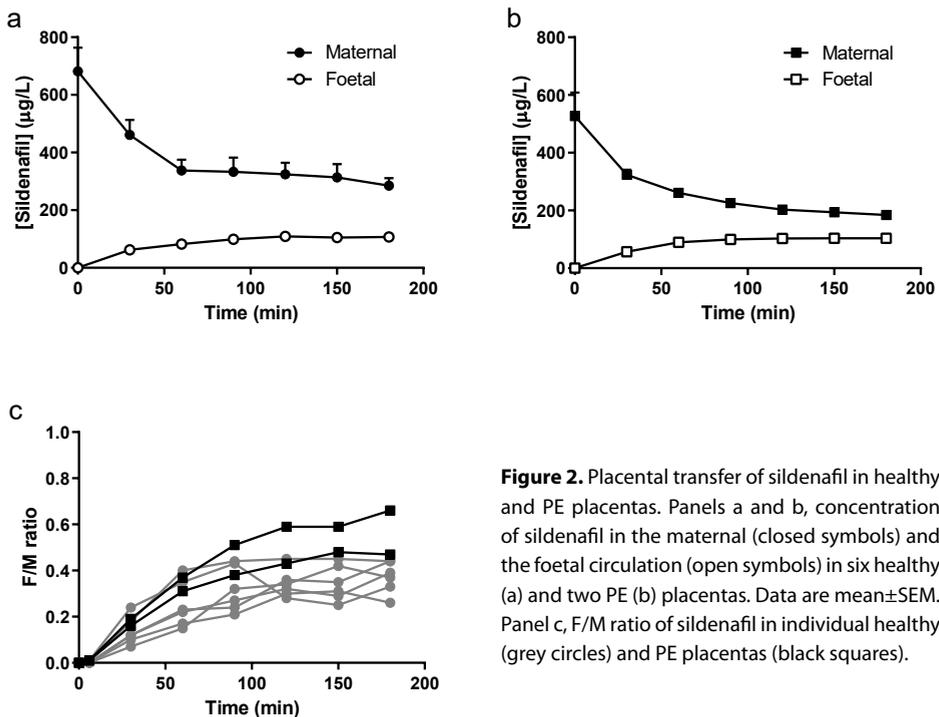DBP = diastolic blood pressure; CS = caesarean section; M = male; F = female.

Figure 2. Placental transfer of sildenafil in healthy and PE placentas. Panels a and b, concentration of sildenafil in the maternal (closed symbols) and the foetal circulation (open symbols) in six healthy (a) and two PE (b) placentas. Data are mean±SEM. Panel c, F/M ratio of sildenafil in individual healthy (grey circles) and PE placentas (black squares).

the maternal and foetal circulations had been reached. After 180 min of perfusion the F/M ratio of sildenafil was 0.37±0.03. In the two PE placentas the placental transfer of sildenafil followed a similar pattern (Figure 2b), and the F/M ratios in these two placentas were the highest of all placentas studied (0.66 and 0.47, Figure 2c). At the end of the experiment, under steady state conditions, in healthy placentas 43±3% and 16±1% of the total amount of added sildenafil were recovered in the maternal and foetal compartments, respectively, while in PE conditions these percentages amounted to 36±8% and 20±1%. This implies that, under healthy conditions, after three hours of perfusion 59% of the total starting amount of sildenafil could be retrieved in the perfusion buffers. After running the system without a placenta, a comparable 52% (mean of two measurements) was retrieved at the end of the experiments, indicating that the loss of sildenafil is largely caused by tube adherence.
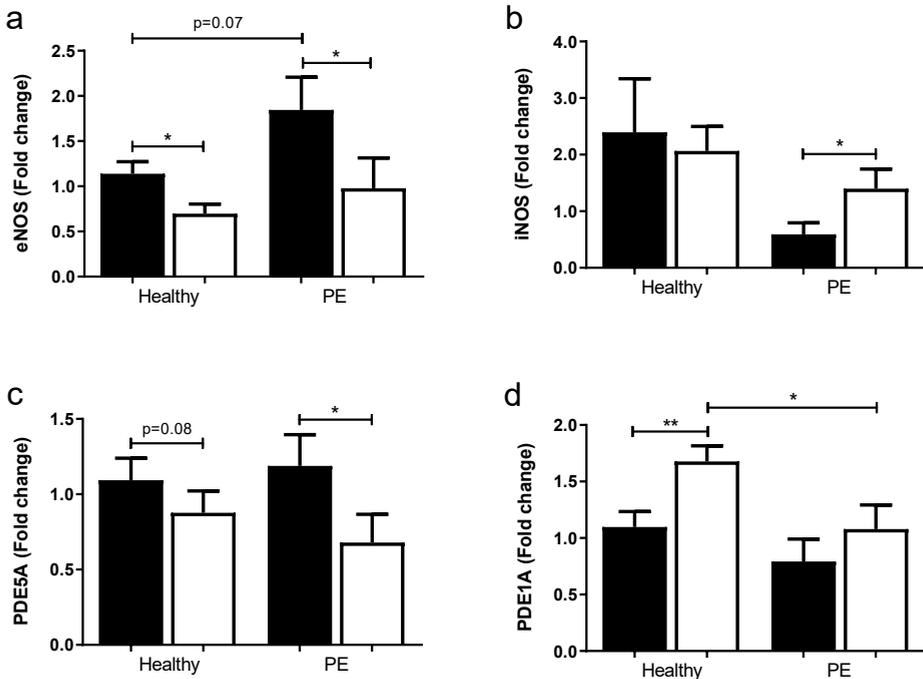

Figure 3. mRNA expression. qPCR analysis of placental biopsies from the maternal (black bars) and foetal side (white bars) of 12 healthy and seven PE placentas. Data (mean±SEM) are expressed as fold change versus the maternal side biopsies of healthy controls. Panels a-d represent data for eNOS, iNOS, PDE5A and PDE1A. *$p < 0.05$; **$p < 0.01$ (Mann-Whitney U test or Wilcoxon matched pairs test).

mRNA and protein expression

Snap frozen samples of 12 healthy and seven PE placentas were used to determine mRNA expression of eNOS, iNOS, PDE4A, PDE10A, PDE5A and PDE1A using qPCR. Clinical char-

acteristics can be found in Table S4. Of all measured PDEs, PDE5A displayed the highest expression (Figure S3). For PDE5A and eNOS, expression on the maternal side was higher than on the foetal side in healthy and PE placentas (Figure 3). PDE1A expression was higher on the foetal side than maternal side in both conditions, although significance was reached in healthy placentas only (Figure 3). For iNOS, expression on the foetal side was higher than on the maternal side in PE placentas only (Figure 3). However, it should be noted that iNOS expression was extremely low and highly variable, and was also not detectable at protein level. No statistically significant differences between patient groups were observed, with the exception of PDE1A, which was lower on the foetal side of PE vs. healthy placentas. In agreement with the gene expression data, protein expression of PDE5A and eNOS did not differ between patient groups (Figure S4 and S5).

DISCUSSION

This study shows that sildenafil decreased baseline pressure in isolated chorionic plate arteries from both healthy and PE placentas. Yet, it enhanced the relaxant effects of the NO donor SNP only in chorionic plate vessels obtained from healthy placentas, and not in vessels from PE placentas, nor when applied at the foetal side in the cotyledon setup. A similar enhancement was observed for the non-selective PDE inhibitor vinpocetine in chorionic plate vessels obtained from healthy placentas, although this drug did not decrease baseline pressure. Placental transfer of sildenafil was highest in the two PE placentas. Finally, while PDE5 expression was higher at the maternal side of the placenta versus the foetal side, its expression was not changed in PE, nor was the expression of other PDEs.

Sildenafil has been proposed to enhance NO availability in FGR and PE, and may thus improve endothelial function and placental perfusion.[39] Exactly this effect was observed when studying NO-induced responses in isolated chorionic plate arteries obtained from healthy placentas: sildenafil facilitated the response to SNP. The fact that this was not observed when adding SNP on top of sildenafil to the foetal circulation of the intact cotyledon setup implies that this effect is limited to a selected subset of placental vessels. Also, it might well be that other pathways than NO are important in regulation of vascular tone in the whole cotyledon setup.[15] The non-selective PDE inhibitor vinpocetine similarly potentiated SNP-induced vasorelaxation in healthy isolated chorionic plate arteries. Yet, no such potentiating effects were observed with either sildenafil or vinpocetine in isolated chorionic plate arteries from PE placentas, suggesting that the potential beneficial effects of PDE inhibition are absent in this condition. In contrast, in ageing vessels, with upregulated PDE levels, sildenafil and vinpocetine did enhance SNP responses,[40] thereby reversing the disturbed endothelial function in this condition.

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In agreement with the lack of effect of sildenafil in PE arteries, we did not detect PDE upregulation, maternally or foetally, at the mRNA or protein level in PE placentas. These data indicate that PDE upregulation is unlikely to underlie the disturbed endothelial function that has been reported in PE. We could even argue that the lost potentiating effect of sildenafil in PE suggests a reduced significance of the NO pathway in vasodilation in these arteries. We stress that in the present study we specifically evaluated NO responsiveness, based on the assumption that the beneficial effect of sildenafil would relate to its capacity to block PDE5 and hence should be seen in the form of NO potentiation. We did not quantify endothelial dysfunction – this would have required the application of an endothelium-dependent vasodilator. Furthermore, we studied foetoplacental vessels rather than spiral arteries, although it is the latter that determine placental perfusion. Unfortunately, acquiring spiral arteries would have required a myometrial biopsy, which was not possible in our hospital. However, even though the development of placental insufficiency starts with aberrant remodelling of the maternal spiral arteries,[10,11] subsequent changes in the foetoplacental vasculature have been described,[41,42] emphasising the relevance to study these vessels. Needless to say, it cannot be said with certainty that the current findings also apply to the maternal spiral arteries.

Sildenafil decreased baseline pressure in isolated chorionic plate arteries, both from healthy and PE placentas. This resembles the vasodilator effect of sildenafil reported by Walton *et al*. in the preconstricted cotyledon setup.[43] Such a decrease was not observed for the non-selective PDE inhibitor vinpocetine, nor for the highly selective PDE5 inhibitor tadalafil (by Walton *et al*.[43]), despite its much greater potency versus sildenafil. Yet, sildenafil and vinpocetine identically potentiated SNP in healthy chorionic plate arteries. Taken together, these data strongly suggest that the acute sildenafil-induced foetal dilation is unrelated to PDE5 inhibition. It may rather represent a non-specific effect of sildenafil. To what degree this still represents interference with PDEs other than PDE5A remains to be investigated. Our data support the presence of PDE5A as the most abundant PDE in placental tissue, and additionally show expression of PDE10A, PDE4A and PDE1A at lower levels. Only foetal PDE1A was altered in PE placentas, being actually lower, i.e., opposing the concept of upregulated PDEs in PE. Importantly, our data do support PE-related vascular changes, since baseline pressure was lower in PE cotyledons, and 5-HT-induced constriction was reduced.

Ex vivo cotyledon perfusion is a safe method to predict placental transfer of drugs *in vivo*, avoiding foetal exposure.[30] In healthy human placentas, Russo *et al*. demonstrated earlier that sildenafil passes the placental barrier, although they observed much higher F/M ratios (0.90 vs. 0.37 here).[31] Applying two different sildenafil concentrations, Russo *et al*. obtained levels in the foetal compartment corresponding with approximately 13-14% of the total added amount of sildenafil,[31] which is identical to what was observed in the current study (16%). Yet, in their setup maternal steady-state levels were as high

as those in the foetal compartment, while in our setup, maternal levels amounted to 43% of the total added amount of sildenafil. To explain the disappearance of sildenafil (~40% in our setup versus ~70% in the Russo paper), one has to assume that the drug is either metabolised, adheres to tubing, and/or accumulates in tissue. Both Russo *et al*. and the present study observed that ~45% of sildenafil adhered to tubing. Russo *et al*. did not observe metabolism, and given the fact that the sum of the percentage adhered to tubing plus the percentages of intact sildenafil in maternal and foetal compartments in our setup equalled the total added amount, our data also do not support metabolism, nor significant accumulation of sildenafil in tissue. Conversely, Russo *et al*. observed significant tissue accumulation, absolute tissue levels being identical at the two applied sildenafil concentrations, despite the fact that these concentrations differed 10-fold. This implies that their tissue levels were up to 30-fold higher than those in the maternal compartment. In the present study, tissue levels (per g tissue), at most corresponding with a few percent of the added amount of sildenafil, would have equalled the maternal levels (per mL). Hence, the much lower maternal levels in the Russo study appear to be entirely due to tissue accumulation of sildenafil, a phenomenon which was not observed in the current study. At this stage we are not aware of data supporting tissue accumulation of sildenafil.

To what degree albumin (present in the perfusion buffer in the study by Russo *et al*., but not in our study) determines tissue accumulation is unknown. Sildenafil binds plasma proteins for ~95%, which *in vivo* influences the concentrations in the maternal and foetal circulations. However, mimicking physiological protein concentrations in the *ex vivo* experimental set-up remains very difficult, since this does not only concern albumin, but also other drug-binding proteins like α1-acid glycoprotein and α-fetoprotein.[30] The concentrations of these proteins vary between maternal and foetal circulations, change with advancing gestational age, and might be altered by conditions like PE.[44] Importantly, the presence or absence of albumin should not affect the F/M ratio of the free drug concentration at steady state.[30] Unfortunately, to our knowledge, no data on sildenafil concentrations in cord blood and maternal plasma of pregnant women are currently available in the literature, making it impossible to compare the observed *ex vivo* transfer ratio to the actual *in vivo* situation.

Of interest, the transfer ratio of sildenafil in the two preterm PE placentas were the highest of all perfused placentas, whilst the general assumption is that placental drug transport increases throughout gestation.[45] Although preterm non-PE placentas would have been the appropriate control for the PE placentas, these data indicate that in PE conditions placental transfer of sildenafil could be enhanced at an early stage. Naturally, these results need to be interpreted with caution, since unfortunately the group size is very limited. This is due to the fact that it is very challenging to successfully perfuse preterm placentas, and especially PE placentas. Hence, extending the PE group, or

including preterm healthy placentas to study the effect of gestational age on sildenafil transfer was not feasible. Notably, the mean measured concentration of sildenafil at t=0 in the maternal circulation was higher in the healthy placentas compared to the PE ones. This seems to be due to measurement variability, as a similar variation is shown by Russo *et al.*[31] However, we believe that the starting concentration is of no influence to the F/M ratio, since it has been shown that even a 10-fold difference in starting concentration produced similar ratios.[31]

Clinical studies reported inconclusive results with sildenafil in the treatment of PE. In a small observational clinical trial sildenafil improved foetal growth in ten women with pregnancies complicated by early FGR,[46] although another study reported no effect on foetal outcome nor prolongation of pregnancy.[47] Recently, the large multinational randomised controlled STRIDER trials evaluating the effects of sildenafil versus placebo on pregnancy outcome in women with severe placental insufficiency were halted prematurely.[25] Interim analysis showed no improvement on foetal outcome in two cohorts[27,29] and there was an increased incidence of pulmonary hypertension of the new-born in the treatment group of the Dutch cohort.[28,29] Given the fact that sildenafil passes the placenta, it might have had unwanted effects in the foetus, e.g. on lung development. One of the vascular changes that is associated with pulmonary hypertension is a lower percentage of peripheral lung vessels, as frequently seen in foetuses with a congenital diaphragmatic hernia (CDH).[48] Russo *et al*. (2016) showed in a rabbit model that, although sildenafil exposure during pregnancy significantly increased the percentage of peripheral lung vessels in offspring with CDH, it had the opposite effect in healthy foetuses without CDH, as it significantly reduced the percentage of peripheral lung vessels compared to placebo treated animals.[48] Furthermore, although sildenafil treatment attenuated wall thickening of peripheral pulmonary vessels in CDH, it has been shown to increase muscularisation of the small pulmonary vessels in mice without CDH.[49] Excessive muscularisation of the foetal pulmonary vasculature could lead to an increased lumen diameter, thereby increasing vascular resistance. Another possibility could be that, since sildenafil reduces pulmonary vascular resistance in the foetus,[48] its placental transfer resulted in acute withdrawal problems after birth in a high-risk group already prone to develop pulmonary hypertension, as treatment was not continued in the neonates. To avoid any unwanted effects on the foetus, neonatal continuation of sildenafil treatment might have been an option. The use of the potent and highly selective PDE5A inhibitor tadalafil could also provide a better option than sildenafil, since it has a longer half-life and is less likely to pass the placenta.[43] However, this needs to be further studied in placental transfer experiments, also given the lack of maturation of the CYP3A pathway in neonates that is crucial for tadalafil metabolism.[50]

In conclusion, in contrast to the general belief, we were unable to demonstrate selective PDE upregulation in PE, nor did sildenafil potentiate NO in PE placentas. It did so

in healthy placentas, and the absence of this potentiation in PE suggests that interference at other levels than PDE5 might be required to improve endothelial dysfunction in this condition, e.g. with sGC activators or stimulators. Importantly, we confirmed the previously described direct vasodilation induced by sildenafil, and observed that this is unrelated to PDE5 inhibition. Furthermore, our data reveal the possibility that sildenafil could reach higher foetal levels during preterm PE treatment. To what degree increased placental transfer and/or PDE5-independent effects of sildenafil may have contributed to its deleterious consequences in the STRIDER trial remains to be determined. We stress that when considering a drug for treatment during pregnancy, its placental transfer, preferably per trimester of gestation, should be investigated first.

ACKNOWLEDGEMENTS

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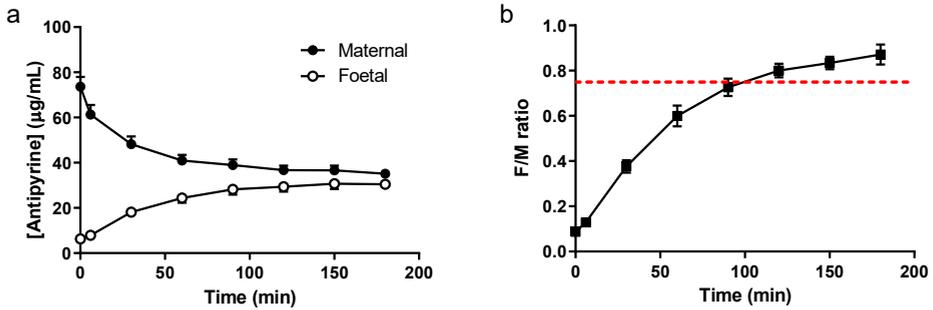
SUPPLEMENTAL INFORMATION

Figure S1. Placental transfer of antipyrine. Panel A, maternal and foetal concentrations. Panel B, F/M ratio, with the cutoff point of 0.75 (dashed red line).
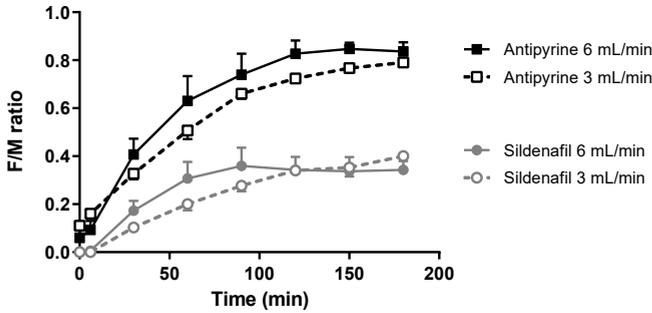

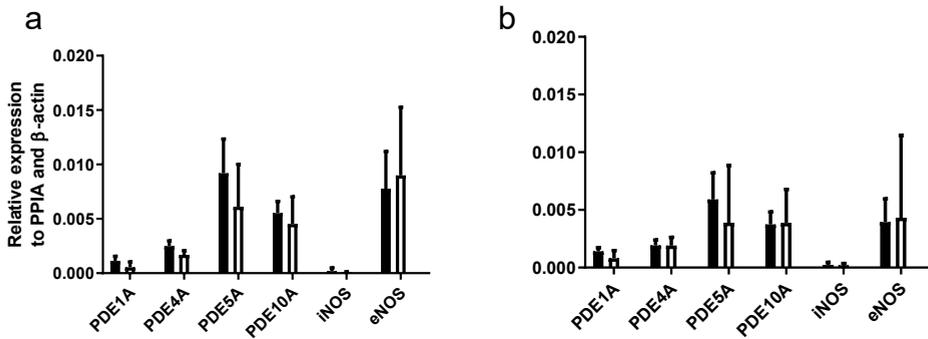**Figure S2.** Lack of different placental transfer outcomes at different foetal flow rates.

Figure S3. mRNA expression of different PDEs on the maternal side (a) and foetal side (b) of healthy (black bars) and PE (white bars) placentas.
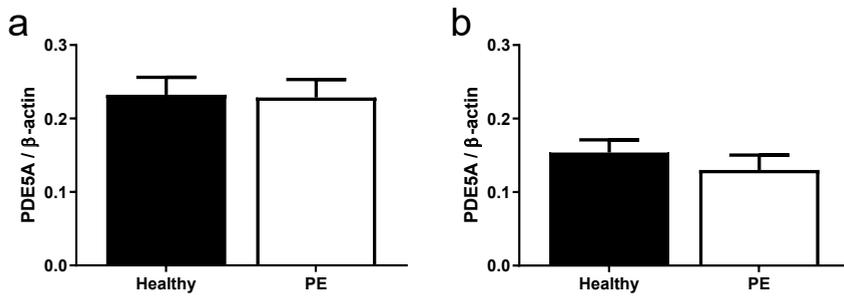
Erasmus University Rotterdam

Figure S4. Protein expression of PDE5A based on Western blot analysis at the maternal (a) and foetal (b) side of the placenta.
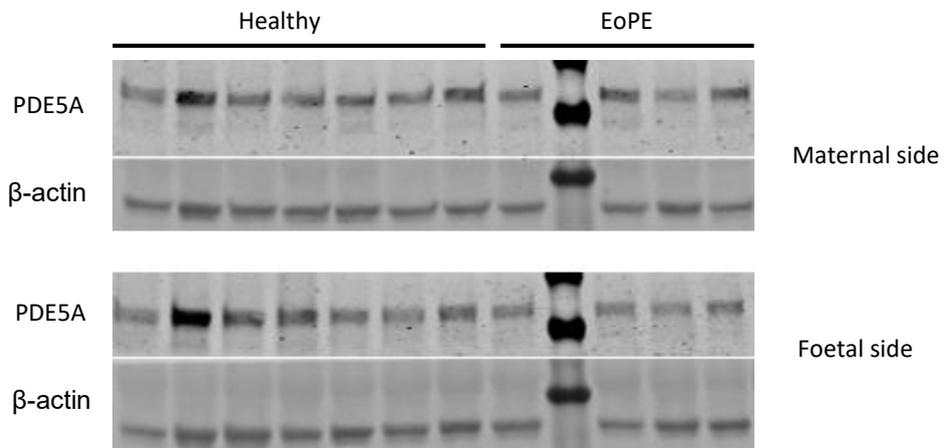

Figure S5. Representative Western blot of PDE5 protein expression.

Table S1. qPCR primer sequences.

Genes	Forward (5' - 3')	Reverse (5' - 3')
eNOS	ACCCACTGGTGTCCTCTTGG	CGAACACACAGAACCTGAGGG
iNOS	GCTTTGTGCGGAATGCCAG	CAAACACCAAGGTCATGCGG
PDE1A	GCCTGAAGGGATTGACAGAGC	TGCAGCTTCCAGGATTTGGC
PDE4A	TCACTCCTGACCAATGTGCC	GTTTCTTCTGACAGCGTGGC
PDE5A	TAGCCCAGGCCATCAACAAG	GGTCAAGCAGCACCTGATTTC
PDE10A	TGGGACATCCTGCTTTGAGC	TGCAGTGTGCTACAGTGACC

Table S2. Clinical characteristics of the placental vasoreactivity study in healthy pregnancy and PE.

Characteristic	Healthy (n=18)	PE (n=8)
Maternal age (y)	33 (29.8-37.3)	30.5 (25.5-31.8)*
Parity	1 (1-1.25)	0 (0-0.75)*
Caucasian ethnicity (n)	12/18	5/8
Body mass index (kg/m^2)	22.4 (20.3-27.5)	26.4 (22.6-30.9)
Smoking (n)	2/18	0/8
Highest DBP (mm Hg)	78 (72-81)	106 (96-110)*
Protein/creatinine ratio (g/mol)	nm	401 (157-908)
Gestational age (weeks)	39 (39-39.2)	30.3 (26.5-31.5)*
Caesarean section (n)	14/18	7/8
Male infants (n)	10/18	4/8
Birth weight (g)	3318 (3101-3558)	1215 (656-1549)*
Placental weight (g)	614 (499-721)	321 (176-357)*

Data are presented as median (interquartile range). DBP = diastolic blood pressure; nm = not measured. *$p < 0.05$.

Table S3. Clinical characteristics of the organ bath study in chorionic plate arteries obtained from healthy and PE placentas.

Characteristic	Healthy (n=7)	PE (n=5)
Maternal age (y)	35 (33-38)	30 (25.5-31.5)*
Parity	1 (1-2)	0 (0-0)*
Caucasian ethnicity (n)	4/7	3/5
Body mass index (kg/m^2)	24.9 (22.9-31.6)	27.8 (21.5-34.5)
Smoking (n)	2/7	0/5
Highest DBP (mm Hg)	80 (70-82)	109 (101-115)*
Protein/creatinine ratio (g/mol)	nm	351 (117-809)
Gestational age (weeks)	38.6 (38.5-39)	30.4 (28.8-31.7)*
Caesarean section (n)	7/7	5/5
Male infants (n)	1/7	4/5
Birth weight (g)	3355 (2985-3555)	1170 (1115-1380)*
Birth weight (centile)	46 (16-74)	9 (3-44)
Placental weight (g)	625 (601-827)	339 (241-402)*

Data are presented as median (interquartile range). DBP = diastolic blood pressure; nm = not measured. *$p < 0.05$.

Table S4. Clinical characteristics of qPCR and Western blot analysis study in placentas obtained from healthy and PE pregnant women.

Characteristic	Healthy (n=12)	PE (n=7)
Maternal age (y)	35.5 (29-38.8)	30 (25-33)
Parity	1.5 (1-2)	0 (0-1)*
Caucasian ethnicity (n)	7/12	4/7
Body mass index (kg/m^2)	24.6 (22.4-33.7)	28.1 (22.8-33.2)
Smoking (n)	0/12	0/7
Highest DBP (mm Hg)	80 (73-82)	108 (95-115)*
Protein/creatinine ratio (g/mol)	nm	202 (31-580)
Gestational age (weeks)	39 (38.5-39.2)	29.6 (28.4-31.6)*
Caesarean section (n)	12/12	6/7
Male infants (n)	7/12	3/7
Birth weight (g)	3673 (3384-3975)	1150 (910-1300)*
Birth weight (centile)	78 (63-91)	13 (9-50)*
Placental weight (g)	711 (639-804)	291 (235-348)*

Data are presented as median (interquartile range). DBP = diastolic blood pressure; nm = not measured. *p<0.05.