# Transfer and vascular effect of endothelin receptor antagonists in the human placenta

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## ABSTRACT

Increasing evidence suggests a role for the endothelin (ET) system in preeclampsia (PE). Hence, blocking this system with endothelin receptor antagonists (ERAs) could be a therapeutic strategy. Yet, clinical studies are lacking due to possible teratogenic effects of ERAs. In this study we investigated the placental transfer of ERAs and their effect on ET-1-mediated vasoconstriction. Term placentas were dually perfused with the selective ET type A receptor ($ET_AR$) antagonists sitaxentan and ambrisentan or the non-selective $ET_AR/ET_BR$ antagonist macitentan and subsequently exposed to ET-1 in the fetal circulation. ET-1 concentration-response curves after incubation with sitaxentan, ambrisentan, macitentan or the selective $ET_BR$ antagonist BQ-788 were also constructed in isolated chorionic plate arteries using wire-myography, and gene expression of the ET-system was quantified in healthy and early onset PE placentas. At steady state, the mean fetal-to-maternal transfer ratios were 0.32±0.05 for sitaxentan, 0.21±0.02 for ambrisentan, and 0.05±0.01 for macitentan. Except for BQ-788, all ERAs lowered the response to ET-1, both in the perfused cotyledon and isolated chorionic plate arteries. Placental gene expression of ECE-1, $ET_AR$ and $ET_BR$ were comparable in healthy and PE placentas, while ET-1 expression was higher in PE. Our study is the first to show direct transfer of ERAs across the term human placenta. Furthermore, $ET_AR$ exclusively mediates ET-1-induced constriction in the fetoplacental vasculature. Given its limited transfer, macitentan could be considered as potential PE therapy. Extending knowledge on placental transfer to placentas of PE pregnancies is required to determine whether ERAs might be applied safely in PE.

## INTRODUCTION

Preeclampsia (PE) is a severe placenta-related pregnancy complication, characterized by de novo hypertension after 20 weeks of gestation, accompanied by evidence of maternal organ damage (e.g. proteinuria, elevated liver enzymes, pulmonary - or cerebral edema) and/or fetal growth restriction.[1] Besides increasing the risk of maternal and fetal morbidity and mortality during pregnancy, PE is associated with maternal and offspring health problems in later life.[2, 3] Over the last years, increased activity of the endothelin (ET) system has been recognized as a key factor in the pathogenesis of PE.[4] ET is a family of three potent vasoconstrictors (i.e. ET-1, -2 and -3), with ET-1 being the most abundantly synthesized by endothelial cells and syncytiotrophoblasts of the placenta.[5] Binding of ET-1 to the ET type A receptor ($ET_AR$) or ET type B receptor ($ET_BR$) on vascular smooth muscle cells leads to vasoconstriction and cell proliferation. In contrast, activation of $ET_BR$ on endothelial cells stimulates vasodilation through the release of nitric oxide and prostacyclin.[6] It has been previously shown that ET-1 plasma levels are increased in women with PE compared to healthy pregnancy, and that ET-1 is an independent predictor of proteinuria in PE.[7, 8] Also, a decrease in $ET_BR$ expression in vascular endothelial cells has been found in women with PE.[9] Similarly, PE animal models have shown a significant increase in the expression of the precursor peptide prepro-ET-1, likely leading to higher levels of ET-1, causing hypertension and renal dysfunction.[10-12] Blocking the effect of ET-1 with endothelin receptor antagonists (ERAs) alleviated maternal PE symptoms and improved fetal growth in animal studies.[13-16] However, developmental toxicity studies have also shown serious teratogenic effects, mainly craniofacial and cardiovascular malformations, in offspring of animals treated with ERAs during pregnancy, arguing against clinical trials in pregnant women.[17-19] It should be noted that teratogenic effects might be species-specific – indeed, certain drugs (e.g. corticosteroids) are known to be teratogenic in mice and rats but safe in humans.[20] Moreover, 39 cases of ERA use during pregnancy in women with pulmonary hypertension have been presented in the literature, and none of these reported teratogenic effects.[21] This raises the possibility that ERA treatment might still be an option for severe PE if applied later in pregnancy, thereby avoiding potential teratogenic effects. Since knowledge is lacking regarding the use of ERAs in human pregnancy, the aim of this study was to investigate the placental transfer of different ERAs making use of an *ex vivo* placental perfusion model, and to evaluate the effect of ERAs on ET-1 mediated vasoconstriction in the fetoplacental vasculature, comparing both healthy and PE placentas.

## METHODS

All supporting data are available within the article and in the Data Supplement.

### Patients and setting

Placentas of women with uncomplicated singleton pregnancies who underwent an elective cesarean section, or women with severe early onset PE (diagnosis ≤ 34 weeks of gestation[22]) were collected immediately after delivery at the Erasmus Medical Center, Rotterdam, the Netherlands. Baseline characteristics were obtained from the digital medical files. The study was exempted from approval by the local institutional Medical Ethics Committee according to the Dutch Medical Research with Human Subjects Law (MEC-2016-418 and MEC-2017-418). All women who donated their placenta provided written informed consent for the use of their placenta and personal data regarding their pregnancy.

### Perfusion experiments

The perfusion model used in the current study was previously described extensively by Hitzerd *et al*.[23] Perfusion experiments were conducted in healthy placentas only, given the extreme difficulty to successfully perfuse a preterm (PE) placenta.[23] In brief, maternal and fetal perfusion media consisted of Krebs-Henseleit buffer at 37ºC, supplemented with heparin (final concentration; 2500 IU/L) and aerated with 95% O2 - 5% CO2. The fetal circulation (closed-circuit; flow rate 6 mL/min) was established by cannulating the chorionic artery and corresponding vein of an intact cotyledon. Maternal circulation (closed-circuit; flow rate 12 mL/min) was created by placing four blunt cannulas in the intervillous space. At t=0, at a concentration of ~10x $C_{max}$, either one of the selective $ET_AR$ antagonists sitaxentan (100 mg/L,[24] a kind gift of dr. M. Iglarz, Actelion, Allschwill, Switzerland) or ambrisentan (10 mg/L,[25] Sigma-Aldrich Chemie, Schnelldorf, Germany), or the non-selective antagonist macitentan (2 mg/L,[26] a kind gift of dr. M. Iglarz) was added to the maternal circulation. Such high concentrations were chosen to prevent underestimation of transfer. To prove good overlap between maternal and fetal circulations antipyrine (100 mg/L) was also added to the maternal buffer. FITC-dextran (40 kDa, 36 mg/L) in the fetal circulation was used as a marker of integrity of the capillary bed. Samples of the maternal and fetal circulations were taken at eight set time points, and immediately stored at -80°C. After 180 min of perfusion, ET-1 (0.1-100 nmol/L) was added to the fetal circulation to construct a concentration-response curve (CRC). These concentrations are higher than the ET-1 concentrations observed in blood,[7] in agreement with the concept that ET-1 normally is synthesized locally, resulting in abluminal concentrations that are far above those in the circulation. An ET-1 CRC was also performed in placentas that were perfused for the same duration without an ERA, to serve

as controls. Changes in pressure were measured by pressure transducers and recorded using acquisition software (Biopac, Goleta, CA, USA).

### Quality control

An experiment was considered successful when the fetal-to-maternal (F/M) ratio of antipyrine was >0.75 and the maternal-to-fetal (M/F) ratio of FITC-dextran <0.03 at t=180.

### Analysis of antipyrine and FITC-dextran

For measuring antipyrine concentration, samples were first deproteinized with perchloric acid 6%, and subsequently 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. Absorption was measured at 350 nm using ultraviolet–visible spectroscopy (Shimadzu UV-1800). 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 endothelin receptor antagonists

Ambrisentan, macitentan and sitaxentan concentrations were measured in the perfusate by using UPLC-MS/MS. The method was validated in a linear range of 20.28 - 2028 µg/L for ambrisentan, 4.052 - 405.2 µg/L for macitentan and 205.4 – 10270 µg/L for sitaxentan. The method was successfully validated according to FDA guidelines and is used in our pharmacy laboratory for research and patient analysis.

### Wire-myography experiments

Second order branches of chorionic plate arteries of both healthy and PE placentas 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 aerated with 95% $O_2$ – 5% $CO_2$. Tension was normalized 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, vessel segments were incubated with sitaxentan (200 µmol/L), macitentan (3 µmol/L), ambrisentan (10 µmol/L) or the selective $ET_BR$ antagonist BQ-788 (10 nmol/L, Sigma-Aldrich Chemie, Schnelldorf, Germany). For sitaxentan, macitentan and ambrisentan the same concentrations were used as in the perfusion experiments (~10x $C_{max}$) and the concentration of BQ-788 was based on previous experiments with this antagonist.[27] Vessel segments without any inhibitor were used as control. After an incubation period of 30 minutes, CRCs to ET-1 (0.1-100 nmol/L) were constructed.

### Quantitative PCR (qPCR) Analysis

Gene expression levels of ET-1 (EDN1), $ET_AR$, $ET_BR$ and endothelin converting enzyme-1 (ECE-1) were measured with qPCR analysis. ECE-1 is an enzyme involved in converting the precursor ET-1 gene into biologically active ET-1.[6] After delivery of the placenta, pieces of placental tissue were immediately dissected from both the decidual ('maternal') and the amniotic ('fetal') side of the placenta, and were subsequently snap frozen in liquid nitrogen. As described previously by Hitzerd *et al.*,[23] small tissue pieces were homogenized in RLT lysis buffer (Qiagen, Venlo, the Netherlands) with β-mercaptoethanol for RNA extraction. Total RNA was extracted (RNeasy Fibrous Tissue Mini Kit, Qiagen) after proteinase K treatment (Invitrogen, Breda, the Netherlands) for ten minutes at 55°C. RNA was eluted in RNase free water and concentration and purity were assessed with a NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific, Bleiswijk, the Netherlands). Complimentary DNA (cDNA) was synthesized 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 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). The primer pairs used in this article are listed in Table S1. Target genes were normalized against the reference genes β-actin and Peptidylprolyl Isomerase A (PPIA) and relative gene expression was calculated by the ΔΔCt method. qPCR was performed according to 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 one min at 60°C.

### Statistical analysis

Data are presented as mean ± SEM for normally distributed data or median (interquartile range) in case of skewed distributions. Statistical analysis was performed with GraphPad Prism (version 5, La Jolla, CA, USA) and SPSS (version 21, SPSS Chicago, IL, USA) on Windows. To compare groups the Student's *t* test or Mann-Whitney U test (in case of non-normally distributed data) were used. For the comparison of continuous variables between more than two groups, one-way ANOVA or Kruskall-Wallis test (in case of skewed distributions) was applied, with a Dunnet or Bonferroni correction for multiple testing. A P-value of $<0.05$ was considered to be statistically significant.

## RESULTS

### Placental transfer of endothelin receptor antagonists

Forty-three women were initially included in the study. Twenty-three out of 43 cotyledons met the quality control criteria and were included in the analysis, leading to a success percentage of 53%, which is comparable to previous research from our lab.[23]

Maternal characteristics as well as clinical characteristics of the placentas and offspring are shown in Table 1. There were no significant differences between groups. At t=180 the mean F/M ratio for antipyrine was 0.92±0.01, indicating adequate overlap between fetal and maternal circulations (Figure S1). shows the placental transfer of sitaxentan, ambrisentan and macitentan. Only in the case of macitentan, the t=0 level was lower than its level thereafter, suggesting that the distribution across the maternal reservoir occurred somewhat slower than that of sitaxentan and ambrisentan. After 180 min of perfusion the F/M ratio for sitaxentan (n=5) was 0.32±0.05 (Figure 1A). At this steady state condition, only 33±4% of the total added sitaxentan concentration was recovered in the fetal and maternal circulations together. Adherence experiments (running the experiment without a placenta) showed only ~9% tube adherence, indicating that ~60% of the added sitaxentan had accumulated in the placental tissue. For ambrisentan (n=5), the F/M ratio was 0.21±0.02 and 80±6% of the starting concentration was retrieved after 180 min of perfusion (Figure 1B). No tubal adherence was observed. Only minimal amounts of macitentan (n=5) passed the placental barrier (F/M ratio 0.05±0.01), resulting in a fetal concentration of around 150 nmol/L (5%) after three hours of perfusion. Most of the added concentration (86±6%) was still detectable in the maternal and fetal circulations at t=180, therefore no tissue accumulation occurred (Figure 1C).

Since no albumin was used in the current setup, corrections for protein binding were applied by adjusting the F/M ratios following the method of Hill and Abramson (for the exact calculation see Supplemental Methods).[28, 29] For this method pKa values of the drugs were subtracted from literature.[30-32] No major changes were seen in the F/M ratios of sitaxentan, ambrisentan and macitentan (adjusted ratios 0.37, 0.25 and 0.06, respectively). The F/M ratios for all drugs are summarized in Table S2.

**Placental vascular reactivity**

After 180 min of perfusion, placentas were exposed to increasing concentrations of ET-1 in the fetal circulation to evaluate the effect of maternally applied ERAs on the fetoplacental vasculature. There was no significant difference in baseline pressure at start of the ET-1 curve between controls and ERA-exposed placentas (Figure 2A). After adding the highest concentration of ET-1 (100 nmol/L) to the fetal side of control placentas, the pressure was increased to 181±16 mm Hg (Figure 2A and 2B). Placentas that had been exposed maternally to sitaxentan, ambrisentan and macitentan all showed attenuated pressure increases to fetally applied ET-1 (pressures of 76±19, 88±32 and 120±15 mm Hg, respectively), which were significant for sitaxentan (P=0.002) and ambrisentan (P=0.01), but not macitentan (P=0.09, Figure 2A and 2B).

**Table 1.** Clinical characteristics of perfused placentas.

| Characteristic | Sitaxentan (n=5) | Ambrisentan (n=5) | Macitentan (n=5) | Control (n=8) |
|---|---|---|---|---|
| Maternal age (y) | 34 (33-37) | 30 (25-37) | 34 (28-35) | 35 (32-36) |
| Parity | 1 (1-2) | 1 (0.5-1) | 1 (1-3) | 1 (1-2) |
| Western ethnicity (n) | 2 | 2 | 3 | 4 |
| Body mass index (kg/m$^2$) | 26.0 (21.2-34.0) | 28.0 (20.8-32.5) | 21.8 (20.8-39.9) | 29.4 (22.5-34.6) |
| Smoking (n) | 0 | 0 | 0 | 1 |
| Highest DBP (mm Hg) | 80 (73-83) | 75 (65-81) | 80 (65-80) | 78 (71-83) |
| Gestational age (weeks) | 39 (39-40) | 39 (39-39) | 39 (39-39) | 39 (38-39) |
| Fetal sex (M/F) | 2/3 | 1/4 | 2/3 | 2/6 |
| Birth weight (g) | 3620 (3363-4078) | 3500 (3258-4085) | 3360 (3320-3828) | 3428 (3153-3610) |
| Birth weight (centile) | 63 (55-95) | 68 (44-94) | 52 (47-87) | 59 (43-63) |
| Placental weight (g) | 645 (597-790) | 618 (543-747) | 773 (616-827) | 631 (610-689) |

Data are presented as median (interquartile range). There were no significant differences between groups (Kruskal-Wallis test). DBP = diastolic blood pressure; F = female; M = male.

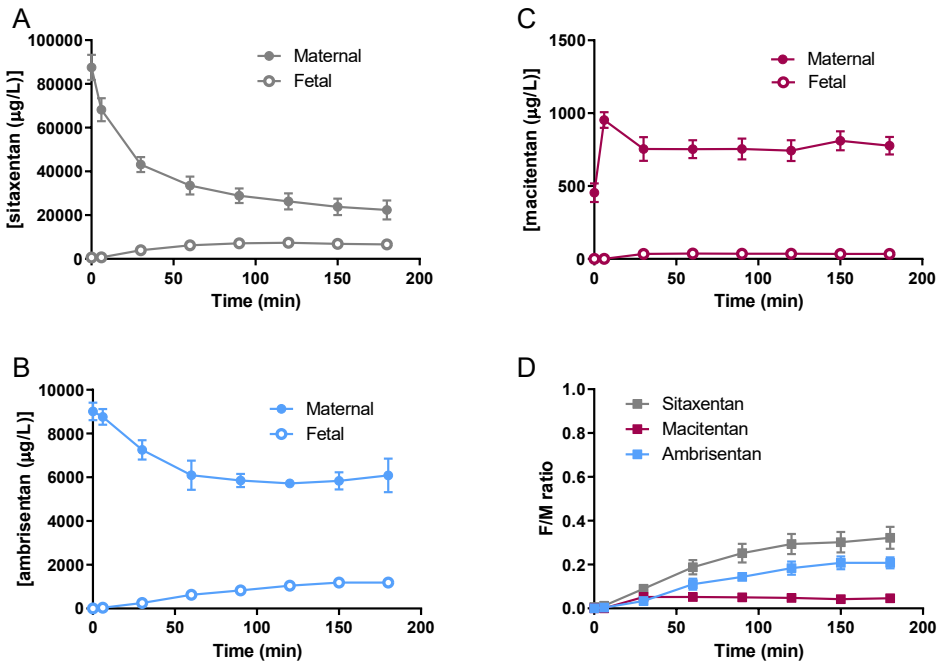


**Figure 1.** Placental transfer of sitaxentan (A), ambrisentan (B) and macitentan (C). Measured concentrations in the maternal (closed circles) and fetal (open circles) circulations are expressed as µg/L, n=5 per group. Panel D shows the fetal-to-maternal (F/M) transfer ratios over time.

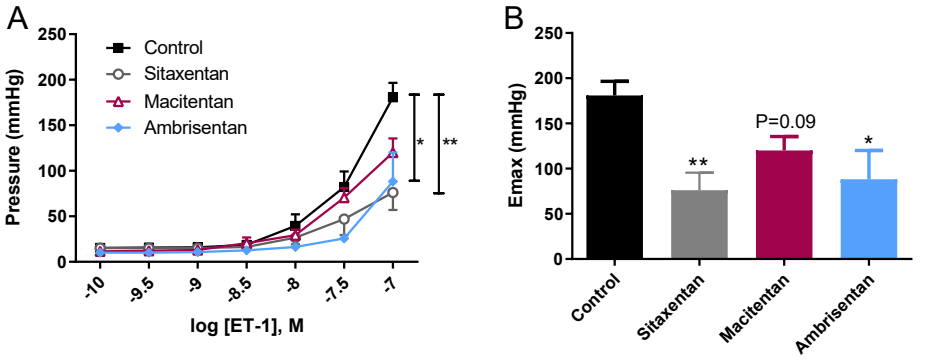


**Figure 2.** Panel A shows the concentration response curves for fetally applied endothelin-1 (ET-1) in the perfused cotyledon without (control, squares) or with prior exposure of the maternal circulation to the endothelin receptor antagonists sitaxentan (circles), macitentan (triangles) or ambrisentan (diamonds). The effect achieved at 100 nmol/L ET-1 is shown in panel B. Responses are expressed as mean±SEM of n=5-8. *P<0.05, **P<0.01 vs. control (one-way ANOVA with Dunnett post-hoc evaluation).

### Wire-myography experiments

Chorionic plate arteries of 11 healthy and five PE placentas were included in these experiments. The clinical characteristics of these placentas are shown in Table 2 and the results of these experiments are shown in Figure 3. At its highest concentration (100 nmol/L), ET-1 elicited a constriction corresponding with 132±9% of KCl constriction in control segments of healthy placentas (Figure 3A). Vessel segments that had been pre-incubated with sitaxentan, ambrisentan and macitentan all displayed a significant decreased response to 100 nmol/L (response 1±1, 2±1 and 10±3% of KCl constriction, respectively, P<0.0001 for all), whereas incubation with BQ-788 did not alter the response to 100 nmol/L ET-1 (130±14% of KCl constriction). Data on vessel segments that had been pre-incubated with different concentrations of macitentan are shown in Figure S2, and confirm the concentration-dependency of its blocking effect. Vessel segments of PE placentas displayed similar ET-1 responses as those of healthy placentas (Figure 3B). The effect of 100 nmol/L ET-1 in control segments was 112±38% of KCl constriction, compared to 1±1, 1±1 and 6±4% of KCl constriction for segments pre-incubated with sitaxentan, ambrisentan and macitentan, respectively (P<0.01 for all). There were no differences in the response to 100 nmol/L ET-1 between healthy and PE placentas (Figure 3C).

### Gene expression

Gene expression of ET-1 (EDN1), $ET_AR$ and $ET_BR$, but not ECE-1, was lower on the amniotic side of the placenta compared to the decidual side, both in healthy and PE placentas (Figure 4). In PE placentas, there was increased gene expression of ET-1 on both the decidual and amniotic side (P=0.02 and 0.06, respectively). No changes in the expression of $ET_AR$, $ET_BR$ and ECE-1 were observed in PE.

**Table 2.** Clinical characteristics of placentas used for wire-myography experiments.

| Characteristic | Healthy (n=11) | PE (n=5) |
|---|---|---|
| Maternal age (y) | 32 (28-34) | 29 (27-32) |
| Parity | 1 (1-3) | 0 (0-0.5)* |
| Western ethnicity (n) | 6 | 4 |
| Body mass index (kg/m$^2$) | 25.7 (22.3-30.5) | 24.9 (19.5-25.7) |
| Smoking (n) | 0 | 0 |
| Highest DBP (mm Hg) | 80 (75-80) | 103 (98-111)* |
| Gestational age (weeks) | 39 (39-40) | 30 (28-31)* |
| Fetal sex (M/F) | 5/6 | 3/2 |
| Birth weight (g) | 3410 (3200-3645) | 920 (708-1281)* |
| Birth weight (centile) | 52 (43-68) | 1 (0.3-2.3)* |
| Placental weight (g) | 654 (610-769) | 285 (243-395)* |

Data are presented as median (interquartile range). *P<0.05, Mann-Whitney U test. DBP = diastolic blood pressure; F = female; M = male; PE = preeclampsia.

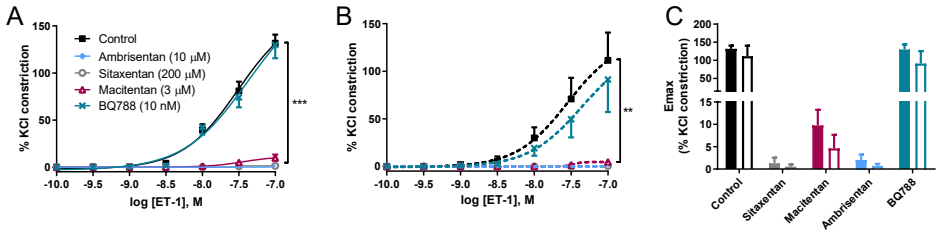


**Figure 3.** Vascular responses of isolated chorionic plate arteries of healthy (A) and preeclamptic (B) placentas to endothelin-1 (ET-1) in the absence (control, squares) or presence of sitaxentan (circles), macitentan (triangles), ambrisentan (diamonds) or BQ-788 (cross marks). Panel C shows the response to 100 nmol/L ET-1 of healthy (closed bars) compared to preeclamptic (open bars) placentas. Responses are expressed as mean±SEM of n=5-11. *P<0.05, **P<0.0001 vs. control (one-way ANOVA with Dunnett post-hoc evaluation).

## DISCUSSION

This study is the first to show direct transfer of ERAs across the human placental barrier. Importantly, the transfer of macitentan was limited (F/M ratio <0.1), while that of sitaxentan and ambrisentan was substantial although no accumulation occurred (F/M ratio <1.0). In line with this, only sitaxentan and ambrisentan (when applied maternally) significantly reduced the contractile effect of fetally applied ET-1 in the cotyledon setup. Yet, all antagonists, when applied at a concentration corresponding with 10 times $C_{max}$ to isolated chorionic plate arteries, were capable of fully blocking the contractile effect of ET-1. In contrast, the selective $ET_BR$ antagonist BQ-788 did not affect the ET-1 response, arguing against a role for $ET_BR$. No differences were observed in the vascular

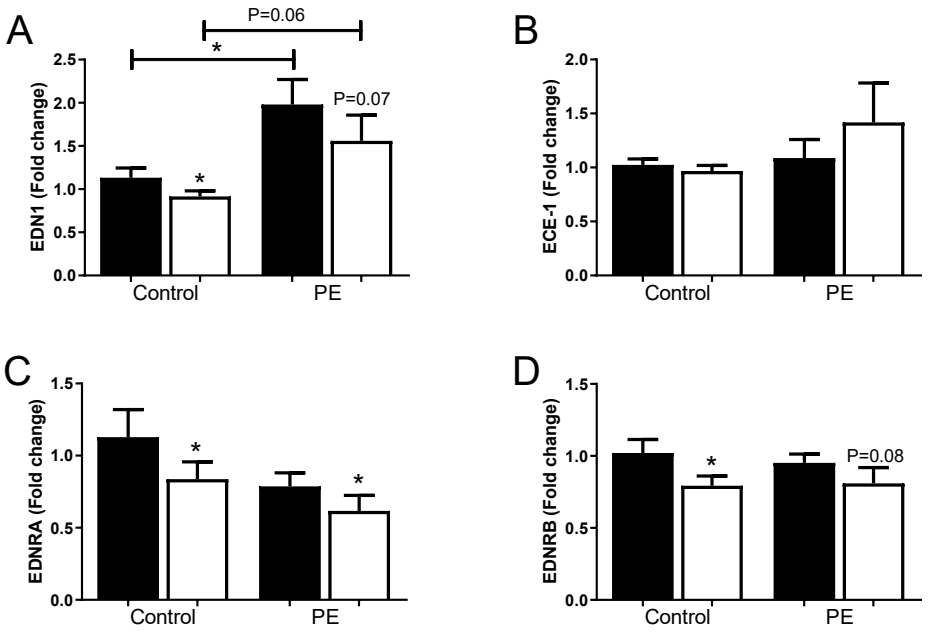


**Figure 4.** Gene expression of endothelin-1 (A), endothelin-converting enzyme-1 (B), endothelin-receptor type A (C) and endothelin-receptor type B (D) in healthy and preeclamptic placentas (n=12 per group). Expression was measured on both the decidual side (black bars) and amniotic side (white bars) of the placenta. Data are expressed as fold change of control samples from the decidual side. *P<0.05 (unpaired or paired Student's t-test where appropriate).

response to ET-1 between healthy and PE placentas. Gene expression of ET-1 and its receptors was lower on the amniotic side of the placenta compared to the decidual side. Furthermore, PE placentas showed an increased expression of ET-1, while no changes in the expression of $ET_AR$, $ET_BR$ and ECE-1 were found.

A significant role for the ET-system has been implicated in the pathogenesis of PE, contributing to hypertension, endothelial- and renal dysfunction with proteinuria.[7, 33] Aside from increased circulating ET-1 levels in preeclamptic women, placental ET-1 levels are similarly elevated, which may account for the increased placental vascular resistance *in vivo*.[8] Thus, administration of ERAs may prove beneficial through alleviation of maternal symptoms and by improving vascular resistance in the placenta. Interestingly, we and others found no difference in ET-1 induced vasoconstriction between chorionic plate arteries of healthy and PE placentas, indicating that there is no altered response to ET-1 during PE. Moreover, while administration of an ERA significantly attenuated ET-1 induced vasoconstriction, this effect was not different between healthy or PE placentas. Taken together, these data, obtained in two different models (the perfusion setup, representing predominantly microcirculatory vasculature, and the myography

setup, involving larger second-order arteries) imply that elevated ET-1 levels, but not an enhanced response, accounts for the increased placental vascular resistance during PE. Indeed, we observed elevated expression of the ET-1 gene in PE placentas, while $ET_AR$ and $ET_BR$ expression did not differ between healthy and PE placentas. In a previous study by Benoit *et al*. blockade of $ET_AR$, but not $ET_BR$ reduced vasoconstriction in healthy placentas when they were exposed to extracts from PE placentas.[34] In the current study, we observed similar results regarding exposure to ET-1, indicating that ET-1-induced vasoconstriction in the fetoplacental vasculature is exclusively mediated by $ET_AR$. It should be mentioned however, that we were not able to extend these studies towards uterine spiral arteries, since we did not obtain myometrial biopsies.

Given the observed fetotoxicity of ERAs in developmental toxicity studies in animals, no clinical trials in pregnant women have been performed with these drugs, and therefore knowledge regarding the placental transfer of ERAs in humans is virtually non-existent. However, the placenta is the most species-specific organ, making direct translation of the results of animal studies to humans challenging.[35] As treatment for PE is generally started in the second or third trimester, i.e. when fetal organogenesis has already been completed, one should not disregard ERAs as potential treatment for PE. Moreover, sporadic cases of pregnant women with pulmonary arterial hypertension (PAH) using ERAs in the second or third trimester did not report an increased incidence of fetal birth defects.[21] Importantly, it should be noted that sitaxentan has been withdrawn from therapeutic use in 2010 due to drug-induced hepatotoxicity, while ambrisentan and macitentan are both registered for the treatment of PAH.

A striking finding of our study is the limited transfer observed of the non-selective antagonist macitentan. Whereas all three ERAs used in this study have low molecular weights (<600 g/mol) and lipophilic properties, which in general would favor placental transfer,[36] macitentan is characterized by sustained receptor binding and enhanced tissue penetration.[37] However, this does not seem to explain the limited transfer, since 86% of the starting concentration was recovered at the end of an experiment in the fetal and maternal circulations together. Although the absence or presence of albumin in the perfusion system should not affect the F/M ratio at steady state,[38] to better predict *in vivo* fetal exposure to ERAs we also corrected the results of the current study for protein binding. Yet, this did not alter the outcome of the study, nor did lowering the fetal pH to 6.9.

Despite its almost absent placental transfer, the fetal vascular bed of the macitentan-perfused placentas did display a (non-significant) decrease in contractile response to ET-1 of approximately 34%. Here it should be noted that we applied a maternal macitentan concentration of 3 µmol/L, resulting in a fetal concentration of around 150 nmol/L (5%) after three hours of perfusion. Wire-myography experiments with chorionic plate arteries in which different concentrations of macitentan were used, revealed that

such a reduction would indeed be expected at 150 nmol/L (Figure S2). The approved macitentan dosage for PAH treatment is 10 mg per day,[39] and with this dosage the $C_{max}$ in plasma of (non-pregnant) Caucasian women is 234 ng/mL, or, at a MW of 588, 0.4 µmol/L.[26] If indeed 5% of this concentration would reach the fetus (~20 nmol/L), it cannot be excluded that a modest degree of blockade occurs in the fetus. Blocking the ET-system in the fetus could lead to undesirable effects, since ET-1 plays an important role in the maintenance of high vascular resistance crucial for fetal lung development.[40] The $ET_AR$ is abundantly expressed in fetal lungs during pre- and postnatal periods, and lung maturation is not completed at the end of third trimester.[41] On the other hand, $ET_AR$ blockade could prove beneficial, since elevated serum ET-1 levels are observed in babies that are born from PE pregnancies.[42] Moreover, one of the standard treatments for neonates with pulmonary hypertension is the non-selective ERA bosentan.[43, 44] Although no long-term follow-up studies have been performed, short-term follow-up showed no adverse effects on lung- and brain development in these children.[43] Another point of concern could be patency of the ductus arteriosus after birth. However, the evidence regarding the effect of ERAs on closure of the ductus is conflicting. Although it has been shown that oxygen-triggered ET-1 release regulates closure of the ductus through binding to $ET_AR$ on vascular smooth muscle cells,[45] $ET_AR$ blockade does not seem to prevent the ductus from closing.[46, 47]

## PERSPECTIVES

Given its key role in the pathogenesis of PE, targeting the ET-system would be an interesting approach in the treatment of this severe placenta-related disease. This study was the first to evaluate transfer of ERAs across the human placenta. Since macitentan only displayed very limited placental transfer, and is already registered for treatment of PAH, it could be a promising drug to further investigate for PE treatment. Expanding this knowledge to early onset PE placentas is needed to further evaluate whether it can be safely applied in pregnancy. Furthermore, third-trimester toxicology studies in animals with a longer gestation than rodents are warranted. Subsequently, as described previously,[21] we would suggest a proof of principle study in women with severe early onset PE (< 24 weeks of gestation), when medically indicated termination of pregnancy is considered because of disease severity, to evaluate the effect on maternal PE symptoms and neonatal outcome.

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## SUPPLEMENTAL INFORMATION

### Methods

*Protein binding adjustment*

Albumin was not added to the experimental system, since it is very difficult to mimic exact physiological concentrations. Although the F/M ratio of the free drug concentration at steady state should not be affected by the presence or absence of albumin,[1,2] the obtained F/M ratios were adjusted for protein binding to estimate the fetal exposure as adequate as possible. The ratios were adjusted using the following formula:[3]

$$\text{F/M ratio} = \frac{\%\,\text{unbound}_M}{\%\,\text{unbound}_F} \times \frac{1+10^{pKa\,-\,pH(F)}}{1+10^{pKa\,-\,pH(M)}} \times \frac{CL_{MF}}{CL_{FM}+CL_f}$$

In this formula the differences in protein binding between both circulations are taken into account, as the % $\text{unbound}_F$ and the % $\text{unbound}_M$ represent the free drug concentrations in the maternal and fetal circulations, respectively. pKa is the acid-base dissociation constant of the drug, and pH(F) and pH(M) are the pH values of fetal (7.35) and maternal (7.40) blood, respectively. The last part of the equation stands for drug clearance (MF = maternal-to-fetal, FM = fetal-to-maternal and f = fetus). In the closed perfusion setup, $CL_{MF}/CL_{FM}$ can be taken as the F/M ratio at steady state. The assumption was made that clearance by the fetus was negligible. Because ERAs are strictly contraindicated, there is no available data regarding the % protein binding in plasma of pregnant women and their fetus. However, it is known that in non-pregnant adults all three ERAs used in the current study bind for ~99% to albumin. With this information, protein binding in maternal and fetal plasma could be estimated using the method of Hill and Abramson:[1]

$$\%\,\text{unbound} = 100 - \frac{100 \times (B/F)}{(B/F)+1}$$

B/F is the ratio between bound (B) and free (F) drug concentrations. This can be calculated for the maternal and fetal circulations, using the known B/F of non-pregnant adults and the plasma protein ratios for albumin (fetal:non-pregnant adult ratio is 0.866 and the maternal:non-pregnant adult ratio is 0.733.[1]

$$(B/F)_{F\text{ or }M} = (B/F)_{\text{non-pregnant}} \times (\text{Plasma albumin ratio})_{F\text{ or }M}$$

All three ERAs have a B/F of 99 in non-pregnant adults. From this we calculated the B/F in the fetal (85.7) and maternal (72.6) circulations, leading to unbound fractions of 1.2% and 1.4%, respectively. The pKa of macitentan is 6.2, of ambrisentan 3.5 and of sitaxentan 5.0,[4-6] leading to corrected F/M ratios of 0.06, 0.25 and 0.37, respectively.

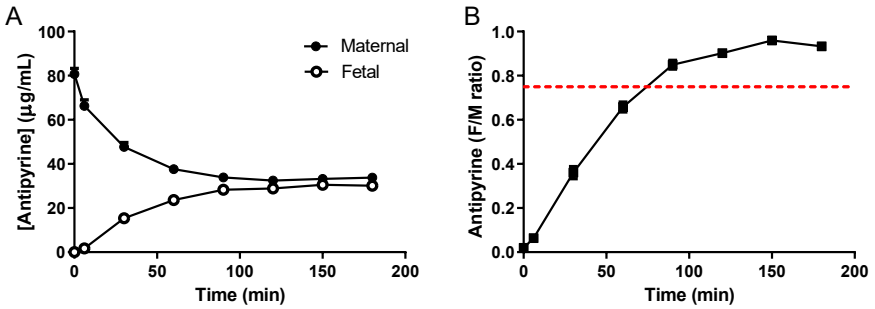


**Figure S1.** Antipyrine transfer. Panel A shows the mean maternal (closed circles) and fetal (open circles) concentrations of antipyrine, proving good overlap between both circulations. Panel B shows the mean fetal-to-maternal transfer ratio, with the cutoff point of 0.75 (red dashed line).

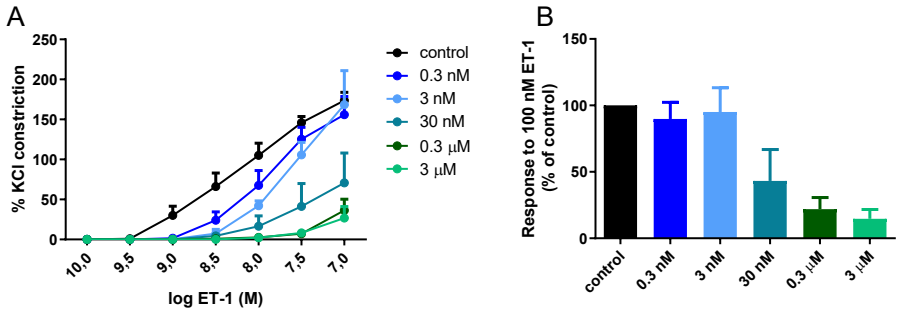


**Figure S2.** Blocking effect of different macitentan concentrations in chorionic plate arteries. Concentration-response curves to endothelin (ET)-1 are shown in the absence (control) or presence of different macitentan concentrations (A). Panel B shows the blocking effect of those different concentrations expressed as % of maximum contraction to 100 nmol/L ET-1 in control segments.

**Table S1.** qPCR Primer Sequences.

| Genes | Forward (5′ - 3′) | Reverse (5′ - 3′) |
|---|---|---|
| EDN1 | AAGACAAACCAGGTCGGAGAC | GTCACCAATGTGCTCGGTTG |
| EDNRA | GTATTTAAGCTGCTGGCTGGG | GAGGTTGAGGACGGTGATCC |
| EDNRB | ATCACCTAAAGCAGAGACGGG | AGAATCCTGCTGAGGTGAAGG |
| ECE-1 | AAGCTCCTTCCTTGACCAGC | GACAGGTCTTCTTGGTCCCG |

**Table S2.** F/M ratios according to different drugs.

| ERA | F/M ratio | Adjusted F/M ratio |
|---|---|---|
| Sitaxentan | 0.32 | 0.37 |
| Ambrisentan | 0.21 | 0.25 |
| Macitentan | 0.05 | 0.06 |

Fetal-to-maternal (F/M) ratios are shown for sitaxentan, ambrisentan and macitentan. Adjusted F/M ratio indicates correction for protein binding using the method of Hill and Abramson.

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