TOWARDS NEW THERAPIES FOR PREECLAMPSIA

Restoring vascular dysfunction

Emilie Hitzerd

Towards New Therapies for Preeclampsia: Restoring Vascular Dysfunction

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Towards New Therapies for Preeclampsia: Restoring Vascular Dysfunction

Op weg naar nieuwe therapieën voor pre-eclampsie: Herstel van vasculaire disfunctie

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CONTENT

Chapter 1

General introduction, aims and outline

GENERAL INTRODUCTION

Preeclampsia (PE) is a severe placenta-related pregnancy disorder, that complicates around 5-8% of pregnancies.[1] PE can have detrimental consequences during pregnancy for the mother, as it increases the risk of for example stroke, liver rupture, and lung edema. It also contributes significantly to perinatal morbidity, as approximately 12% of PE cases is complicated by fetal growth restriction, and around 20% results in preterm birth.[2] Besides these short term consequences, PE is also associated with health risks later in life for both mother and child. Women who have suffered from PE have a 2-4 times higher risk of developing cardiovascular diseases.[3, 4] Furthermore, prematurity and low birth weight increase the risk of cardiopulmonary and neurological impairment in children.[5, 6]

Nowadays PE is defined as de novo hypertension after the 20th week of gestation, along with evidence of maternal organ damage and/or fetal growth restriction.[7] However, as early as 400 BC, Hippocrates already wrote that 'a headache accompanied by heaviness and convulsions during pregnancy is considered bad'.[8] In that time it was thought that all female diseases resulted from an imbalance in body fluids, and therefore treatments existed of dietary changes, purging and blood-letting.[9] In the 17th century, the word 'eclampsia' arose and the disease was systematically described for the first time. In an attempt to prevent convulsions, phlebotomies during pregnancy were recommended.[10] In the 19th century, the disease was called 'toxemia', as it was thought that it resulted from the inability to eliminate an increase in waste products.[11] Treatment still mainly existed of bleeding and purging, in order to eliminate the excess of toxic elements. It was not until the 20th century that the pathophysiology of PE was linked to the abnormal placentation in early pregnancy.[12] Although still not fully elucidated, it is now known that increased placental vascular resistance leads to an imbalance in pro - and antiangiogenic factors, causing generalized endothelial dysfunction.[13, 14] The latter is characterized by disturbances in different vascular pathways, e.g. decreased activity of the nitric oxide pathway and increased activity of the endothelin system.[15, 16] Furthermore, there is an imbalance in the immune system response, with a shift towards pro-inflammatory conditions.[17, 18]

To date, still no cure is available for PE, except for delivery of the placenta. Patients are often stabilized with antihypertensive drugs, such as methyldopa and/or calcium antagonists, and magnesium sulfate to prevent further complications and prolong pregnancy as long as possible. Targeting the vascular endothelial dysfunction in PE is a promising strategy in developing new therapeutic options.

The *ex vivo* placental perfusion system

For the research described in this thesis we used dual-sided *ex vivo* placental perfusion of isolated cotyledons, an established experimental model to study the transfer of drugs across the human placenta, their subsequent effects and placental metabolism. It is the only reliable method to predict *in vivo* fetal exposure to maternally administered compounds without imposing risks on mother or child.[19, 20] The model was first described in 1967 by Panigel *et al.*[21] and later modified by Schneider *et al.* in 1972.[22] The model used for the studies in this thesis has been adapted from the model described by Schalkwijk *et al.*[23] It consists of a plastic perfusion chamber and two peristaltic roller pumps. Heating devices and a water bath keep the temperature in the setup at 37 °C. Maternal and fetal perfusion media are aerated continuously with 95% O_2 – 5% CO_2. After arrival at the lab directly after birth, an intact cotyledon is selected and the corresponding chorionic artery and vein pair is cannulated to establish the fetal circulation. Subsequently, the cotyledon is cut from the rest of the placenta and placed inside the perfusion chamber. Maternal circulation is created by placing four blunt cannulas in the intervillous space and outflow from the intervillous space is collected in a reservoir. Changes in fetoplacental pressure and pH are recorded using acquisition software. A schematic overview of our placental perfusion model is shown in Figure 1.

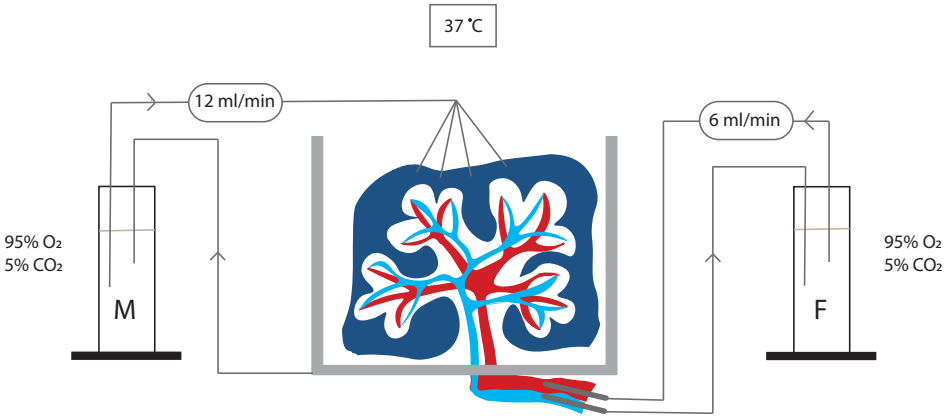

Figure 1. Schematic overview of the placental perfusion model in our lab.

AIMS AND OUTLINE OF THE THESIS

In this thesis new treatment strategies for PE are discussed, targeting the placental vascular dysfunction that plays a key role in the pathogenesis of this disease. By using *ex vivo* techniques, such as perfusion of the isolated cotyledon and wire-myography, the human placenta is studied without the risk of harming either mother or fetus.

The aims of this thesis are:

1. To understand placental vascular reactivity in health and disease
2. To evaluate possible new treatment options for PE, using *ex vivo* placental perfusion

Chapter 2 provides an extensive overview of the vascular reactivity profile of the human placenta. It summarizes the most important pathways and factors that are involved in the regulation of (placental) vascular function during healthy pregnancy and changes associated with PE. Furthermore, potential treatment strategies interfering with these changes are discussed. **Chapters 3-6** focus on experimental therapeutic strategies targeting the vascular dysfunction seen in PE. In **Chapter 3** we study the transfer and effect of the phosphodiesterase-5 inhibitor sildenafil in placentas from healthy and PE pregnancies. In **Chapter 4** we try to elucidate the vasodilator mechanism of pentoxifylline by studying its effect in porcine coronary arteries and human chorionic plate arteries. **Chapter 5** discusses the possibility of using endothelin receptor antagonists for PE treatment. It includes an overview of all cases reported in literature of women who were exposed to these drugs during pregnancy. Following this, in **Chapter 6** we examine the transfer and effects of the endothelin receptor antagonists macitentan, sitaxentan and ambrisentan in the human placenta. **Chapter 7** explores the associations between first-trimester *in vivo* placental vascular parameters and *ex vivo* placental vascular function at term. **Chapter 8** provides a general discussion and suggestions for future research, and in **Chapter 9** this thesis is summarized.

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Chapter 2

Human placental vascular reactivity in health and disease: implications for the treatment of preeclampsia

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ABSTRACT

Adequate development of the placenta is essential for optimal pregnancy outcome. Pre-eclampsia (PE) is increasingly recognized to be a consequence of placental dysfunction and can cause serious maternal and fetal complications during pregnancy. Furthermore, PE increases the risk of neonatal problems and has been shown to be a risk factor for cardiovascular disease of the mother later in life. Currently, there is no adequate treatment for PE, mainly because its multifactorial pathophysiology remains incompletely understood. It originates in early pregnancy with abnormal placentation and involves a cascade of dysregulated systems in the placental vasculature. To investigate therapeutic strategies it is essential to understand the regulation of vascular reactivity and remodeling of blood vessels in the placenta. Techniques using human tissue such as the *ex vivo* placental perfusion model provide insight in the vasoactive profile of the placenta, and are essential to study the effects of drugs on the fetal vasculature. This review highlights the different pathways that are involved in the vascular regulation of the human placenta, changes that occur during PE and the importance of focusing on restoring these dysfunctional systems when studying treatment strategies for PE.

INTRODUCTION

The placenta is an essential regulatory organ that provides the fetus with nutrients and oxygen, determines the passage of pharmacological and toxic agents, and regulates the endocrine and immune systems.[1] Optimal placental function is crucial for fetal health and subsequent neonatal outcome. Insufficient development of the placenta is increasingly recognized to underlie serious pregnancy complications such as preeclampsia (PE) and fetal growth restriction (FGR), thereby contributing to both maternal and perinatal morbidity and mortality.[2-4]

PE is a multi-system disorder, clinically characterized by de novo onset hypertension and proteinuria after 20 weeks of gestation, which complicates approximately 3-8% of pregnancies.[3] Two forms of PE can be distinguished: early onset PE, manifesting before the 34th week of gestation, and late onset PE, manifesting after the 34th week of gestation.[5] PE can have serious maternal consequences, such as kidney failure, liver disease, cerebral hemorrhage and lung edema, but can also result in FGR and/or premature birth. Evidence is accumulating that placenta-related pregnancy complications increase the risk of maternal and neonatal health problems in later life. For example, women who have suffered from PE have a higher chance of developing cardiovascular diseases,[6, 7] and children born prematurely or with low birth weight have an increased risk of impaired cardiopulmonary and neurological development.[8-10] Currently, there is no appropriate therapy available for PE. Treatment is aimed at prolonging pregnancy by symptom relief and prevention of further complications, but the only cure is delivery. However, this is often harmful for the preterm fetus, and therefore development of novel treatment options to safely prolong pregnancy is very important.

Although the exact etiology of PE remains unknown, a large body of evidence indicates that it originates in the first weeks of pregnancy and is the result of abnormal placentation. Initially, impaired trophoblast invasion leads to aberrant remodeling of the spiral arteries, resulting in higher placental vascular resistance and hypo-perfusion, with oxidative stress related to ischemia-reperfusion damage and increased placental production of anti-angiogenic factors. The combination of these factors induces generalized vascular dysfunction, thereby contributing to the hypertension and proteinuria occurring in PE patients.[11-13] Since early- and late onset PE show distinct histopathological differences,[5] separate pathogenic pathways have been suggested, proposing that late onset PE might be a predominant maternal syndrome, rather than a placental disorder.[14, 15]

Many vasoactive pathways have been proposed to be altered in PE, although data on the human placental vasculature are largely inconclusive. To investigate therapeutic strategies it is essential to understand the regulation of vascular tone and remodeling of blood vessels in the healthy placenta, and changes in PE. Research using the *ex vivo*

placental perfusion system or wire-myography, two models to study human tissue, may provide insight into vasoactive characteristics of the placenta. *Ex vivo* dual-sided placental perfusion is an experimental model to study vascular reactivity of the fetal side of the placenta in a single cotyledon, and using wire-myography similar experiments can be performed in isolated vessel segments.

The purpose of this review is to summarize the vascular reactivity profile of the fetal side of the human placenta, by describing its most important pathways and factors, being vascular endothelial growth factor (VEGF), the endothelin (ET) system, the renin-angiotensin system (RAS), prostaglandins, nitric oxide (NO) and NO-dependent vasodilators, serotonin and tryptophan (Trp), and calcitonin gene-related peptide (CGRP). Relevant studies were identified by making use of a systematic search in literature on 20 April 2018. We highlight changes that occur during, and/or contribute to the pathophysiology of PE, and discuss possible treatment strategies based on interfering with the regulation of placental vascular tone.

DEVELOPMENT OF THE FETOPLACENTAL VASCULATURE

Adequate development of the placental vasculature is essential for normal growth and development of the fetus during pregnancy.[1] Many pregnancy complications are associated with disturbed placentation, such as PE, FGR, preterm birth and spontaneous abortion.[16] At term, the placenta consists of cotyledons, i.e., villous trees that are supplied by arteries branching off the umbilical circulation. These are surrounded by intervillous space filled with maternal blood coming from spiral arteries, to enable the exchange of oxygen and nutrients between mother and fetus.[1]

During embryonic implantation, the outer layer of the blastocyst, the trophectoderm, invades deep into the uterine wall. The trophectoderm differentiates into multiple types of trophoblast cells, including cytotrophoblast cells and, when these cells fuse, syncytiotrophoblast cells.[17] The process of differentiation is closely regulated by multiple growth factors, hormones and environmental factors such as oxygen tension.[18] The syncytiotrophoblast layer covers the cytotrophoblast and lines the villous trees, making direct contact with the maternal circulation and thereby playing an important role in the supply of oxygen and nutrients from mother to fetus, and waste products and carbon dioxide from fetus to mother. Furthermore, these cells are involved in pregnancy-related hormone production.[17] In early gestation, when there is no direct exchange of oxygen and nutrients between the fetal and maternal circulations, the embryo is provided with nutrients via diffusion from the decidua.[19] At this stage, cytotrophoblast plugs occlude the spiral arteries, allowing diffusion but preventing perfusion of the intervillous space, this way keeping a low oxygen environment.[20] Around 10 weeks of gestation, extravillous

cytotrophoblast cells invade around the spiral arteries, initiating their remodeling and unplugging. This remodeling encompasses a 5- to 10-fold increase in terminal lumen diameter and structural changes of the vessel wall, such as demuscularization, creating a low resistance circulation.[12, 18] Because of these changes, the perfusion capacity is increased and the blood velocity into the intervillous space is reduced to protect the vulnerable villous tree and to optimize exchange of oxygen and waste products.[12] After the vascular network has been formed in early pregnancy, capillary growth continues until delivery, mediated by various growth factors. From mid-gestation onwards, there is an exponential growth in vascular volume of placental vessels to accommodate the needs of the growing fetus.[1] Unlike most other blood vessels, vessels of the fetoplacental circulation are not innervated. Local vascular tone and fetal cardiac output are the main determinants of the blood flow through these vessels, regulated by circulating and locally produced hormones and vasoactive compounds, such as estrogen, prostaglandins, ET-1 and NO.[21] The most important pathways that are involved in the regulation of placental vascular tone and changes occurring in PE, as will be discussed in this review, are summarized in Table 1. Drugs targeting these systems and their potential relevance in the treatment of PE are described in Table 2.

VASCULAR ENDOTHELIAL GROWTH FACTOR

The VEGF pathway plays a pivotal role in the formation and development of blood vessels during growth of the human placenta.[22] The VEGF system has not only been implicated in the promotion of angiogenesis, but is also crucial to maintain vascular endothelial function. VEGF induces the release of NO and prostacyclin (PGI_2) from endothelial cells, indicating its potential in the regulation of vascular tone (Figure 1).[23, 24] VEGF acts in a paracrine manner on endothelial cells, and its expression has been localized to villous trophoblast cells, maternal and fetal macrophages, decidual cells and the fetal membranes of the placenta.[23, 25] Its biological actions are elicited upon binding to tyrosine kinase receptors (VEGFRs), of which VEGFR-1 and VEGFR-2 are considered the main functional receptors in the placenta. VEGFR-2 plays a major role in angiogenesis through promoting endothelial cell proliferation and vascular formation. Its location is restricted to fetal endothelial cells and syncytiotrophoblasts.[26] VEGFR-1 is expressed in fetal and umbilical vein endothelial cells, decidual cells and (extra)villous trophoblast cells.[27] While the function of VEGFR-1 is not entirely understood, it is thought that it regulates angiogenesis either positively or negatively through mechanisms involving VEGF-trapping and homo- or heterodimerization with VEGFR-2.[28-30] Placental growth factor (PlGF), which is homologous to VEGF, is considered equally important in the regulation of placental angiogenesis, through its interaction with VEGFR-1.[23] In addition,

Table 1. Vascular reactivity pathways in the human placenta and changes occurring during preeclampsia.

Pathway	Function in normal pregnancy	Changes in maternal plasma during PE	Changes in placental tissue during PE
VEGF	Promotion of angiogenesis;	Decreased levels of VEGF and PlGF;	Increased gene expression of VEGF;
	Maintaining vascular endothelial function;	Increased levels of sFlt-1	No change in PlGF gene expression;
	Stimulating release of NO and PGI_2		Increased gene expression of sFlt-1
Endothelin	Vasoconstriction;	Increased levels of ET-1, MMPs and ECE	Increased gene expression of ET-1 and MMP-2;
	Promotion of trophoblast proliferation and invasion;		Decreased gene expression of ET_A receptor;
	Initiation of uterine contractions		Increased/decreased gene expression of ET_B receptor
RAS	Regulation of vascular tone;	Decreased levels of renin, Ang I, Ang II and aldosterone;	Increased levels of angiotensinogen and Ang II;
		Increased Ang II sensitivity;	
	Sodium homeostasis	Increased levels of AT_1R-AA;	Increased expression of AT_1R;
		Decreased AT_2R expression	Upregulation of ACE and chymase
Prostaglandins	Vasoconstriction (TxA_2, PGE_2, $PGF_{2\alpha}$);	Increased levels of TxA_2 and lipid peroxides;	Increased production of TxA_2;
	Stimulation of platelet aggregation and uterine contractility (TxA_2);		Reduces release of PGI_2;
	Vasodilation and inhibition of platelet aggregation and uterine contractility (PGI_2)	Decreased levels of PGI_2	Increased expression of COX-1
Nitric oxide	Vasodilation	Decreased levels of NO metabolites and NO-mediated vasodilators;	Increased gene expression of eNOS
		Increased levels of ADMA	Increased levels of peroxynitrite
Bradykinin	Vasodilation through stimulation of NO and PGI_2 release;		Reduction of gene - and protein expression of B2 receptor
	Vasoconstriction through stimulation of TxA_2 production;		
	Stimulation of cell migration and trophoblast invasion		
Acetylcholine	Influencing placental transfer of amino acids;		Decreased synthesis of acetylcholine;
	Influencing placental hormone release		Decreased density of mAChR;
			Increased expression of nAChR

Table 1. Vascular reactivity pathways in the human placenta and changes occurring during preeclampsia. (continued)

Pathway	Function in normal pregnancy	Changes in maternal plasma during PE	Changes in placental tissue during PE
Histamine	Promotion of trophoblast proliferation and invasion; Vasoconstriction (through H1 receptor); Vasodilation (through H2 receptor)		Increased tissue concentrations of histamine; Higher mast cell density; Reduced sensitivity to histamine
Serotonin	Vasoconstriction	Increased levels of serotonin	Increased sensitivity to serotonin in microvasculature
Tryptophan	Vasodilation through metabolism by IDO1		Reduced expression and activity of IDO1
CGRP	Influencing vascular adaption; Maintaining uterine relaxation during pregnancy; Vasodilation	Decreased levels of CGRP	Reduced mRNA and protein expression of CRLR and $RAMP_1$

Abbreviations: ACE = angiotensin converting enzyme; ADMA = asymmetric dimethylarginine; Ang I = angiotensin I; Ang II = angiotensin II, AT_1R = angiotensin II type 1 receptor; AT_1R-AA = angiotensin II type 1 receptor auto-antibodies; AT_2R = angiotensin II type 2 receptor; B2 = bradykinin receptor; CGRP = calcitonin gene-related peptide; COX-1 = cyclooxygenase-1; CRLR = calcitonin receptor-like receptor; ECE = endothelin converting enzyme; eNOS = endothelial nitric oxide synthase; ET-1 = endothelin-1; ET_A = endothelin-1 type A; ET_B = endothelin-1 type B; H1 = histamine type 1; H2 = histamine type 2; IDO1 = indolamine 2,3-dioxygenase; mAChR = muscarinic acetylcholine receptor; MMP = matrix metalloproteinase; nAChR = nicotinic acetylcholine receptor; NO = nitric oxide; PE = preeclampsia; PGI_2 = prostacyclin; PlGF = placental growth factor; $RAMP_1$ = receptor activity modifying protein-1; sFlt-1 = soluble Fms-like tyrosine kinase-1; TxA_2 = thromboxane A_2; VEGF = vascular endothelial growth factor.

Table 2. (Potential) Treatment strategies to target the dysfunctional placental vascular reactivity in preeclampsia.

Target	Therapy	Mechanism of action	Preclinical studies	Clinical trials
VEGF	Recombinant VEGF	Supplementation of VEGF	Attenuated hypertension in rats	NA
	Relaxin	Upregulation of VEGF	Attenuated hypertension in rats	NA
sFlt-1	Dextrane sulphate apheresis	Extracorporeal removal of sFlt-1	Absorbed recombinant sFlt-1 in human whole blood	Alleviated PE symptoms
	HO-1	Inhibition of sFlt-1 release	Attenuated hypertension in rats	NA
	Proton pump inhibitors	Upregulation of HO-1	Inhibited sFlt-1 secretion in placental explants	No effect on PE symptoms
	Statins	Upregulation of HO-1	Inhibited sFlt-1 secretion in placental explants	NA
	Ouabain	Downregulation of placental sFlt-1 production	Inhibited sFlt-1 secretion in placental explants and human trophoblast	NA
	Metformin	Downregulation of placental sFlt-1 production	Inhibited sFlt-1 secretion in placental explants and primary human tissue	Reduced incidence of PE
PlGF	Administration of PlGF	Supplementation of PlGF	Attenuated hypertension and proteinuria in rats	NA
Endothelin	ERAs	Blocking ET-1 receptors	Attenuated hypertension and proteinuria in rats	NA
	small interfering RNA	Silencing sFlt-1 mRNA	Attenuated PE symptoms in baboons	NA
RAS	ACE inhibitors	Inhibition of the conversion of Ang I into Ang II	Increased risk of IUFD and stillbirth	Teratogenic effects on fetus
	AT receptor blockers	Blocking the AT_1R	Increased risk of IUFD and stillbirth	Teratogenic effects on fetus
	n7AAc	AT_1R-AA antagonism	Attenuated hypertension and reduced sFlt-1 and preproET-1 levels	NA
Prostaglandins	Low dose acetylsalicylic acid	Inhibition of TxA_2 synthesis through COX-inhibition		Reduced the risk of developing PE in high risk patients
	Celecoxib	Selective COX-2 inhibition	Attenuated hypertension and improved fetal growth	NA

Table 2. (Potential) Treatment strategies to target the dysfunctional placental vascular reactivity in preeclampsia. (continued)

Target	Therapy	Mechanism of action	Preclinical studies	Clinical trials
Nitric oxide	Organic nitrates/ S-nitrosothiols	Exogenous NO-donors		Decrease in blood pressure, no effect on maternal or fetal outcome and significant side-effects
	N-acetylcysteine	Antioxidant	Improved NO-mediated vasodilation in fetoplacental vasculature	No improvement in severe early onset PE
	YC-1/Riociguat	sGC activation or stimulation	Inhibited sFlt-1 production and endothelial dysfunction in PE tissue	NA
	Sildenafil	Reducing cGMP degradation through PDE5 inhibition	Reduced maternal PE symptoms and improved fetal outcome	Halted due to lack of beneficial effects and a possible increase of neonatal complications
Bradykinin	ACE inhibitors	Inhibition of BK degradation	Increased risk of IUFD and stillbirth	Teratogenic effects on fetus
Acetylcholine	Nicotine	Stimulation of the nAChR	Stimulated production of VEGF and PlGF	NA
Histamine	H1-antagonist	Inhibition of vasoconstriction	NA	NA
	H2-agonist	Stimulation of vasodilation	NA	NA
Serotonin	Ketanserin	5-HT2 receptor antagonist	Decreased blood pressure and placental blood flow in hypertensive rats	Led to persistent hypertension and there was no beneficial effect on pregnancy outcome
CGRP	Administration of CGRP	Supplementation of CGRP shortage	Reduced maternal hypertension and pup mortality in rats	NA
	Rutaecarpine	Potentiation of endogenous CGRP release	Reduced blood pressure in hypertensive rats	NA
	αCGRP analogue	Supplementation of CGRP shortage	Antihypertensive effects in cardiovascular murine studies	NA

Abbreviations: 5-HT2 = 5-hydroxytryptamine-2; ACE = angiotensin converting enzyme; Ang I = angiotensin I; Ang II = angiotensin II, AT_1R = angiotensin II type 1 receptor; AT_1R-AA = angiotensin II type 1 receptor auto-antibodies; CGRP = calcitonin gene-related peptide; COX = cyclooxygenase; ERA = endothelin receptor antagonist; ET-1 = endothelin-1; H1 = histamine type 1; H2 = histamine type 2; HO-1 = heme-oxygenase-1; IUFD = intrauterine fetal death; nAChR = nicotinic acetylcholine receptor; NA = not applicable; NO = nitric oxide; PDE5 = phosphodiesterase-5; PE = preeclampsia; PGI_2 = prostacyclin; PlGF = placental growth factor; sFlt-1 = soluble Fms-like tyrosine kinase-1; sGC = soluble guanylate cyclase; TxA_2 = thromboxane A_2; VEGF = vascular endothelial growth factor.

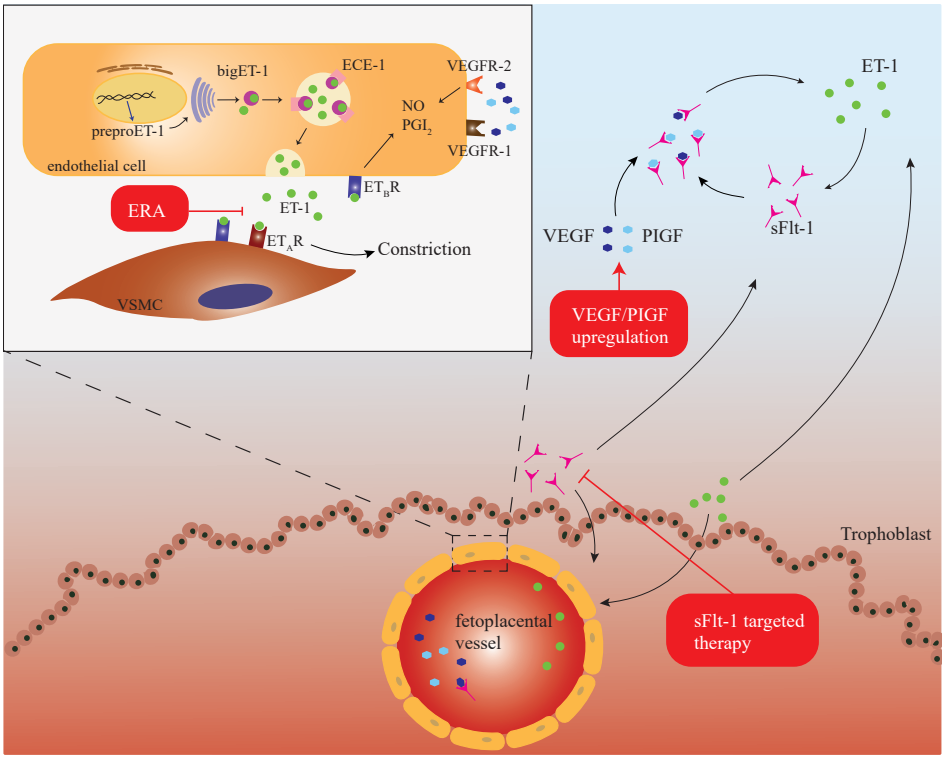

Figure 1. VEGF and ET-1 system in the human placenta and therapeutic strategies targeting this pathway. During PE there is an increase in sFlt-1 and ET-1, whereas VEGF and PlGF are decreased. Abbreviations: bigET-1 = big-endothelin-1; BM = basal membrane; ECE-1 = endothelin converting enzyme-1; ERA = endothelin receptor antagonist; ET-1 = endothelin-1; ET_AR = endothelin type A receptor; ET_BR = endothelin type B receptor; NO = nitric oxide; PGI_2 = prostacyclin; PlGF = placental growth factor; preproET-1 = preproendothelin-1; sFlt-1 = soluble Fms-like tyrosine kinase-1; VEGF = vascular endothelial growth factor; VEGFR = vascular endothelial growth factor receptor; VSMC = vascular smooth muscle cell.

soluble Fms-like tyrosine kinase (sFlt-1), a soluble form of VEGFR-1 generated through alternative splicing, binds with high affinity to its natural ligands VEGF and PlGF, thereby preventing interaction with their receptors (Figure 1).[23] It is produced and released into the maternal circulation by villous trophoblast cells of the placenta.[31] Interestingly, sFlt-1 levels found in the venous perfusate of the maternal side in the perfused cotyledon of healthy human placentas closely reflect *in vivo* serum values, indicating that sFlt-1 is predominantly secreted by the placenta.[27] Moreover, Saleh *et al.* observed a rapid postpartum decline by >99% in circulating sFlt-1 levels of women with suspected or confirmed PE, further suggesting the placenta is the major source of sFlt-1 secretion.[32]

In healthy pregnancy, VEGF is highly expressed by placental tissue during the first trimester[13] and is thought to act as a chemoattractant promoting trophoblast invasion.[23] In contrast, circulating sFlt-1 levels are relatively low in early pregnancy and begin to rise

during the third trimester, reflecting an antiangiogenic shift toward the end of pregnancy.[13] Excess sFlt-1 produced by preeclamptic villous tissue attenuates fetoplacental angiogenesis *in vitro*,[33] implicating that the physiological rise in sFlt-1 levels during healthy pregnancy is essential for adequate control of placental angiogenesis. Studies examining the release of VEGF in the *ex vivo* perfused cotyledon of term placentas are consistent with the former findings, showing a bilateral release of total VEGF, predominantly in the maternal circulation, with a high ratio of sFlt-1 to free VEGF, resulting in near complete VEGF binding. Interestingly, free VEGF levels were only detected on the fetal side. This finding may not be too surprising, as sFlt-1 levels are marginally secreted on this side, so that free VEGF will not be sequestered.[27] In the fetoplacental vasculature, VEGF acts as a potent vasodilator via the interaction with VEGFR-2. *Ex vivo* administration of free VEGF to the fetal side of the perfused cotyledon after pre-constriction with U46619 caused a dose-dependent vasodilation in the fetal circulation, which was partially attenuated by NO synthase inhibition.[27] Similar observations were made in isolated human chorionic plate arteries; VEGF evoked a concentration-dependent vasodilator response in the fetal circulation following pre-constriction with phenylephrine.[24] Importantly, the concentrations that elicit a vasodilator response in the fetoplacental vasculature are within the physiological range of fetal serum levels of VEGF. With regard to PlGF, no changes in fetal perfusion pressure were observed in the fetoplacental vasculature, while it significantly provoked vasodilation in isolated human chorionic plate arteries.[24, 27] These apparent opposite findings suggest that large arteries contribute relatively little to total resistance of the placental vasculature.

In recent years, excessive placental production of sFlt-1 has been implicated to be involved in the pathogenesis of PE. Multiple studies have reported elevated sFlt-1 levels in the circulation along with decreased free VEGF and free PlGF levels in women with PE,[34, 35] while PE placentas displayed increased expression of sFlt-1 mRNA.[36] In pregnant mice, sFlt-1 infusion or injection of an adenovirus encoding the sFlt-1 gene, elicits the hallmark features of PE, including hypertension, proteinuria and glomerular endotheliosis.[36, 37] Furthermore, treatment of cancer patients with VEGF antagonists induces a PE-like syndrome, with hypertension and proteinuria.[38] Consequently, reduced circulating levels of free VEGF and free PlGF promote the maternal syndrome of PE. Moreover, significant alterations of the VEGF system have been implicated in the fetoplacental vasculature during PE, which may actively contribute to increased blood flow resistance in the placental circulation.[27, 39] The increase in placental sFlt-1 production may be triggered by several factors during PE, including inflammatory cytokines, angiotensin II type 1 receptor auto-antibodies (AT_1R-AA) and placental hypoxia, most likely involving hypoxia inducible factor-1α (HIF-1α) as a mediator.[40, 41] Subsequently, sFlt-1 mediates hypertension through impairing endothelial NO production, which is normally upregulated by VEGF, thereby inducing endothelial dysfunction.[42] This is accompanied by upregulated

ET-1 production,[43] most likely as a consequence of impaired inhibition of ET-production by NO, and further reflecting endothelial dysfunction. Brownbill *et al*., making use of the *ex vivo* dual-sided placenta perfusion model, observed an enhanced vasodilator effect in the fetal circulation in response to exogenous VEGF administration in PE placentas as compared with healthy controls.[39] However, this response was only seen at high VEGF doses, which fall outside the physiological range of fetal serum concentrations. Moreover, diminished fetal serum concentrations of free VEGF were reported, in accordance with higher fetal sFlt-1 levels during PE, probably contributing to a reduced vasodilator response of the fetoplacental vasculature *in vivo*. Where the higher fetal sFlt-1 levels arise from is largely unknown, although one could hypothesize that the trophoblasts either secrete more sFlt-1 into the fetal circulation, or that the excessive maternal sFlt-1 levels are, at least in part, transported to the fetal side. Notably, an important role for VEGF localized to decidual tissue of the placenta has been identified. Fan *et al*. reported that endometrial-specific VEGF overexpression in mice significantly induced sFlt-1 release.[44] Combined with the increased decidual expression of VEGF mRNA in human PE placentas, this observation may indicate that VEGF regulates sFlt-1 release in the placenta at the maternal-fetal interface.[44]

Several studies have focused on improving the angiogenic imbalance in PE, for instance, through the extracorporeal removal of sFlt-1 using dextrane sulphate apheresis.[45] This resulted in significant alleviation of PE symptoms.[45] Furthermore, treatment with recombinant VEGF both *in vitro* and *in vivo* partially reduced the antiangiogenic consequences of excess sFlt-1 in an animal study.[46] With regard to the fetoplacental vasculature, administration of recombinant VEGF might aid in reducing placental vascular resistance during PE. However, concern has been raised regarding potential harm to the fetus by VEGF that passes the placental barrier. The recent development of VEGF fused to elastin-like polypeptide, a biopolymer carrier that does not cross the placental barrier, could overcome this problem.[46] Similarly, administration of PlGF in a PE rat model has shown promising results in reducing hypertension and proteinuria,[47] while there are no studies assessing the vasoreactive effects of PlGF in the fetoplacental vasculature during PE. Another potential target for improving the VEGF pathway may be through the vasodilator hormone relaxin, a local upregulator of VEGF, that could potentially improve fetoplacental blood flow during PE.[31] The safety profile of relaxin is currently being investigated for the treatment of women with PE.[31]

Inhibition of sFlt-1 secretion in the placenta itself forms an ideal approach towards restoring the angiogenic imbalance and improving placental blood flow. Moreover, given that sFlt-1 induces a rise in ET-1, such treatment would be able to target the ET-1 system. Some may argue that this method is not desirable, since sFlt-1 appears to be required to maintain placental health.[45] Several research groups have focused on inhibiting the release of sFlt-1 upstream. For instance, heme-oxygenase-1 (HO-1) attenuated

sFlt-1 induced hypertension *in vivo*.[48] Onda *et al.* observed decreased sFlt-1 secretion from placental explants treated with proton pump inhibitors (PPI), which are known to upregulate HO-1.[49] In keeping with this data, our group has found lower sFlt-1 and ET-1 levels in women with (suspected) PE that were on PPI treatment.[50] Recently, Cluver *et al.* conducted a randomized controlled trial involving 119 women with PE, and found no difference in median sFlt-1 levels between women treated with PPI and women treated with placebo.[51] These conflicting findings demand future studies to establish the role of PPI treatment on sFlt-1 in women with PE. Statins also inhibit sFlt-1 secretion in placental explants through the stimulation of HO-1.[52] While the safety profile of the latter is still under investigation, PPIs have been proven safe during pregnancy.[51, 53] Ouabain, a cardiac glycoside, downregulates placental sFlt-1 production *in vitro* through inhibition of the HIF-1α pathway, while similar effects were observed with metformin, possibly by blockade of the mitochondrial electron transport chain.[54, 55] Several other agents may be under evaluation for their potential sFlt-1 inhibiting properties, however the safety of their use during human pregnancy remains a main point of concern.

In summary, PE is characterized by increased circulating levels of sFlt-1, and decreased levels of VEGF and PlGF, causing an anti-angiogenic imbalance. Restoring this imbalance showed promising results in animal studies, and clinical trials are now awaited.

ENDOTHELIN

Over the last 3 decades, a dominant role for the ET system has emerged in the modulation of vascular tone in many organ tissues, including the human placenta. ETs belong to a family of three 21-amino acid peptides (ET-1, ET-2, ET-3), with ET-1 being the predominant vascular isoform and currently one of the most potent vasoconstrictors known.[56] ET-1 is secreted by endothelial cells and trophoblast cells towards the basolateral side of these cells, indicating its role as a paracrine or autocrine peptide.[57, 58]

Derived from its precursor peptide known as preproendothelin-1 (preproET-1), big-endothelin-1 (bigET-1) is cleaved by endothelin converting enzymes (ECEs) and other enzymes, including matrix metalloproteinases (MMP) and chymase, into biologically active ET-1.[59] Once synthesized, ET-1 modulates vascular tone through two cell surface G-protein-coupled receptors; ET type A (ET_A) receptors located on the vascular smooth muscle cell (VSMC) and ET type B (ET_B) receptors located on endothelial cells and VSMCs (Figure 1). Whereas ET_A receptors and ET_B receptors on VSMCs both induce vasoconstriction, ET_B receptors on endothelial cells account for the vasodilator effects of ET-1 through the release of NO and prostaglandins, and additionally provide the means for clearing ET-1 from the circulation.[59, 60] Receptor localization studies have localized ET_A receptors in the veins and arteries of the chorionic plate, while ET_B receptors were identi-

fied in decidual cells, veins in stem villi, and blood vessels in distal regions of the villous tree.[61] ET-binding sites have also been localized in trophoblastic tissue.[62] Stimuli for ET-1 release include ET-1 itself, hypoxia, inflammatory cytokines, along with other vasoactive substances such as Ang II.[59]

The functional role of the ET system in healthy pregnant women is not fully understood. ET-1 has been demonstrated to exert mitogenic effects, promoting first trimester trophoblast proliferation and invasion *in vitro*.[63] In addition, expression studies have shown an increased ratio of ET_B to ET_A receptors in the human placenta of healthy pregnant women compared to other vessel beds, which may account for the enhanced local vasodilatory state during pregnancy.[64] While maternal serum levels of ET-1 remain similar to those of non-pregnant women during gestation, a substantial rise is detected during labor.[65, 66] Increased uterine expression of ET_A and ET_B receptors was also observed during parturition, implicating ET-1 is involved in the initiation of uterine contractions.[67] High concentrations of ET-1 were also found in amniotic fluid and the fetal circulation including the umbilical vessels, being almost three-fold higher in the umbilical vein when compared to maternal serum levels during labor.[68] Furthermore, ET-1 was found to be a potent vasoconstrictor of human umbilical vessels.[69] Subsequently, earlier studies hypothesized that ET-1 is an important regulator of placental vascular tone. Wilkes *et al.* were the first to report a dose-dependent increase in perfusion pressure caused by ET-1 in the *ex vivo* perfusion model,[70] implying vasoconstriction. Furthermore, a long-lasting, strong concentration-dependent vasoconstrictor response to ET-1 was observed in isolated chorionic plate vessels, stem villous and umbilical vessels, albeit to a higher degree in veins than in arteries.[71, 72] However, blockade of the ET_A receptor in placental veins resulted in little inhibitory effect on ET-1 mediated contractions, implying a high abundance of constrictor ET_B receptors in placental veins.[73] Hence, differences in receptor distribution could account for the increased ET-1 sensitivity in placental veins, and may contribute to different vasopressor responses within the placenta. In placental arteries, the contractile response to ET-1 was significantly abolished by blocking ET_A and to a lesser degree ET_B receptors, indicating both subtypes mediate contractions in response to ET-1. Administration of the NO-donor nitroglycerin, counteracted these contractions for the most part, confirming that NO is able to neutralize the effects of ET-1 during physiological conditions.[68, 74] The concentration at which ET-1 provokes a vasocontractile response in these experimental models is higher than its *in vivo* plasma levels.[68, 70, 71] Thus, consistent with a predominantly abluminal release of ET-1, local production of ET-1 in the placenta determines the vasopressor responses to this peptide. The potency of ET-1 is maintained throughout the fetoplacental vascular tree,[71] unlike those of serotonin and thromboxane A_2 (TxA_2), which decline dramatically when reaching stem villous arteries, making ET-1 an important substance in maintaining placental vascular resistance.[71, 75]

A role for ETs has been proposed in cardiovascular disease including hypertension.[76] Moreover, activation of the ET system has been implicated as a key final pathway in the pathogenesis of PE. Indeed, most studies have reported elevated plasma ET-1 levels in women with PE as compared to normotensive pregnant women, while some studies even demonstrate a positive correlation with disease severity.[66, 77-79] Benoit *et al.* investigated the vasoconstrictive function of ET-1 in PE placentas by exposing healthy isolated chorionic plate arteries to conditioned medium prepared from PE placentas. In their study, enhanced vasoconstriction was shown in response to this medium, which was in part inhibited by an ET_A receptor blocker, while no effect was seen when blocking the ET_B receptor.[80] The latter finding suggests that an altered response to ET-1 is not the major contributor to the elevated vasoconstriction observed in PE. In keeping with these findings, both in the *ex vivo* placental perfusion model and in isolated placental arteries, no differences were observed in vascular response to ET-1 between healthy and PE placentas.[81, 82] Nevertheless, elevated levels of ET-1 in the placenta may still account for increased placental vascular resistance *in vivo* and could further play a part in the maternal endothelial dysfunction observed in PE. Indeed, as discussed earlier, women with PE display elevated circulating concentrations of ET-1. Similarly, increased ET-1 mRNA expression has been observed in placental tissue[83, 84] of PE pregnancies. Thus, the main question that needs to be answered is: what causes this uniform rise in ET-1 in women with PE? One prevailing theory is that elevated placental ET-1 production is released into the maternal circulation and triggers the manifestations of PE. The augmented release of this vasoactive peptide could be triggered by placenta-derived factors resulting from placental ischemia. In preclinical studies, pregnant mice infused with tumor necrosis factor-α (TNF-α) or AT_1R-AA display significantly increased expression of preproET-1 in the placenta, and in the case of TNF-α, also in the maternal aorta.[60] In addition, the sFlt-1 concentration is positively correlated with a rise in ET-1 plasma levels.[43] Intriguingly, administration of an ET_A receptor antagonist in sFlt-1-infused mice significantly abolished hypertension.[37] Hence, it is possible that placental sFlt-1 directly induces local ET-1 production. Experimental animal studies were not able to confirm this theory, as exogenous administration of sFlt-1 in pregnant mice only induced preproET-1 mRNA levels in the kidney but not in the placenta.[37] The observation that treatment with VEGF inhibitors in cancer patients or rats induces a PE-like syndrome with a rise in ET-1,[38] further indicates that the ET-1 elevation in the maternal circulation is a direct consequence of VEGF inactivation by sFlt-1.[59] Taken together, it is plausible to assume that the elevated sFlt-1 levels rather than local ET-1 production in the placenta are the main source of the increased circulating ET-1 levels in PE. Since ET-1 triggers oxidative stress in placental tissue,[85] a vicious cycle may arise, where ET-1 production in the placenta contributes to the release of placenta-derived factors including sFlt-1 into the maternal circulation.

This hypothesis, which suggests that placental ET-1 overproduction may contribute to enhanced release of sFlt-1 into the maternal circulation, was recently investigated by Li *et al.,* making use of mice with a modified ET-1 gene that causes high ET-1 production.[86] Their study demonstrates that maternal overproduction of ET-1 in pregnant mice is responsible for the development of full spectrum PE-like symptoms. However, placental overproduction of ET-1 was significantly associated with high plasma sFlt-1 levels in the maternal circulation, and contributed to elevated blood pressure. These findings confirm the concept that local ET-1 production in the placenta could contribute to PE through increasing sFlt-1 release in the maternal circulation. In turn, as discussed, sFlt-1 binds to VEGF, thereby inducing endothelial dysfunction and increasing plasma ET-1 production. Additionally, the decreased circulating volume and decreased placental perfusion[59] in PE women might further elevate placental ET-1 levels. Alterations in the expression or localization of ET-1 receptors in the placenta may also enhance placental vascular resistance in PE. Rutherford *et al.*, using affinity binding assays, observed no difference in either density or affinity of the ET-1 receptors in PE placentas.[61] Faxen *et al.* reported reduced ET_A receptor mRNA expression in PE placentas, while in another study this was only observed in late-onset PE. Downregulation of these receptors due to elevated ET-1 levels may underlie these findings.[87, 88] On the other hand, studies investigating ET_B receptor expression reported both increased and decreased mRNA levels in PE placentas.[88, 89] Altogether, the evidence regarding changes in placental ET receptor expression during PE remains ambiguous. Lastly, it has been hypothesized that MMPs, enzymes that can cleave bigET-1 into ET-1, are significantly increased in women with PE.[60] An elevated expression of MMP-2 in placental tissue, which has been reported recently, could account for increased ET-1 production in the placenta.[90] Also, increased ECE levels in serum of women with PE has been observed,[91] although its expression level in PE placentas has not yet been determined.

Endothelin receptor antagonists (ERAs) are clinically implemented for the treatment of cardiovascular diseases such as pulmonary hypertension, renal failure and cancer.[60] Concerning their actions in the treatment of the maternal syndrome of PE, most of our understanding comes from experimental animal models of PE. Particularly in the reduced uterine perfusion pressure (RUPP) model or sFlt-1-induced hypertension in rats, selective blockade of the ET_A receptor resulted in attenuated hypertension.[37, 92] In spite of these reports, ERAs are contraindicated in pregnancy, as genetic ET_A knockout models or treatment with ET_A receptor antagonists have led to fetal malformations or death in rodents.[93] Thaete *et al.* determined the levels of a selective ET_A receptor antagonist in fetal plasma of pregnant rats following long-term maternal exposure, and observed that only 2% of plasma levels reached the fetus.[94] Despite these findings, it is currently unknown whether ERAs cross the placental barrier in humans. Given the low molecular weight and lipophilic characteristics of these drugs, (partial) placental transfer seems

probable, since it is long known that lipophilic drugs with a molecular weight below 600 in general rapidly cross the placental barrier.[95] However, specific knowledge on ERAs in the human placenta is imperative to determine their usefulness in reducing placental vascular resistance in PE, because if ERAs are indeed to pass the placental barrier in humans, their potential teratogenic effects would possibly compromise their use in pregnancy. Linking ERAs to elastin-like peptides that do not cross this barrier, could provide an alternative approach to target the ET-1 pathway in the maternal circulation without affecting the fetus.[60] In addition, as observed by Thaete *et al.*,[94] administration of ERAs during late pregnancy, after completion of fetal organogenesis, might be harmless. Which ET-receptor subtype should be blocked requires further investigation, as few studies have investigated the role of ERAs in PE placental vessels. In healthy placental arteries, ET_A receptor antagonism and to a lesser degree ET_B receptor antagonism both diminished the vasoconstrictor response of ET-1.[68] Obviously, changes in receptor expression may influence the response to ERAs during PE.

In conclusion, the ET system plays seems to play an important role in the pathogenesis of PE. Compared to healthy pregnancy, increased circulating levels of ET-1 are found in PE. To target this system, future studies making use of the *ex vivo* human placental perfusion model should first determine the passage of ERAs through the human placental barrier. In addition, alternative mechanisms to target the ET system such as inhibiting ET-1 release, inactivating ET-1 in the circulation or suppressing endothelial ET-1 synthesis either directly (e.g with small interfering RNA) or indirectly (through inhibition of sFlt-1) should be further explored in future studies.

RENIN-ANGIOTENSIN SYSTEM

The RAS contributes to the long-term regulation of arterial pressure via its effects on vascular tone and sodium homeostasis. Angiotensinogen, the precursor to all angiotensins, is converted via renin into Ang I. Ang I is not biologically active and is cleaved primarily by angiotensin-converting enzyme (ACE) to form Ang II, the main effector peptide of the RAS. Of note, Ang II can also be generated via chymase which is produced by villous syncytiotrophoblasts, although evidence that this occurs *in vivo* is lacking.[96] Once formed, Ang II can stimulate both the angiotensin II type 1 receptor (AT_1R) and the angiotensin II type 2 receptor (AT_2R) (Figure 2). Although the affinity of Ang II is ~15-fold greater for the AT_2R than the AT_1R,[97] the AT_1R is the dominant receptor expressed following fetal life.[98] Activation of the AT_1R elicits the classical actions of the RAS including aldosterone release, sodium retention, vasoconstriction and pro-inflammatory effects. Conversely, activation of the AT_2R induces natriuresis, vasodilation and anti-inflammatory effects (Figure 2). The discovery of novel angiotensin fragments and receptors has led

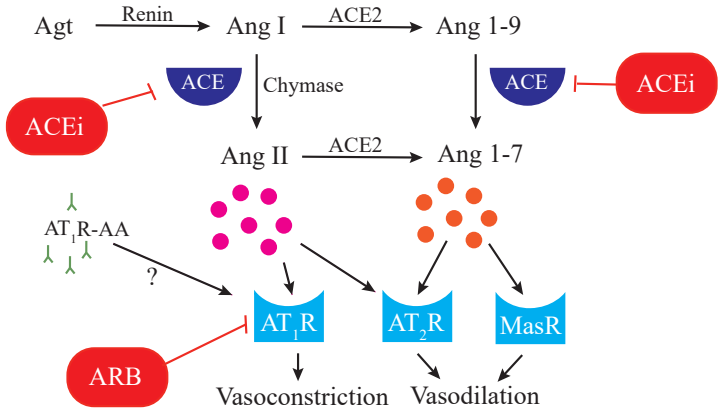

Figure 2. The RAS in human pregnancy and therapeutic strategies targeting this pathway. During PE there is a decrease in renin, Ang I, Ang II and Ang 1-7, whereas circulating levels of AT_1R-AA are increased. Abbreviations: ACE = angiotensin converting enzyme; ACEi = ACE inhibitor; Agt = angiotensinogen; Ang = angiotensin; ARB = angiotensin II type 1 receptor blocker; AT_1R = angiotensin II type 1 receptor; AT_2R angiotensin II type 2 receptor; AT_1R-AA; angiotensin II type 1 auto-antibodies; MasR = Mas receptor.

to the identification of depressor RAS pathways, which encompasses the AT_2R and the angiotensin-converting enzyme 2 (ACE2)/Ang 1-7/Mas receptor axis, that counteract the classical actions of AT_1R stimulation.[98]

The maternal RAS undergoes major changes during pregnancy. All of the components of the RAS are increased in uncomplicated pregnancies, except for ACE.[99] During early gestation, the ovaries and maternal decidua contribute to an increase in extra-renal renin release.[100] In addition, the high estrogen level maintained by the placenta promotes hepatic angiotensinogen synthesis. Consequently, circulating Ang II and aldosterone levels are increased during pregnancy.[101] Yet, despite the increase in circulating Ang II, pregnant women and gravid animals are less sensitive to the pressor effects of Ang II.[102, 103] In a landmark study by Gant *et al.* from 1973, it was demonstrated that normotensive pregnant women require twice the dose of Ang II to elicit a 20 mmHg increase in diastolic arterial pressure as compared to non-pregnant women.[104] This refractoriness to Ang II has been attributed to increases in progesterone and PGI_2 which can decrease sensitivity to Ang II-induced vasoconstriction,[105] but may also be, at least in part, mediated by an upregulation of the depressor RAS pathways. For example, it has been demonstrated that during normotensive pregnancies circulating ACE2 is upregulated,[103, 106] plasma Ang 1-7 increases by 20-fold[103] and that the AT_2R/AT_1R balance rises.[103] Similarly, in murine studies, genetic and/or pharmacological AT_2R[107-110] or ACE2[111] deficiency leads to pregnancy induced hypertension, supporting a key role for the depressor RAS pathways in the normal cardiovascular adaptations to pregnancy.

Compared to the characteristic refractoriness to Ang II observed during normal pregnancy, PE is characterized by increased sensitivity to Ang II and altered expression of RAS components.[102, 104, 112, 113] Circulating renin, Ang I, Ang II and aldosterone levels are lower in women with PE as compared to women with uncomplicated pregnancies.[99, 114] Moreover, the pregnancy-induced increase in plasma Ang 1-7 in women with uncomplicated pregnancies is not observed during PE.[99] Although little is known about AT_2R expression during PE, women with a history of PE have a lower AT_2R/AT_1R ratio than women who had uncomplicated pregnancies,[115] suggesting that AT_2R expression is decreased during PE. Conversely, during PE, the presence of AT_1R-AA[116] and the increase in AT_1R-bradykinin receptor (B2) heterodimers[117, 118] potentiate the pro-hypertensive effects of the AT_1R. Moreover, in PE women, AT_1R-AA titer is proportional to sFlt-1, suggesting that AT_1R-AA may stimulate additional pathways (for example ET-1 and anti-angiogenetic factors (sFlt-1 and soluble endoglin (sEng)) in addition to directly activating the AT_1R.[119]

Within the placenta, all of the components necessary for a functional RAS are expressed on both the maternal and fetal sides.[120-128] The AT_1R is the predominant AT receptor expressed throughout the placenta.[120] Within the placental villi, it has been demonstrated that AT_1R are localized in and around the blood vessels.[120] Consequently, Ang II induces a potent dose-dependent vasoconstrictor effect within the fetoplacental circulation,[129-131] with isolated chorionic plate vessels being more sensitive to Ang II than segments from umbilical[132, 133] and uterine[131] arteries. Similarly, in animals, the infusion of Ang II markedly increases fetal-placental pressure.[134, 135] Using the dually perfused *ex vivo* human placenta model, Glance *et al.* demonstrated that administration of Ang II to the maternal circulation induces a dose-related increase in fetal perfusion pressure.[130] Conversely, administration of Ang II to the fetal circulation does not induce an increase in maternal perfusion pressure,[130] indicating one-way direction (maternal to fetal) of Ang II. This suggests that high circulating maternal Ang II concentration could result in elevated fetoplacental Ang II concentration and enhanced pressor responsiveness to Ang II. Moreover, sensitivity of the fetoplacental vasculature to Ang II may also be dependent on thromboxanes and/or prostaglandins since selective cyclooxygenase (COX)-1 inhibition with low dose acetylsalicylic acid (via infusion into the intervillous space)[136] or dual COX-1 and COX-2 inhibition with meclofenamate[137] attenuates Ang II-induced vasoconstriction. Interestingly, refractoriness to Ang II-induced vasoconstriction is even greater in the fetoplacental circulation than the maternal systemic circulation during late gestation.[138] Furthermore, tachyphylaxis to Ang II has been reported in the fetoplacental circulation of term placentas from uncomplicated pregnancies.[139] This reduced sensitivity to Ang II may serve to protect the fetoplacental circulation from the normally occurring increases in maternal Ang II concentration.

In contrast to uncomplicated pregnancies, angiotensinogen, Ang II and AT_1R are significantly higher in chorionic villous tissue from nulliparous third trimester PE pregnan-

cies.[140] While these authors did not observe any differences in placental ACE, ACE2, or Ang 1-7 levels between PE and uncomplicated pregnancies, there is evidence that ACE expression and activity[141] and chymase[96] are upregulated in PE placentas, which would result in an increase in placental Ang II generation. In agreement with these studies, when isolated chorionic plate arteries from uncomplicated pregnancies are perfused with placental conditioned media from PE placentas, vasoconstriction (equal to 50% of the response to KCl 100) is blunted by 40% by chymase inhibition and 20% by ACE inhibition.[80] Moreover, AT_1R blockade with losartan attenuated the contractile response to PE-conditioned media by 30%, while AT_2R blockade with PD123319 only reduced the contractile response by 16%.[80] Collectively these data suggest that elevated Ang II acting via the AT_1R may favor vasoconstriction in placental chorionic villi during PE, which may contribute to impaired fetal blood flow and poor fetal outcomes. However, to date, studies using isolated chorionic vessels or the dually perfused *ex vivo* placental perfusion model to investigate Ang II sensitivity in PE placentas have reported that the pressor response to Ang II is decreased[133, 142] or unchanged[143] as compared to placentas from uncomplicated pregnancies. The reason for the discrepancy between these studies is unclear, but may relate to the use of magnesium sulfate ($MgSO_4$) or the combined use of cesarean and vaginally delivered placentas. Previous studies have demonstrated that $MgSO_4$, which is used clinically to prevent eclampsia, attenuates the sensitivity of the fetal-placental vasculature to Ang II.[143, 144] Differential expression of RAS components in PE and uncomplicated placentas has not been observed in vaginally delivered placentas,[145] suggesting that the expression of the RAS is altered in placentas during labor.

Drugs that inhibit the RAS (ACE inhibitors (ACEi) and AT_1R blockers (ARBs)), which are a mainstay for the treatment of hypertension, are contraindicated during pregnancy due to the high risk of teratogenic effects. In preclinical studies, ACEi use was associated with increased risk of intrauterine death and still births.[146, 147] In women, ACEi during second and third trimesters of pregnancy has been shown to have adverse effects on the fetus,[148] including FGR, respiratory, renal and circulatory abnormalities, and patent ductus arteriosus.[149-151] Similar fetal abnormalities are seen with maternal ARB treatment during the second and third trimesters of pregnancy.[152] An alternative approach to antagonize the effects of AT_1R activation during PE might be to antagonize AT_1R-AA with the help of a high affinity 7-amino acid sequence ('n7AAc'). Cunningham *et al.*, have recently demonstrated that in the reduced uterine perfusion pressure model of PE, AT_1R-AA blockade with this inhibitory peptide prevents PE symptoms, including the normalization of arterial pressure and reduces sFlt-1, preproET-1, pro-inflammatory and generation of reactive oxygen species.[153] However, there is still doubt whether AT_1R-AA truly activate AT_1R *in vivo*,[154] and thus it cannot be excluded that inhibiting AT_1R-AA has effects that are unrelated to the RAS.

Taken together, in pregnancies complicated by PE the RAS is altered, featuring an increased sensitivity to Ang II and suppressed maternal plasma levels of most RAS components. Yet, the levels of angiotensinogen and Ang II in the placenta are increased. Unfortunately, drugs that directly inhibit the RAS are contraindicated in pregnancy. New approaches, such as AT_1R-AA antagonism, could be of therapeutic value in the future, once it has been established what exactly such treatment interferes with.

PROSTAGLANDINS

Two of the most important prostaglandins in pregnancy are TxA_2 and PGI_2. They both originate from the common precursor prostaglandin H_2 (PGH_2), which is formed by conversion of arachidonic acid under the influence of COX-1 and/or COX-2.[155] To form TxA_2 and PGI_2, which act as each other's natural antagonist, PGH_2 is converted by thromboxane synthase or prostacyclin synthase respectively (Figure 3). PGI_2 is a vasodilator, and a very potent inhibitor of platelet aggregation.[156] Furthermore, in pregnancy PGI_2 inhibits uterine contractility.[157] In contrast, TxA_2 is a potent vasoconstrictor, which additionally induces platelet aggregation and stimulates uterine contractility.[156, 157] The placenta has been shown to produce both TxA_2 and PGI_2, as well as prostaglandins E_2 and F_{2a}, which both exert vasoconstrictor effects in the fetoplacental vasculature (Figure 3).[131, 158-161] TxA_2 might also mediate the constrictor effects of ET-1, 5-hydroxytryptamine (5-HT) and Ang II.[162]

Compared to the non-pregnant state, serum concentrations of PGI_2 metabolites are increased during normal pregnancy, with a peak in the first trimester.[163] In PE, the production of PGI_2 in the placenta and umbilical vessels is reduced,[164, 165] while the levels of PGI_2 and its metabolites are also diminished in plasma, urine and amniotic fluid.[163, 166, 167] This indicates a possible role for decreased PGI_2 activity in the pathogenesis of PE. Remarkably, the response of the *ex vivo* perfused PE placenta to PGI_2 is diminished compared to healthy controls.[142] As one of the first, Walsh *et al.* found that there is an imbalance between TxA_2 and PGI_2 in the PE placenta, with a three-fold increase in TxA_2 production, and a 50% reduction in PGI_2 release.[168] This TxA_2/PGI_2 imbalance causes a shift towards vasoconstriction, possibly leading to hypertension and reduced uterine flow. A factor that possibly contributes to the TxA_2/PGI_2 imbalance in PE is an elevation in circulating levels of lipid peroxides.[169] Lipid peroxides stimulate TxA_2 production, and simultaneously inhibit PGI_2.[170] Wang *et al.* showed that placental perfusion with peroxides indeed leads to a rise in the TxA_2/PGI_2 ratio.[171] Trophoblast cells are the main source of this increased TxA_2 synthesis, and accordingly, they display increased expression of COX-1 in PE,[172, 173] which may further amplify the elevation of the TxA_2 plasma levels are elevated in severe PE versus normal pregnancy.[174]

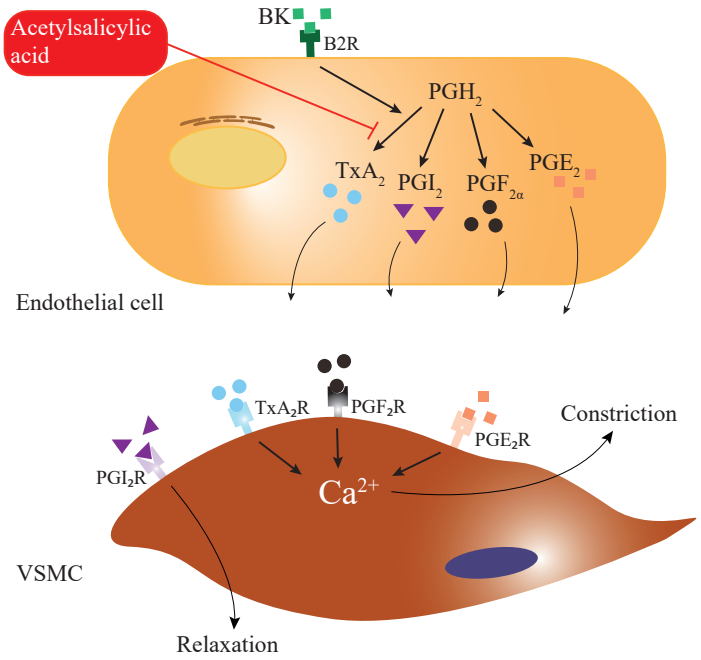

Figure 3. The prostaglandin pathway in the human placenta and therapeutic strategies targeting this pathway. During PE circulating levels of TxA_2 and $PGF_{2\alpha}$ are increased, while PGI_2 levels are decreased, resulting in an imbalance. Abbreviations: B2R = bradykinin receptor; BK = bradykinin; PGE_2 = prostaglandin E_2; PGE_2R = PGE_2 receptor; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; PGF_2R = $PGF_{2\alpha}$ receptor; PGH_2 = prostaglandin H_2; PGI_2 = prostacyclin; PGI_2R = PGI_2 receptor; TxA_2 = thromboxane A_2; TxA_2R = TxA_2 receptor; VSMC = vascular smooth muscle cell.

Of all prostaglandins, TxA_2 exerts the most powerful vasoconstrictive effect in the placenta, as shown by many *ex vivo* studies.[131, 175] Administration of the TxA_2 agonist U46619 in the isolated perfused cotyledon or chorionic plate arteries elicits a potent dose-dependent response.[176-182] However, the response differs greatly among different vessel types, in contrast to other prostaglandins (e.g. E_2 and $F_{2\alpha}$) that are also known to constrict fetoplacental vasculature through binding different receptors.[75, 131] Broegger *et al.* found a positive correlation between vessel sensitivity to the TxA_2 agonist U46619 and the inner vessel diameter,[75] suggesting differences in receptor numbers and/or distribution along placental vasculature, however this still needs to be confirmed through quantification methods. These findings critically question the general opinion that TxA_2 is one of the most important regulators of placental vascular tone, since the smallest vessels are presumed to be the most contributory to resistance.[183] In PE placentas, the release of TxA_2 in response to 5-HT exposure during *ex vivo* placental perfusion is increased.[184] However, the vasoconstrictive response to U46619 is significantly attenuated,[82, 142] possibly to compensate for the higher circulating levels of TxA_2 during PE.

Targeting the TxA_2/PGI_2 imbalance as a preventive strategy for PE has been performed for a long time by studying the effects of low-dose acetylsalicylic acid. Low-dose acetylsalicylic acid selectively inhibits the synthesis of TxA_2, without interfering with the production of PGI_2.[185] This is thought to be the due to compartmentalization of the production sites of TxA_2 and PGI_2 in the placenta, as TxA_2 is primarily produced by trophoblast cells near the maternal circulation and PGI_2 by endothelial cells near the fetal circulation.[185] Studies on the efficacy of low-dose acetylsalicylic acid have shown contradictory results. Some of these differences could be explained by timing of treatment initiation or patient selection. A systematic review and meta-analysis including 10 randomized controlled trials comparing first trimester low-dose acetylsalicylic acid versus placebo or no treatment in women at risk for PE found a significant reduction in the overall risk ratio (RR 0.35; 95% CI 0.13-0.94) of developing early onset PE.[186] However, early identification of women who are at high risk for PE remains very difficult, and when started after 16 weeks of gestation the beneficial effects of acetylsalicylic acid seem no longer evident.[187, 188] Acetylsalicylic acid use is regarded safe throughout pregnancy, as no associations with adverse fetal outcome (e.g. congenital abnormalities, intraventricular hemorrhage, premature closure of the ductus arteriosus) or maternal outcome (e.g. postpartum hemorrhage, placental abruption) have been found.[189, 190] Although an increased risk of vaginal bleeding has been reported, this was not associated with an increased risk of pregnancy loss.[189] Another interesting method could be to selectively target COX-2. Sones *et al*. showed upregulation of COX-2 in a PE mouse model. Treating these mice with a single dose of the selective COX-2 inhibitor celecoxib during decidualization prevented maternal hypertension and normalized placental- and fetal growth.[191] However, the effects on PE in human pregnancy remain to be investigated. Moreover, although short term exposure to celecoxib in the third trimester showed no increase in maternal or neonatal complications,[192] the risks of long-term exposure during pregnancy are not yet known.

In conclusion, there is a distinct imbalance between TxA_2 and PGI_2 in PE, causing a preponderance towards the vasoconstrictive function of the prostaglandin axis. Until now, targeting this axis has been only proven useful in the prevention of PE in high-risk patients.

NITRIC OXIDE

NO, previously identified as endothelium-derived relaxing factor,[193] plays a key-role in vasodilation. It is synthesized by a family of nitric oxide synthases (NOS), most importantly endothelial NOS (eNOS) and inducible NOS (iNOS), that are present in various cell types including endothelial cells and fetal trophoblasts. These NOS enzymes cause ca-

talysis of L-arginine, resulting in production of NO, after stimulation by different factors (e.g. endothelial shear stress, estrogen, bradykinin, acetylcholine). By activating soluble guanylate cyclase (sGC), NO causes an increase in intracellular free cyclic guanosine 3',5'-monophosphate (cGMP), leading to vasodilation through closure of Ca^{2+} channels (Figure 4).[194-196]

In normal pregnancy, circulating levels of NO are increased,[197] as well as the blood plasma levels of hormones and growth factors that stimulate NO release, like estrogen, VEGF and PlGF.[198, 199] Apart from blood pressure regulation, NO also influences cytotrophoblast invasion and spiral artery remodeling in early gestation.[200] It has been shown in *ex vivo* placental perfusion experiments that inhibition of the NO pathway causes an increased response to the TxA_2 agonist U46619,[201] indicating that there is NO release in response to vasoconstriction, possibly due to increased shear stress.

The role of the NO pathway in PE has been extensively discussed in literature, and the mechanisms contributing to its dysfunction seem multifactorial.[194, 195] In animal models, systemic inhibition of NO synthesis leads to development of a PE-like syndrome, with maternal hypertension and proteinuria, as well as fetal growth restriction.[202, 203] Furthermore, studies using rat models with RUPP, causing PE symptoms, showed a decrease in NO synthesis and/or release from endothelial cells.[204] Altogether, these studies suggest that indeed loss of NO can contribute to development of PE. In women with PE, circulating NO metabolites have been found to be significantly decreased versus healthy pregnant women.[197, 205-207] Similarly, lower circulating levels of the NO-dependent vasodilators estrogen, VEGF and PlGF have been reported.[36, 208, 209] In addition, plasma levels of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, are elevated in PE patients,[210] further contributing to impaired eNOS activity. Protein expression of eNOS seems unchanged in placental tissue of PE pregnancies,[206, 211] although eNOS mRNA expression has been reported to be increased, possibly as a compensatory mechanism.[212] Oxidants, such as reactive oxygen species (ROS), are produced during oxidative stress and are known to scavenge NO, resulting in the formation of peroxynitrite (Figure 4). It is thought that in PE there is an increase in ROS, explaining, at least partly, the reduced bioavailability of NO despite increased eNOS expression.[196] Indeed, peroxynitrite has been shown to be increased in vessels of women with PE.[213] Accordingly, Bisseling *et al.* found that the antioxidant N-acetylcysteine improved the NO-mediated effects in the fetoplacental circulation during *ex vivo* placental perfusion in healthy and PE placentas to a similar extent.[196] However, in a randomized controlled trial where women with severe early onset PE received either N-acetylcysteine or placebo, no improvement of maternal disease and no differences in neonatal outcome were observed.[214] Other *ex vivo* placental perfusion studies showed that administering N(ω)-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, induced a significant increase in the baseline perfusion pressure of healthy placentas.[196, 215, 216] However, in PE placentas this pressure increase

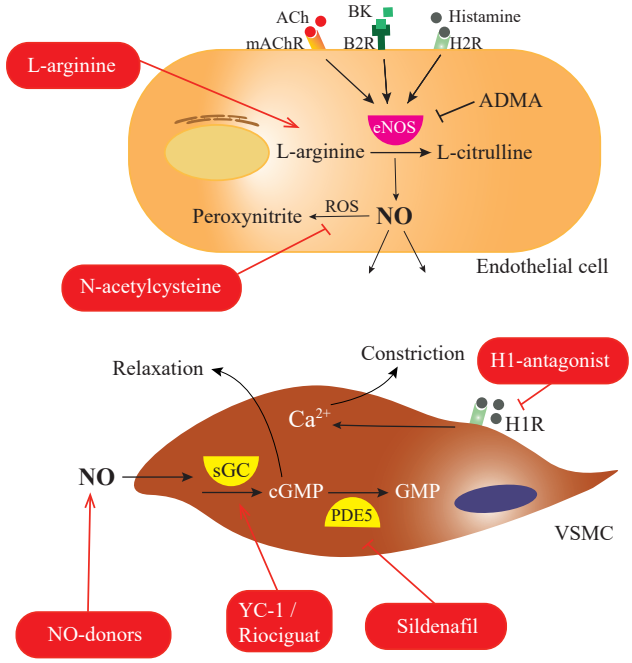

Figure 4. The NO-pathway and NO-dependent vasodilators in the human placenta and therapeutic strategies targeting this pathway. During PE there is a decrease of NO and ACh, while levels of ROS, peroxynitrite, ADMA and histamine are increased. Abbreviations: ACh = acetylcholine; ADMA = asymmetric dimethylarginine; B2R = bradykinin receptor; BK = bradykinin; cGMP = cyclic guanosine 3',5'-monophosphate; eNOS = endothelial nitric oxide synthase; GMP = guanosine 3',5'-monophosphate; H1R = histamine type 1 receptor; H2R = histamine type 2 receptor; mAChR = muscarinic acetylcholine receptor; NO = nitric oxide; PDE5 = phosphodiesterase-5; ROS = radical oxygen species; sGC = soluble guanylate cyclase; VSMC = vascular smooth muscle cell.

is much less pronounced, suggesting that NO-mediated vasodilation is decreased in PE.[196, 216] Another intriguing possibility is that placental eNOS is uncoupled in PE, and produces superoxide rather than NO. However, to our knowledge, this has not been investigated to date.

Many clinical trials have evaluated whether drugs that affect the NO pathway, like NO donors (e.g. organic nitrates and *S*-nitrosothiols) and NO precursors (e.g. L-arginine) are capable of restoring the NO-pathway in PE. Although they caused a significant decrease in blood pressure, there were no effects on maternal or fetal outcome.[217-221] Therefore, evidence for their effectiveness is limited, and they are additionally known to quickly develop drug tolerance and to have significant side-effects.[222] Indeed, the vasodilator response to various NO-donors is attenuated in PE placentas.[142] Studies investigating the reactivity of isolated chorionic plate arteries showed similar results. In this set-up, NO-donors result in potent vasodilation of pre-constricted healthy placental arteries,[179, 223, 224] an effect that is significantly reduced in vessels of PE placentas.[225] This lack of

efficacy of NO-donors could be due to increased scavenging of NO and/or disruptions more downstream in the NO-pathway. Interestingly, not much is known about directly modulating sGC, through either activators (e.g. YC-1) or stimulators (e.g. riociguat), in the placenta. These compounds potentiate the effects of the NO-pathway in an NO-independent way.[226, 227] Brownfoot *et al.* showed that YC-1 inhibits placental production of sFlt-1 and sEng, and that it reduces endothelial dysfunction in preeclamptic tissue.[228] Until now, no (pre)clinical trials with sGC-modulators in PE have been published, but it could be a novel therapeutic target. Another way to potentiate the action of the NO-pathway, is to inhibit the degradation of the active cGMP into the inactive GMP by phosphodiesterases. A drug that is currently under investigation is the phosphodiesterase-5 (PDE5) inhibitor sildenafil. In PE animal models, sildenafil improved fetal outcome and additionally diminished maternal symptoms.[229, 230] *Ex vivo* studies showed a significant vasodilator response of chorionic plate arteries to sildenafil.[231, 232] However, this has not been studied in placentas from PE pregnancies. Samangaya *et al.* performed a small randomized controlled trial of sildenafil versus placebo in women with PE, but found no improvement in duration of pregnancy or neonatal outcome.[233] Currently, a large multicenter randomized controlled trial is further evaluating the effects of sildenafil on pregnancy outcome in extreme FGR due to placental insufficiency.[234] Unfortunately, the first cohort of this study, from the United Kingdom, reported that sildenafil did not prolong pregnancy and did not improve fetal outcome.[235] Furthermore, the Dutch cohort has recently been halted since sildenafil did not show beneficial effects and there was an increase of neonatal complications in the treated group.[236]

To summarize, NO-mediated vasodilation is impaired in PE compared to normal pregnancy, possibly due to a decrease in NO synthesis and/or release, increased NOS inhibition, or increased NO scavenging by ROS. Although many drugs targeting this pathway have been evaluated in PE, none of them improved fetal outcome. Possibly direct stimulation and/or activation of sGC could provide a favorable treatment option.

NITRIC OXIDE-DEPENDENT VASODILATORS

Bradykinin

Bradykinin (BK) is known to be a powerful stimulator of the release of both NO and PGI_2 (Figure 4), inducing vasodilation and increasing vascular permeability through binding of the G-protein-coupled B2 receptors. In contrast, binding the B1 receptor, that is mostly expressed in the central nervous system in response to injury, induces typical signs of inflammation.[237]

In the placenta, the B2 receptor has been documented in decidua, placental and extravillous trophoblasts and in the fetal endothelium.[238] Although generally considered

to be a vasodilator, BK is also known to induce vasoconstriction in placental vessels, probably through the stimulation of TxA_2 production (Figure 3).[239] However, most *ex vivo* studies were unable to show responses of chorionic plate arteries to BK, possibly because it is rapidly metabolized in placental tissue.[177, 179, 225] After a single passage of the fetoplacental circulation, BK loses 98% of its biological activity,[240] indicating effective clearance of BK in placental vasculature. The most important enzyme responsible for BK degradation is ACE,[241] and indeed the effect of BK on placental vessels is potentiated in the presence of an ACEi.[242] BK stimulates cell migration and plays an important role in trophoblast invasion through the B2 receptors.[238] Consistent with the alterations in PE, there is a significant reduction of the B2 receptor gene- and protein expression, compared to healthy placentas.[243, 244] Possibly, this contributes to impaired trophoblast invasion. However, the precise role of BK in PE remains unknown and therapeutically targeting this system would be difficult, as for example ACEi are severely fetotoxic and, as outlined in section 5, also impact the RAS.[148]

Acetylcholine

Even though the placenta is a non-neuronal and non-innervated tissue, acetylcholine (ACh), as well as its synthesizing hormone choline acetyltransferase, are present in large concentrations in the human placenta.[245] ACh is continuously synthesized in the syncytiotrophoblast cells from where it is released into both the maternal and fetal circulations, as shown by *ex vivo* placental perfusion studies. However, it does not seem to have an effect on fetoplacental vascular tone.[246] ACh exerts its action through 2 types of cholinergic receptors: the muscarinic (mAChR) and nicotinic receptors (nAChR), both of which are expressed in the human placenta.[247, 248] It has been suggested that ACh plays an important role in the trans-placental passage of amino acids, and in placental hormone release.[249] Furthermore, it is involved in the development of placental vessels and syncytiotrophoblasts.[250]

In the PE placenta, ACh synthesis and output are decreased,[251, 252] as is the density of mAChR.[253] In contrast, the expression of nAChR is increased.[254] Interestingly, binding of nicotine to nAChR stimulates the production of growth factors, such as VEGF, leading to increased angiogenesis.[255] In line with this, smoking during pregnancy seems to be protective against PE,[256, 257] although it is well known that smoking increases the risk of many other adverse pregnancy outcomes. To investigate the therapeutic potential of nAChR agonists in PE, Mimura *et al.* tested the effect of nicotine on damaged endothelial cells. They found that nicotine significantly increased PlGF levels and counteracted the endothelial dysfunction caused by increased sFlt-1 levels, suggesting a possible therapeutic target for restoring the anti-angiogenic imbalance as seen in PE.[258]

Histamine

Histamine is an angiogenic and vasoactive mediator, that is derived from mast cells.[259] It plays an important role in allergic and inflammatory processes, and is also involved in pregnancy.[260] Histamine exerts its biological actions through binding to four receptors, two of which are involved in vascular reactivity: H1 and H2. Binding to the H1 receptor causes contraction of smooth muscle cell, whereas activation of the H2 receptor stimulates vasodilation (Figure 4).[260]

In first trimester, maternal serum levels of histamine are highest, decreasing with progressing gestation.[261] Histamine has been shown to promote trophoblast invasion.[262] During placental development, hypoxia is an important trigger for mast cell activation, stimulating angiogenesis through production of HIF-1α and VEGF. HIF-1α activity leads to increased synthesis of histamine within mast cells, and their degranulation.[263] Isolated vessel studies show that in human placental arteries and veins, histamine predominantly causes vasoconstriction,[264-266] an effect that is attenuated by adding a H1 receptor blocker.[264] However, a vasodilator effect on placental arteries through the H2 receptor has also been recorded.[267] Both effects indicate that histamine is involved in regulation of placental vascular tone. Histamine is predominantly metabolized by diamine oxidase (DAO), an enzyme that is highly expressed in, and produced by the placenta.[268] An increase in circulating DAO levels during advancing gestation causes a decrease of circulating histamine levels. In animal studies, histamine injection and/or DAO inhibition had fatal consequences, indicating a protective role for DAO against reaching harmful histamine levels.[269, 270] Also in human pregnancy, reduced activity of DAO has been linked to unfavorable outcome.[268]

In pregnancies complicated by PE, placental tissue concentrations of histamine and mast cells are significantly higher as compared to normal pregnancy.[271, 272] Furthermore, higher mast cell density and a lower vascular/extravascular index of histamine have been identified, indicating a change in mast cell distribution, as well as in circulating histamine concentration.[272] On the contrary, isolated placental arteries of PE placentas show a decreased responsiveness to histamine *ex vivo* indicating reduced placental sensitivity for histamine.[266] Whether blockade of the H1 receptor, or stimulation of the H2 receptor could offer treatment potential for PE has not yet been investigated.

SEROTONIN AND TRYPTOPHAN

Tryptophan (Trp) is an essential amino acid and, in addition to its requirement in protein synthesis, is the precursor to several vasoactive metabolites. Two main Trp metabolizing pathways are present in the placenta, being the serotonin pathway initiated by conversion of Trp by tryptophan hydroxylase (TPH) and the kynurenine pathway, initiated by

conversion of Trp by either tryptophan 2,3-dioxygenase (TDO), indolamine 2,3-dioxygenase-1 (IDO1), or indolamine 2,3-dioxygenase-2 (IDO2).[273]

Serotonin

Serotonin, also known as 5-HT, is mainly known for its functions in the central nervous system. Furthermore, it regulates blood vessel tone and blood pressure. For these functions outside the central nervous system 5-HT might originate from platelets.[274] Additionally, locally produced serotonin might aid in regulation of placental blood vessel tone. Serotonin is a metabolite of Trp, and the responsible enzyme, Trp hydroxylase, is expressed in the placenta.[275] In blood vessels 5-HT can exert different effects, ranging from constriction to dilation, depending on the receptor(s) it binds to.[276] Watts *et al.* concluded that 5-HT is able to induce vasoconstriction in virtually every isolated blood vessel, mediated predominantly by 5-HT2A (Figure 5), and partly by 5-HT1B/1D receptors.[276] Results from wire-myography experiments reveal that segments from umbilical and chorionic plate arteries and veins show a strong dose dependent vasoconstrictive response to 5-HT, dependent on 5-HT2 and not 5-HT1 or 5-HT3 receptors.[131, 177, 277-280]

Although placental arteries and veins respond similarly to 5-HT, sensitivity to 5-HT decreases with vessel size.[71, 132] Like with prostaglandins, this could indicate differences in receptor density and/or distribution. Given the wide variety of constrictor and dilator 5-HT receptor,[276] it is not surprising that in perfused vessel segments 5-HT often elicits a biphasic pressure response, with a pressure decrease preceding vasoconstriction.[281] In *ex vivo* placental perfusion experiments infusion with 5-HT results in a dose-dependent elevation in perfusion pressure.[82, 162, 216, 282-284] However, these rises in pressure vary greatly between experiments and often decline slowly.

Responses to 5-HT are largely dependent on calcium (Ca^{2+}). Hence, both the extracellular Ca^{2+} concentration and Ca^{2+} antagonists affect the 5-HT response in chorionic arteries and veins.[279] Other factors involved in the 5-HT response are prostaglandins, more specifically TxA_2. This prostanoid was detectable in the perfusate of *ex vivo* perfused placentas after 5-HT induced vasoconstriction, supporting the concept that placental vessels are able to produce TxA_2 locally (Figure 5). Moreover, both the COX-inhibitor acetylsalicylic acid and the TxA_2 antagonist GR32191 significantly attenuate the vasoconstriction in response to 5-HT.[162] Not only TxA_2, but also prostaglandin $F_{2\alpha}$ potentiates the 5-HT response in chorionic veins in both wire-myography and vessel segment perfusion experiments.[278] These results show that prostaglandins potentially regulate vascular resistance by modifying the 5-HT response.

Blood levels of 5-HT change during pregnancy and are affected by PE. Whereas the platelet 5-HT concentration increases during both healthy and PE pregnancies, the free 5-HT concentration is elevated during PE pregnancies only, in both the maternal and fetal circulation.[285-287] Moreover, this free 5-HT plasma concentration directly correlates

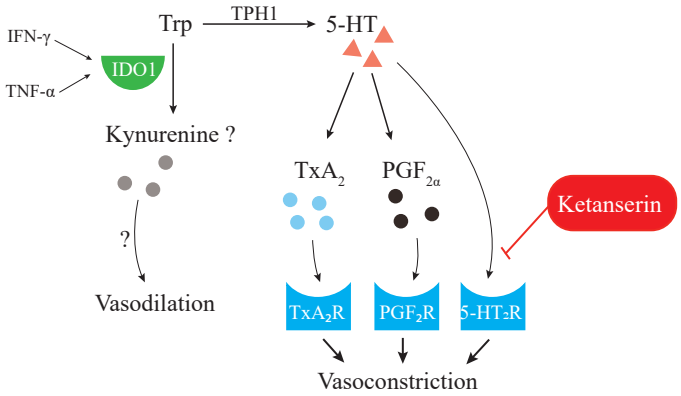

Figure 5. 5-HT and tryptophan in human pregnancy and therapeutic strategies targeting this pathway. During PE there is a decrease of IDO1, while circulating levels of TxA_2, $PGF_{2\alpha}$ and 5-HT are increased. Abbreviations: 5-HT = 5-hydroxytryptamine; 5-HT2R = 5-HT type 2 receptor; IDO = indolamine 2,3-dioxygenase; IFN-γ = interferon-γ; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; PGF2R = PGF_2 receptor; TNF-α = tumor necrosis factor-α; TPH1 = tryptophan hydroxylase-1; Trp = tryptophan; TxA_2 = thromboxane A_2; TxA_2R = TxA_2 receptor.

with systolic and diastolic blood pressure in women suffering from severe PE.[287] *Ex vivo* placental perfusion experiments showed unaltered or increased vascular resistance in response to 5-HT in PE as compared to healthy placentas.[82, 184] Conversely, in isolated umbilical vessels and placental veins from PE placentas, the sensitivity to 5-HT did not increase throughout the third trimester like in healthy placentas, with a reduced 5-HT vasoconstrictor response as compared to healthy placental veins as a result.[266, 288]

Research on therapeutic possibilities of interfering with the 5-HT pathway has mainly focused on the role and safety of antidepressants such as selective serotonin reuptake inhibitors (SSRI), serotonin-norepinephrine reuptake inhibitors (SNRI), and tricyclic antidepressants. Studies have not been conclusive, possibly because PE has been defined in various ways, and effects of antidepressants might depend on the timing of exposure during pregnancy.[289] Platelets of PE patients have a higher transport rate of 5-HT compared to healthy pregnant women.[290] Importantly, since a large prospective population-based study has shown that depression per se results in an increased risk of developing PE in women suffering from depression, independently of treatment,[291] effects of antidepressants on PE should be convincingly distinguished from the effect of the depression itself. Indeed, women who took antidepressant medications, but without depression, tended to display an increase in the risk of PE.[292] It could be reasoned that inhibition of the responsible serotonin transporter by SSRI could contribute to a reduced platelet 5-HT delivery in the periphery, and prevention of undesired vasoconstriction. The use of another drug, the 5-HT2 receptor antagonist ketanserin, was aborted in a double-blind randomized trial due to persistent hypertension in severe early-onset PE, despite promising results in earlier studies.[293, 294] Out of the women that received

ketanserin, 73.3% experienced persistent hypertension, which was treated successfully with the rescue medication nicardipine.[293] Besides the disappointing effects on blood pressure reduction, the use of ketanserin could also not aid in the prevention of further complications or pregnancy prolongation.[293]

In conclusion, whereas 5-HT induced vasoconstriction is equal or potentially even increased in the microvasculature of PE placentas, larger vessels seem to be less sensitive to 5-HT compared to those in healthy pregnancy. This difference between the larger and smaller vessels might be due to the presence or absence of different 5-HT receptors, that can have opposing effects. Nonetheless, the combined increased plasma levels and increased microvascular sensitivity to 5-HT can have a detrimental effect on vascular resistance *in vivo* and thus complicate placental blood flow and cause progressive worsening of PE.

Tryptophan

Trp conversion occurs for 95% via the kynurenine pathway into kynurenine and other metabolites (Figure 5). These metabolites have functions in inflammation and regulation of vascular tone.[295] The first and rate-limiting step of the pathway is the conversion of Trp to N-formylkynurenine, by either TDO, IDO1, or IDO2. While TDO is mainly expressed in the liver, IDO1 is highly expressed in the placenta during pregnancy. Its expression increases with the duration of pregnancy, and in closer proximity of the fetal-maternal interface.[296] Despite several conflicting reports, cell types in which IDO1 seems to be present include trophoblasts and decidual immune- and stromal cells.[297] In placental tissue, the microvascular endothelial cells together with the ends of maternal spiral arteries are the main locations of IDO1 expression.[298, 299] The high IDO1 expression is generally believed to be essential for maintenance of fetal-maternal tolerance.[297, 300] However, studies in animals have shown that Trp metabolism via IDO1 might be involved in regulation of vascular tone as well.[301] Knowledge on the effects of Trp in the human placental vasculature is very limited. A recent study showed that Trp can induce vascular relaxation in isolated vessel segments of the chorionic plate arteries. IDO1 seems to play a critical role in this process, since its inhibition attenuates the vascular relaxation in the presence of Trp. However, for isolated chorionic plate arteries to relax in response to Trp, it was necessary to stimulate them overnight with TNF-α and interferon (IFN)-γ,[302] which might be due to the fact that chorionic plate arteries do not express IDO1 under normal circumstances[298] and therefore, it needs to be upregulated by these inflammatory cytokines first before an effect can be seen. The *ex vivo* perfused cotyledon responded to Trp with a decrease in fetal perfusion pressure, even without pre-constriction of the placental vasculature,[302] suggesting that IDO1 metabolites contribute to regulation of placental microvascular resistance vessels. Since microvascular endothelial cells of the placenta were reported to express IDO1 under normal physiological conditions, it is

likely that these cells contribute to the vascular relaxation in response to Trp.[298] IDO1 is also present in vascular endothelial cells of the decidua basalis,[298] and thus it could be speculated that endothelial IDO1 has a vasodilatory effect in the maternal decidua. Trp is an endothelium dependent vasodilator, and the metabolite kynurenine is proposed to contribute to this vasodilator response (Figure 5).[301, 303] However, there might also be other metabolites with vasodilator effects besides kynurenine. Although these exact metabolites and mechanisms that contribute to vasodilation remain to be identified, the metabolism of Trp by IDO1 plays an important role in the regulation of vascular tone in placental microvasculature.

Altered Trp metabolism through the IDO1 pathway may be involved in the development and/or progression of PE and FGR. In these conditions, expression and activity of IDO1 is reduced.[299, 302, 304, 305] Yet, after overnight stimulation of chorionic arteries of PE and FGR placentas with TNF-α and IFN-γ, Trp still induced vascular relaxation.[302] However, these results should be interpreted with caution, since experiment numbers were very low and the added doses of Trp substantially exceeded physiological levels. *Ex vivo* perfusion experiments measuring the response of PE placentas to Trp in the presence and absence of IDO1 inhibitors are required to establish whether indeed IDO1 plays a critical role in PE.

If reduced Trp metabolism due to low IDO1 expression is causal for increasing placental vascular resistance, finding ways to increase Trp metabolism by IDO1 could be a potential target for treatment of the reduced placental blood flow in PE and FGR. However, it should be noted that, in contrast to PE, which is associated with reduced IDO1 activity, many other diseases are associated with increased Trp metabolism, and IDO1 inhibitors are now being tested in clinical trials as anticancer therapy. Extreme caution is warranted for use of these inhibitors during pregnancy and in women at child bearing age, as they might have detrimental effects on pregnancy progression.

Altogether, the metabolism of Trp by IDO1 seems to be crucial for a healthy pregnancy progression. The reported functions of Trp metabolism in regulation of vascular tone, together with the fact that IDO1 is mainly expressed in microvascular endothelial cells, imply a role for IDO1 in regulation of vascular tone in the placenta. Finally, given the fact that 95% of Trp is metabolized through the kynurenine pathway, it is possible that even a small reduction of IDO1 in PE may substantially increase the amount of Trp that is metabolized through the serotonin pathway, which, as outline above, is more activated in PE.

CALCITONIN GENE-RELATED PEPTIDE

CGRP is a neuropeptide, widely distributed in the central and peripheral nervous system, that has a potent vasodilator effect on vascular tone. CGRP acts through binding of the G protein-coupled calcitonin receptor-like receptor (CRLR), in the presence of the receptor activity modifying protein-1 ($RAMP_1$).[306]

In the human placenta, both CRLR and $RAMP_1$ are abundantly expressed in the vascular endothelium and underlying smooth muscle cells of the umbilical, chorionic and stem villous vessels, as well as in the villous trophoblast.[307] Stevenson *et al.* showed a significant increase in maternal plasma CGRP levels throughout normal pregnancy, with a rapid drop after delivery, indicating the placenta as a source of CGRP production.[308] Furthermore, it has been shown that there is a weight- and gestational age-dependent increase in neonatal plasma CGRP levels.[309] This suggests a role for CGRP in vascular adaptation in pregnancy and fetal growth and development. It has also been suggested that CGRP is involved in maintaining uterine relaxation during pregnancy.[310] Isolated chorionic plate arteries show a dose-dependent relaxation response to CGRP, an effect that is attenuated in the presence of a CGRP receptor antagonist, further indicating that CGRP plays a role in the control of fetoplacental vascular tone.[307, 311] In placental arteries of pregnancies complicated by PE or FGR the vasodilator effect of CGRP is significantly reduced.[311-313] In line with this finding, Dong *et al.* showed that mRNA expression of CRLR and $RAMP_1$ in placental vessels of PE pregnancies is reduced, which was accompanied by a decrease in CRLR and $RAMP_1$ protein expression and CGRP binding sites in vascular and trophoblast tissue of PE placentas.[313] Furthermore, maternal serum levels of CGRP are significantly lower in women with PE compared to uncomplicated pregnancies.[314] This evidence supports a potential role of compromised CGRP-mediated vasodilation in the pathogenesis of PE. In animal models of PE, administration of CGRP reduced maternal hypertension and pup mortality in rats.[315] In agreement with this finding, infusion of a CGRP receptor antagonist induced maternal hypertension and caused a significant decrease in pup weight.[316]

Unfortunately, knowledge on the underlying mechanisms of CGRP on fetoplacental development is still lacking and direct targeting of the CGRP-pathway remains complicated. CGRP itself can only be administered parenterally, has a very short half-life and is costly. Rutaecarpine, a traditional Chinese drug that potentiates the release of endogenous CGRP, could be a therapeutic option. However, its effect in pregnancy is unknown.[317] Also, an αCGRP analogue with a longer half-life has been described in murine studies, investigating its application in cardiovascular disease, where it showed antihypertensive effects.[318]

CONCLUSION AND PERSPECTIVES

Since the blood vessels of the fetoplacental vasculature lack autonomous innervation, circulating and locally produced hormones are essential in regulating vascular tone. As has been shown in this review, mechanisms behind this regulation are very intricate, as they involve many pathways, and are influenced by numerous factors. Adequate development of the placenta is essential for an optimal course of pregnancy and subsequent maternal, fetal and neonatal outcome. The pathophysiology of placental diseases such as PE seems multi-factorial and complex, including a cascade of dysregulated systems. Finding new treatment options that safely prolong pregnancy is essential for reducing the risk of fetal, neonatal and maternal complications. This review has highlighted the importance of focusing on restoring the dysfunctional vascular regulatory systems when studying treatment strategies for PE. Techniques using human tissue, such as the *ex vivo* placental perfusion model and wire-myography, are indispensable in unraveling the vasoactive profile of the human placenta, to help understand the pathological changes occurring during PE.

Future research should focus on both efficacy and safety, by performing well designed dose-finding studies before starting clinical trials. Targeting the ET-axis by blocking the ET-1 mediated effects or restoring the sFlt-1/PlGF imbalance currently seems to have the greatest therapeutic potential. Here a first step might be to determine trans-placental passage of ERAs and to quantify their effects on sFlt-1 release in the *ex vivo* perfusion model. Another promising treatment option would be increasing NO-mediated vasodilation through sGC stimulators or activators. Although such drugs showed beneficial effects on placental tissue, results of clinical trials are still not available. When considering new drugs for treatment, the first step should be to determine their trans-placental transfer and effect on the fetal vasculature, for which the *ex vivo* placental perfusion model is a very reliable method. Parallel studies with isolated arteries may help to obtain a more detailed mechanistic insight. With this, differences in pathophysiology between early- and late onset PE and the changes in pharmacokinetics that occur during pregnancy should be kept in mind. Furthermore, individual differences in pathway disturbance may call for a more personalized therapeutic approach.

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Chapter 3

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 nitroprusside (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-maternal 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 placentas, 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),

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.

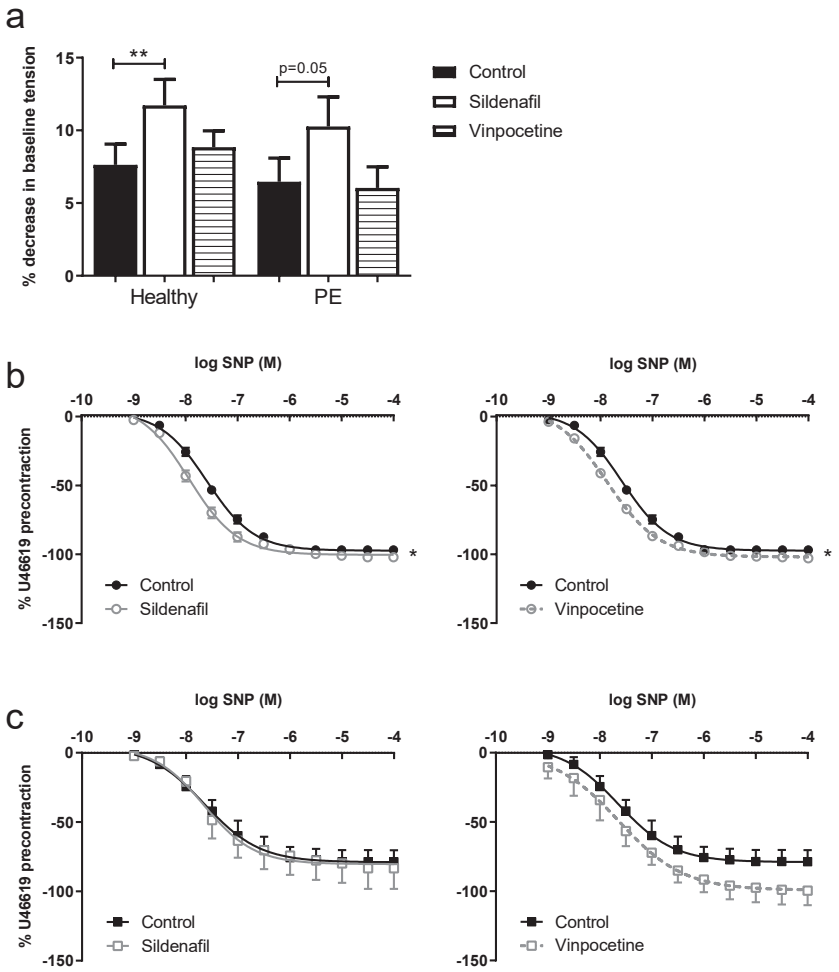

Figure 1. Wire-myography experiments. Panel a, decrease of baseline tension in response to PDE inhibitors. Panels b and c, effect of PDE inhibition with sildenafil or vinpocetine on SNP-mediated relaxation of chorionic plate vessels of seven healthy (b) and five PE (c) placentas. Data (mean±SEM) are 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).

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.

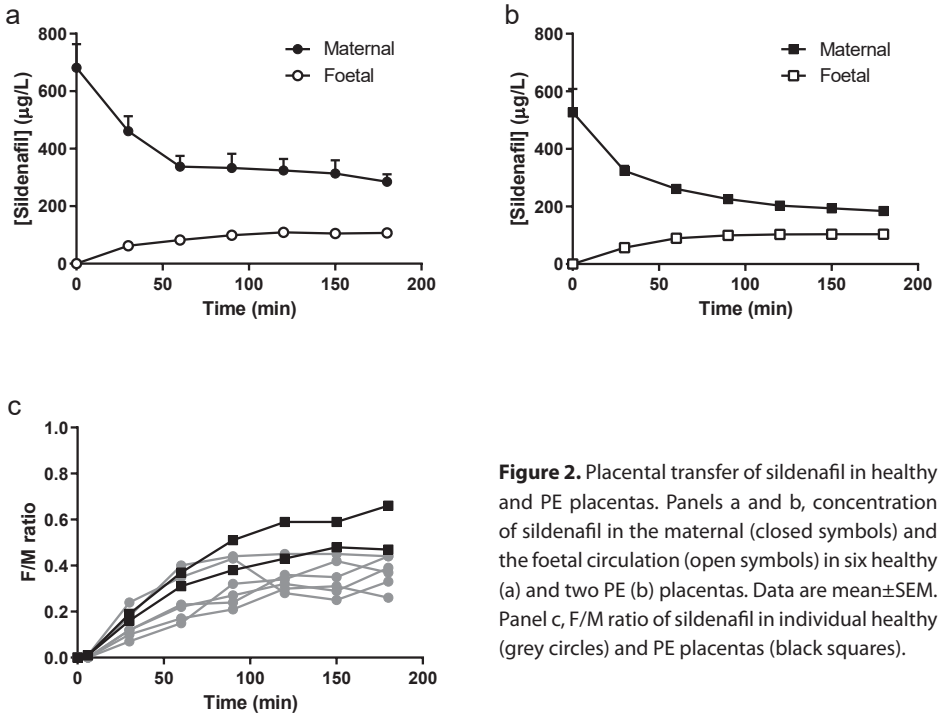

Figure 2. Placental transfer of sildenafil in healthy and PE placentas. Panels a and b, concentration of sildenafil in the maternal (closed symbols) and the foetal circulation (open symbols) in six healthy (a) and two PE (b) placentas. Data are mean±SEM. Panel c, F/M ratio of sildenafil in individual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

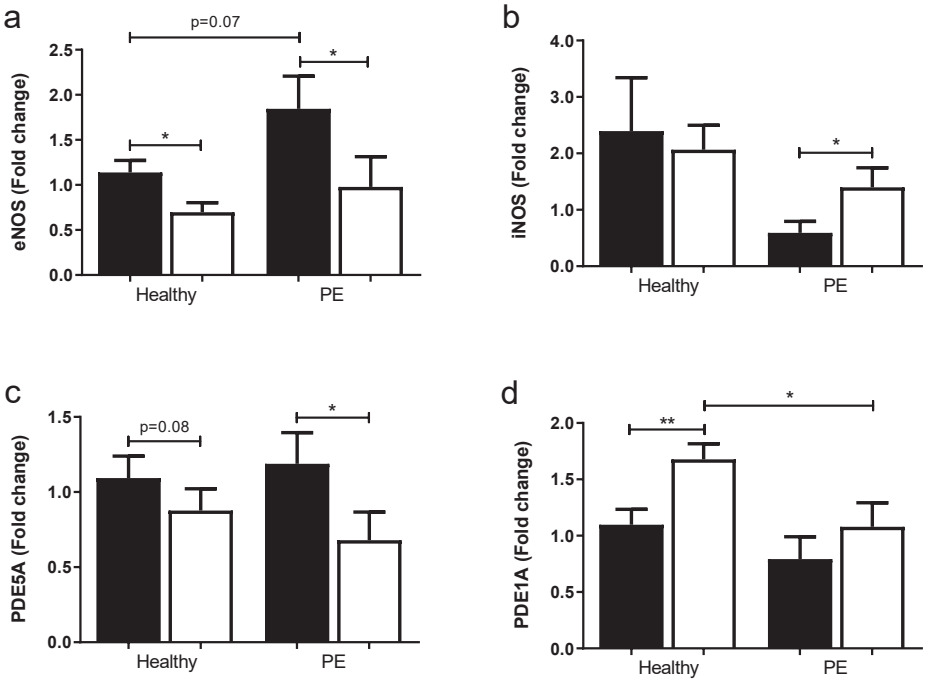

Figure 3. mRNA expression. qPCR analysis of placental biopsies from the maternal (black bars) and foetal side (white bars) of 12 healthy and seven PE placentas. Data (mean±SEM) are expressed as fold change versus the maternal side biopsies of healthy controls. Panels a-d represent data for eNOS, iNOS, 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.

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.

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SUPPLEMENTAL INFORMATION

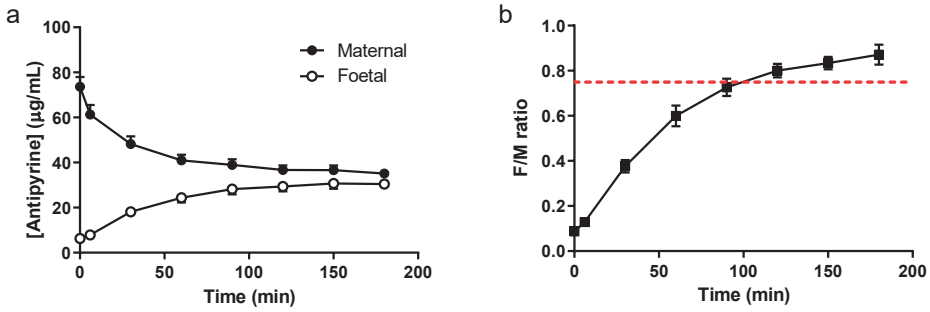

Figure S1. Placental transfer of antipyrine. Panel A, maternal and foetal concentrations. Panel B, F/M ratio, with the cutoff point of 0.75 (dashed red line).

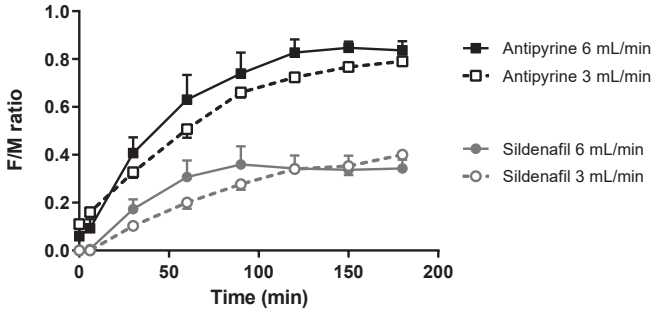

Figure S2. Lack of different placental transfer outcomes at different foetal flow rates.

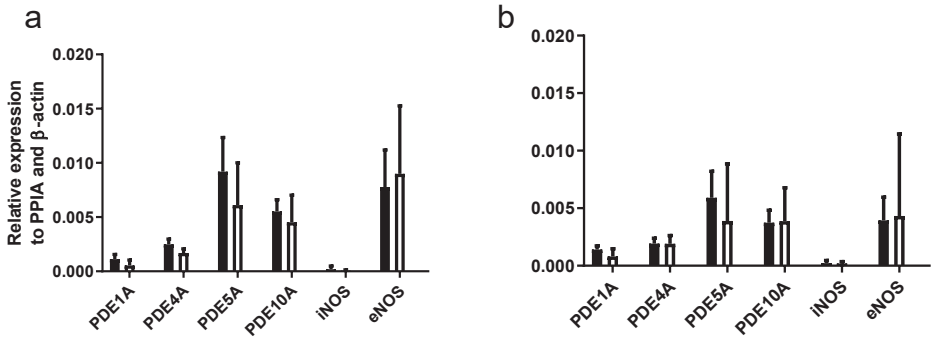

Figure S3. mRNA expression of different PDEs on the maternal side (a) and foetal side (b) of healthy (black bars) and PE (white bars) placentas.

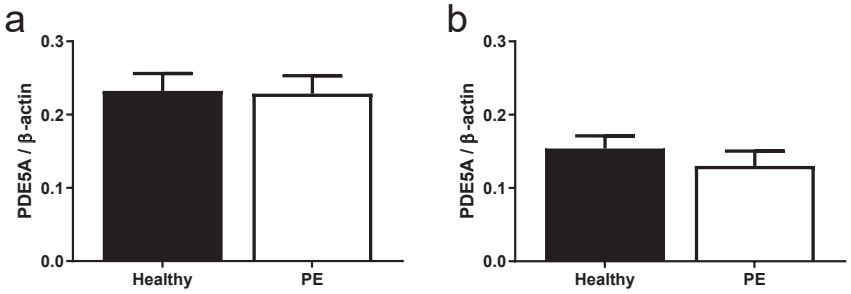

Figure S4. Protein expression of PDE5A based on Western blot analysis at the maternal (a) and foetal (b) side of the placenta.

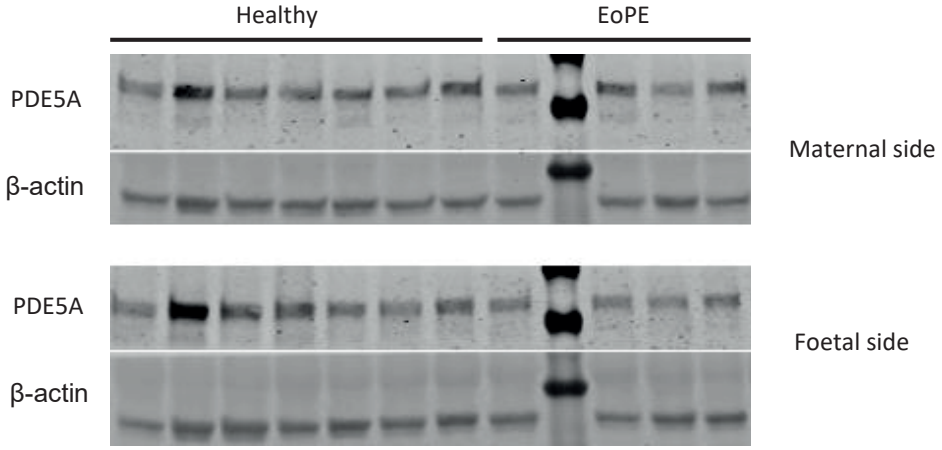

Figure S5. Representative Western blot of PDE5 protein expression.

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. *$p<0.05$.

Chapter 4

Vascular effects of pentoxifylline; towards a novel therapeutic option for preeclampsia

Hitzerd E, Broekhuizen M, de Vries R, Danser AHJ, Reiss IKM, Simons SHP

In preparation

ABSTRACT

Recently, the non-selective phosphodiesterase inhibitor pentoxifylline (PTX) has gained interest as a possible therapeutic that could target both the generalized endothelial dysfunction and the immune system imbalance in preeclampsia (PE). However not much is known about its effects on the placenta. Therefore, in this study the vascular effects of PTX were evaluated. PTX concentration-response curves after incubation with SQ22536 or L-NAME were constructed in porcine coronary arteries and human chorionic plate arteries of both healthy and PE placentas. The effect of PTX-incubation on vasodilation through the cGMP - and cAMP pathways was studied in segments of the same vessels using the endothelium-independent NO donor sodium nitroprusside and the adenylyl cyclase activator forskolin, respectively. PTX exerted direct vasodilator effects on both porcine coronaries and human chorionic plate arteries, that could be blocked by L-NAME, indicating that this effect is mainly NO/cGMP-mediated. This dilator effect was increased in arteries of PE placentas. PTX enhanced the vasodilator effects of sodium nitroprusside and forskolin in chorionic plate arteries derived from healthy, but not PE placentas. Since PTX induced direct vasodilation in chorionic plate arteries, and even to a greater extent in PE, it could be a beneficial treatment option to improve placental perfusion. As a next step placental transfer of PTX should be studied.

INTRODUCTION

Preeclampsia (PE) is a serious placenta-related pregnancy disorder, affecting approximately 5-8% of all pregnancies.[1] It is characterized by hypertension with an onset 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.[2] Early onset PE, i.e. diagnosis before the 34th week of gestation,[3] is not only associated with an increased risk of maternal and fetal complications during pregnancy, but it can also lead to health problems later in life for both mother and child.[1, 4, 5] Different pathophysiological mechanisms have been proposed for early onset PE, all involving impaired placentation in early pregnancy, leading to increased vascular resistance, generalized endothelial dysfunction and endovascular inflammation. Previous studies showed that PE patients have increased plasma levels of pro-inflammatory cytokines, such as interleukin(IL)-6, interferon(IFN)-γ and tumor necrosis factor(TNF)-α. On the other hand, the anti-inflammatory cytokines IL-5 and IL-10 seem to be suppressed.[6, 7] Furthermore, increased concentrations of pro-inflammatory cytokines have been found in placental tissue of PE patients compared to healthy controls.[8] This imbalance in the immune response attributes to the impaired placentation, endothelial damage and ischemic-reperfusion injury seen in PE.[9-11] It would therefore be very interesting to target both the generalized endothelial dysfunction and the immune system imbalance to treat early onset PE, for example by using the methylxanthine derivative pentoxifylline (PTX). PTX is a non-selective phosphodiesterase (PDE) inhibitor, that increases the intracellular concentration of cAMP.[12] It has been shown to have anti-inflammatory properties, scavenge oxygen radicals, improve endothelial function, induce vasodilation, increase erythrocyte flexibility and inhibit platelet aggregation.[13, 14] Although currently only registered for intermittent claudication, PTX has been suggested as a possible preventive or therapeutic intervention for inflammatory placental diseases such as PE and preterm birth.[15, 16] Previously, Speer *et al*. showed that PTX reduces inflammation in placental explants.[12] Furthermore, it has been indicated that PTX has a beneficial effect on the fetoplacental circulation in patients with imminent preterm labor.[16] However, the mechanisms behind these vascular effects are not well understood. Therefore, the aim of this study was to evaluate the vascular effects of PTX, using both porcine coronary arteries as well as human chorionic plate arteries of healthy and PE placentas.

METHODS

Tissue collection

Placentas of women with uncomplicated singleton pregnancies who underwent an elective cesarean section were collected immediately after delivery at the Erasmus MC

University Medical Center, Rotterdam, the Netherlands. Also, placentas of women with early onset PE (diagnosis before the 34th week of gestation) were collected. 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), and all patients gave written consent to use of their placenta and data prior to the experiments. Additionally, porcine hearts were collected from the slaughterhouse.

Wire-myography experiments with porcine coronary arteries

Coronary arteries were dissected from the hearts and stored overnight in cold, oxygenated 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; pH 7.4). Vessel segments of 4 mm were suspended on stainless steel hooks in 15-ml organ baths, filled with Krebs-Henseleit buffer at 37°C and aerated with 95% O_2 – 5% CO_2. After a period of equilibration, vessel segments, stretched to a stable force of about 15 mN, were exposed to 30 mmol/l KCl twice. Subsequently, maximum contractile responses were determined using 100 mmol/l KCl. After washout of the KCl, segments were pre-incubated for 30 min in the absence or presence of one of the following inhibitors: the adenylate cyclase inhibitor SQ22536 (100 µmol/l), the NOS-inhibitor L-NAME (100 µmol/l) or PTX (100 µmol/l). All vessel segments were then pre-constricted using the thromboxane A2 agonist U46619 (1 µmol/l). Segments that were pre-incubated with SQ22536 or L-NAME were used to construct concentration-response curves to PTX (1 nmol/l - 300 µmol/l), and segments that were pre-incubated with PTX were exposed to sodium nitroprusside (SNP, 1 nmol/l - 100 µmol/l) or forskolin (1 nmol/l - 30 µmol/l). Vessel segments without any additives were used as a control for all concentration-response curves. Changes in tissue contractile force were recorded with a Harvard isometric transducer (South Natick, MA, USA).

Wire-myography experiments with human chorionic plate arteries

Segments of second order branches of chorionic plate arteries (2 mm in length) were 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 38 mmHg effective transmural pressure to mimic the physiological circumstances of placental vessels. Maximum contractile responses were determined using 100 mmol/l KCl. Experimental protocols were similar to those used for the porcine coronary arteries. After washout of KCl, all vessel segments were pre-constricted with U46619 (10 nmol/l). Concentration-response curves for PTX (1 nmol/l - 300 µmol/l) were performed in a subset of segments, that had been pre-incubated for 30 min with either SQ22536 (100 µmol/l), L-NAME (100 µmol/l) or both. Again vessel segments without any additives were used as a control. Concentration-response curves for SNP (1 nmol/l - 100 µmol/l) and forskolin (1 nmol/l - 30 µmol/l) were constructed in the presence or absence of PTX (100 µmol/l).

Chemicals

PTX (Trental®) was acquired from the hospital pharmacy of Erasmus MC, Rotterdam, the Netherlands. All other compounds came from Sigma-Aldrich, Schnelldorf, Germany.

Data analysis

Data are presented as mean±SEM. Vascular responses are expressed as a percentage of the contractile response to 100 mmol/l KCl or U46619. Log10-transformed values at which the half-maximal response (pEC_{50}) occurred were individually estimated using sigmoid curve fitting software (GraphPad Prism 5, La Jolla, CA, USA). In case of normally distributed data, groups were analyzed using 1-way ANOVA with Bonferroni post-hoc evaluation. When 1-way ANOVA was significant, individual comparisons were made with Student's *t* test. A p-value of <0.05 was considered statistically significant.

RESULTS

Porcine coronaries

Figure 1A shows the concentration-response curves to PTX in porcine coronary arteries. The maximum relaxation (E_{max}) induced by PTX was 68±5% of U46619 pre-constriction, with a pEC_{50} of -4.2±0.1. Incubation with L-NAME or SQ22536 reduced E_{max} to 30±9% and 55±7% respectively, however this was only significant for L-NAME (p=0.003, Table 1). Incubation with either antagonist shifted the PTX curve to the left, evidenced by a decrease in pEC_{50} (p=0.05, Table 1). Incubation with PTX tended to shift the SNP (p=0.09) and forskolin (p=0.06) curves to the left, without altering E_{max} (Figures 1B and 1C and Table 1).

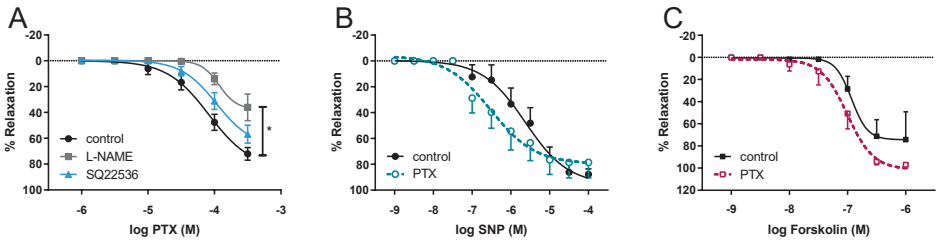

Figure 1. Results of the wire-myography experiments using porcine coronary arteries. Panel A shows the concentration-response curves to pentoxifylline (PTX) in control segments (circles) or after incubation with SQ22536 (triangles) or L-NAME (squares). Panel B shows the concentration-response curves to sodium nitroprusside (SNP) in control segments (closed circles) or after incubation with of 100 µmol/l PTX (open circles). Panel C shows the concentration-response curves to forskolin in control segments (closed squares) or after incubation with 100 µmol/l PTX (open squares). Data are mean±SEM (n=5-9). *p<0.01, 1-way ANOVA for repeated measures with Bonferroni post-hoc evaluation.

Table 1. Organ bath experiments with porcine coronaries.

			Incubation	
CRC	**Control**	**SQ22536**	**L-NAME**	**PTX**
pEC_{50}				
PTX	-4.2±0.1	-4.0±0.1	-3.9±0.1*	
SNP	-5.7±0.3			-6.4±0.3
Forskolin	-6.6±0.2			-7.1±0.2
E_{max}				
PTX	68±5	55±8	30±9**	
SNP	91±4			79±12
Forskolin	99±3			98±2

pEC_{50}: log10-transformed concentrations at which the half-maximum response occurred. E_{max}: maximum effect expressed as % relaxation of U46619 constriction. *p<0.05 and **p<0.01 compared to control, 1-way ANOVA for repeated measures with Bonferroni post-hoc evaluation.

Human chorionic plate arteries

The concentration-response curves to PTX, SNP and forskolin in human chorionic plate arteries are shown in Figure 2. In chorionic plate arteries of healthy placentas, PTX induced an E_{max} of 74±8% of U46619 pre-constriction, with a pEC_{50} of -4.4±0.1 (Table 2). Incubation with L-NAME, SQ22536, or both did not affect E_{max} (Table 2, Figure 2A), but L-NAME with or without SQ22536 did shift the PTX curve to the left (p=0.02 and p=0.04, respectively). Incubation with PTX potentiated both SNP and forskolin, evidenced by a decreased pEC_{50} (p=0.002 and p=0.008, respectively, Figure 2B and 2C, Table 2). In chorionic plate arteries of PE placentas, the E_{max} induced by PTX tended to be larger than in healthy placentas (p=0.07, Table 3). None of the antagonists altered the pEC_{50} of

Table 2. Organ bath experiments with human chorionic plate arteries of healthy placentas.

			Incubation		
CRC	**Control**	**SQ22536**	**L-NAME**	**SQ22536 + L-NAME**	**PTX**
pEC_{50}					
PTX	-4.4±0.1	4.3±0.1	-4.1±0.1*	4.1±0.1*	
SNP	-7.9±0.1				-8.7±0.2**
Forskolin	-5.8±0.2				-6.6±0.1**
E_{max}					
PTX	74±8	64±10	59±9	53±10	
SNP	90±6				90±4
Forskolin	88±8				113±10

pEC_{50}: log10-transformed concentrations at which the half-maximum response occurred. E_{max}: maximum effect expressed as % relaxation of U46619 constriction. *p<0.05 and **p<0.01 compared to control, Student's *t* test.

PTX in PE vessels (Figure 2D, Table 3). Furthermore, PTX did not alter the response to SNP and forskolin in PE arteries (Figure 2E and 2F, respectively).

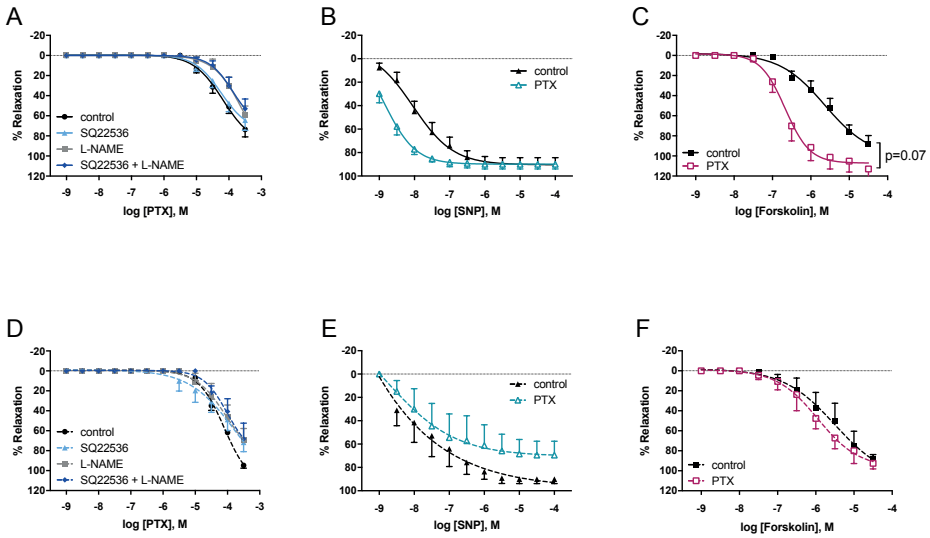

Figure 2. Results of the wire-myography experiments using human chorionic plate arteries. The upper part of the figure (Panels A, B and C) represent the results of chorionic plate arteries of healthy placentas (solid lines, n=6-10) and the lower part (Panels D, E and F) the results of chorionic plate arteries of preeclamptic placentas (dashed lines, n=3-5). Panels A and D show the concentration-response curves to pentoxifylline (PTX) in control segments (circles) or after incubation with SQ22536 (triangles), L-NAME (squares) or both (diamonds). Panels B and E show the concentration-response curves to sodium nitroprusside (SNP) in control segments (closed triangles) or after incubation with of 100 µmol/l PTX (open triangles). Panels C and F show the concentration-response curves to forskolin in control segments (closed squares) or after incubation with 100 µmol/l PTX (open squares). Data are mean±SEM.

Table 3. Organ bath experiments with human chorionic plate arteries of preeclamptic placentas.

		Incubation			
CRC	**Control**	**SQ22536**	**L-NAME**	**SQ22536 + L-NAME**	**PTX**
pEC_{50}					
PTX	-4.1±0.2	-4.0±0.2	-4.1±0.3	-4.0±0.1	
SNP	-7.9±0.5				-7.5±0.6
Forskolin	-5.8±0.3				-5.6±0.6
E_{max}					
PTX	95±3	72±9	69±11	68±16	
SNP	90±4				70±12
Forskolin	89±5				93±6

pEC_{50}: log10-transformed concentrations at which the half-maximum response occurred. E_{max}: maximum effect expressed as % relaxation of U46619 constriction. *p<0.05 and **p<0.01 compared to control, Student's *t* test.

DISCUSSION

This study shows that PTX has direct vasodilator effects on both porcine coronaries and human chorionic plate arteries, and that these effects can be blocked by L-NAME. Besides directly inducing vasodilation, PTX also enhanced the vasodilator effects of the NO donor SNP and the adenylate cyclase activator forskolin in chorionic plate arteries derived from healthy, but not PE placentas.

In line with previously reported effects of PTX on for example rat mesenteric arteries and rabbit aorta,[18, 19] PTX evoked concentration-dependent relaxation in the current study. Similar to the results of Hansen *et al*.[19], who showed inhibition of the PTX response by L-NAME in rat mesenteric vessels, we saw a blocking effect on PTX response by L-NAME in porcine coronary arteries and human chorionic plate arteries. The fact that incubation with the adenylate cyclase inhibitor SQ22536 did not result in similar blocking effects indicates that, at least in these vessels, the acute dilator PTX response is mainly cGMP-mediated. PTX did enhance the vasodilator effects of both SNP and forskolin in healthy chorionic plate arteries, confirming that it affects both cGMP and cAMP degradation. In contrast, no such potentiation was seen in the rabbit aorta,[18] emphasizing the difficulties of translating results from animal studies to humans.

PTX tended to induce stronger relaxant effects in PE arteries, yet was unable to potentiate forskolin or SNP in these arteries. The latter is reminiscent of what was observed for the PDE inhibitors sildenafil and vinpocetine in PE arteries,[17] and suggest that the larger relaxant effects of PTX are unrelated to PDE inhibition. Hence, PTX might still be a beneficial treatment option to improve placental perfusion, acting via a non-PDE-dependent mechanism.

Besides its vasodilator properties, PTX has been shown to reduce inflammation. In placental explants with LPS-induced inflammation, PTX reduced expression and production of the pro-inflammatory cytokines TNF-α, IFN-γ and IL-1β.[12] It could therefore be speculated that PTX is a good candidate to treat placental inflammatory conditions *in vivo*. PTX is already studied as anti-inflammatory treatment in preterm born infants with suspected late-onset sepsis or necrotizing enterocolitis, with promising results.[20, 21]

Before starting a clinical trial in pregnancy, it is essential to gain knowledge on the placental transfer of PTX. No teratogenic effects of PTX have been described in animal studies, and clinical trials with PTX as treatment to prevent preterm labor did not report negative effects, although no long-term follow-up has been performed.[16] Furthermore, in preterm infants PTX treatment was well tolerated without significant adverse effects.[20]

CONCLUSION

Since it has vasodilator and anti-inflammatory properties, PTX is a very interesting drug that could target both the generalized endothelial dysfunction and the immune system imbalance seen in PE. As a next step we suggest to study placental transfer of PTX in both healthy and (preterm) preeclamptic placentas. Furthermore, we should expand our knowledge on the anti-inflammatory effects of PTX in the PE placenta, using placental explants and/or trophoblast cell culture. With this, possible differences between early – and late onset PE, with or without fetal growth restriction should be kept in mind. Furthermore, a pharmacokinetic model of PTX in pregnant women should be made before starting a clinical trial.

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Chapter 5

Endothelin receptor antagonism during preeclampsia: a matter of timing?

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ABSTRACT

Preeclampsia (PE) is a pregnancy complication, featuring elevated blood pressure and proteinuria, with no appropriate treatment. Activation of the endothelin system has emerged as an important pathway in PE pathophysiology based on experimental PE models where endothelin receptor antagonists (ERAs) prevented or attenuated hypertension and proteinuria. Hence, ERAs have been suggested as potential therapy for PE. However, developmental toxicity studies in animals have shown severe teratogenic effects of ERAs, particularly craniofacial malformations. Nonetheless, sporadic cases of pregnancy in women using ERAs to treat pulmonary hypertension have been described. In this review we give an overview of cases describing ERA use in pregnancy and critically address their possible teratogenic effects. A systematic search in literature yielded 18 articles describing 39 cases with ERA exposure during human pregnancy. In most cases there was only exposure in the first trimester, but exposure later or throughout pregnancy was reported in 5 cases. Elective termination of pregnancy was performed in 12 pregnancies (31%), two ended in a spontaneous miscarriage (5%) and no fetal congenital abnormalities have been described in the remaining cases. These preliminary findings support the idea that ERA treatment for severe, early onset PE might be an option if applied later in pregnancy, when organogenesis is completed to avoid teratogenic risks. However, third trimester toxicology studies are warranted to evaluate drug safety. Subsequently, it remains to be established whether ERA treatment is effective for alleviating maternal symptoms, as demonstrated in preclinical PE models, allowing pregnancy prolongation without leading to adverse neonatal outcomes.

INTRODUCTION

Preeclampsia (PE), a syndrome featuring de novo hypertension accompanied by proteinuria and/or evidence of maternal acute kidney injury, liver dysfunction, neurological features, hemolysis or thrombocytopenia, and/or fetal growth restriction (FGR), is the most frequently encountered medical complication during pregnancy.[1] Ultimately, PE may lead to severe complications, thereby increasing maternal, fetal and neonatal morbidity and mortality.[2, 3] While randomized trials have tested interventions with different antihypertensive agents including methyldopa and various calcium antagonists, these drugs only show a temporary effect on stabilization of the clinical manifestations of PE, but not on hard clinical outcomes such as mortality.[4-6] To date, the only effective treatment of PE is delivery of the placenta and hence the baby, often severely premature. Consequently, novel treatment strategies to prevent or alleviate PE are urgently needed. Achieving an additional week of gestational age in premature infants enhances fetal maturity and hereby leads to a decrease in fetal mortality and enhances neonatal outcome.[7] There is strong experimental evidence that activation of the endothelin (ET)-system plays a key role in the pathophysiology of PE.[8] In addition, increased ET-1 levels have been reported in PE compared to healthy pregnancies.[9] A possible therapeutic strategy could therefore be to target the activated ET-axis by using endothelin receptor antagonists (ERAs).[8] However, the latter approach remains controversial since developmental toxicity studies have shown serious teratogenic effects in offspring of animals treated with ERAs during pregnancy. Nonetheless, it is important to note that this teratogenicity observed in animals does not necessarily translate to humans. The aim of this review is to provide a complete overview of ERA use in pregnancy, based on evidence from humans and animals, and to critically address their potential teratogenic effects.

ENDOTHELIN-1 AS THE ROOT CAUSE OF PREECLAMPSIA

Though not fully elucidated, evidence suggests that the activation of the ET-axis is strongly linked to the manifestations of PE.[10, 11] The ET-system consists of a family of 3 structurally closely related amino acid peptides (ET-1, ET-2 and ET-3). The main ET synthesized and secreted by endothelial cells is ET-1, this peptide is also synthesized and secreted by the syncytiotrophoblasts of the placenta.[12] ET-1 exerts its effect by binding to the cell-membrane G-protein-coupled ET type A receptors (ET_AR) and ET type B receptors (ET_BR).[13] Activation of ET_AR and ET_BR on vascular smooth muscle cells initiates prolonged vasoconstriction as well as cell proliferation. Conversely activation of the ET_BR on endothelial cells mediates vasodilation by releasing nitric oxide (NO) and prostacyclin (Figure 1). ET_BR activation may therefore counterbalance the ET_AR-mediated

vasoconstrictive response.[14, 15] In addition, the ET_BR serves as a clearance receptor for endothelins.[16]

ET-1 in pregnancy is regulated by numerous factors as recently reviewed by Granger *et al*.[8] The functional role of the ET-system in healthy pregnant women is not well known, it seems to promote first trimester trophoblast proliferation and invasion,[17] and an increased ratio of ET_BR to ET_AR has been observed in human placental villi during their development (Figure 1).[18] Furthermore, while maternal serum levels of ET-1 during healthy gestation remain similar to those of non-pregnant women, a substantial rise is detected during labor,[19, 20] implicating ET-1 involvement in the initiation of uterine contractions.[21] In addition, high concentrations of ET-1 in the amniotic fluid and in the fetal circulation are observed, being almost 3-fold higher in the umbilical vein when compared to maternal serum levels around labor.[22]

In animal models inducing placental ischemia, chronic elevation of soluble Fms-like tyrosine kinase-1 (sFlt-1), infusion of tumor necrosis factor-alpha (TNF-α) or agonistic autoantibodies to the angiotensin II type I receptor (AT1-AA), all induce a PE-like syndrome. In all these animals a significant increase in the expression of the precursor peptide prepro-ET-1 is seen in the kidney, as well as in the maternal vasculature in the case of AT1-AA and in the case of TNF-α in both the maternal vasculature and the placenta.[23-25] This enhanced prepro-ET-1 expression likely translates in higher ET-1 levels, underlying the rise in blood pressure in PE, as this rise can be prevented by ERAs. Furthermore, in these animals the ET_BR is downregulated in endothelial cells.[26] Similarly, decreased ET_BR expression in vascular endothelial cells has been found in pregnant women suffering from PE.[27] Subsequently, a wide range of clinical studies in pregnant women has observed a 2- to 3-fold rise of circulating ET-1 in PE compared to normal pregnancies.[9, 10, 28, 29] In addition, multiple regression analysis revealed that ET-1 is an independent determinant of urinary protein in PE and of the suppression of the renin-angiotensin aldosterone system in this condition.[10]

Regarding ERA effects in the treatment of the maternal syndrome of PE, knowledge comes from experimental animal models of PE. For example, in the reduced uterine perfusion pressure model in rats, ET_AR blockade reduced maternal blood pressure.[30] This is suggestive for an upregulation of the ET-1-ET_AR pathway in this model, which when blocked allows counter regulatory factors (like NO) to lower blood pressure.[8, 31] In rats infused with sFlt-1 and soluble endoglin, treatment with selective ET_AR antagonists also significantly decreased hypertension, urinary protein-to-creatinine ratio, hemolysis and liver enzymes, and increased platelets (Table S1, online-only data supplement).[32] Moreover, compared to placebo, FR-139317, an ET_AR antagonist (Table 1), potentially by improving placental perfusion, prevented FGR in rats placed in a hypoxic environment (Table S1).[33]

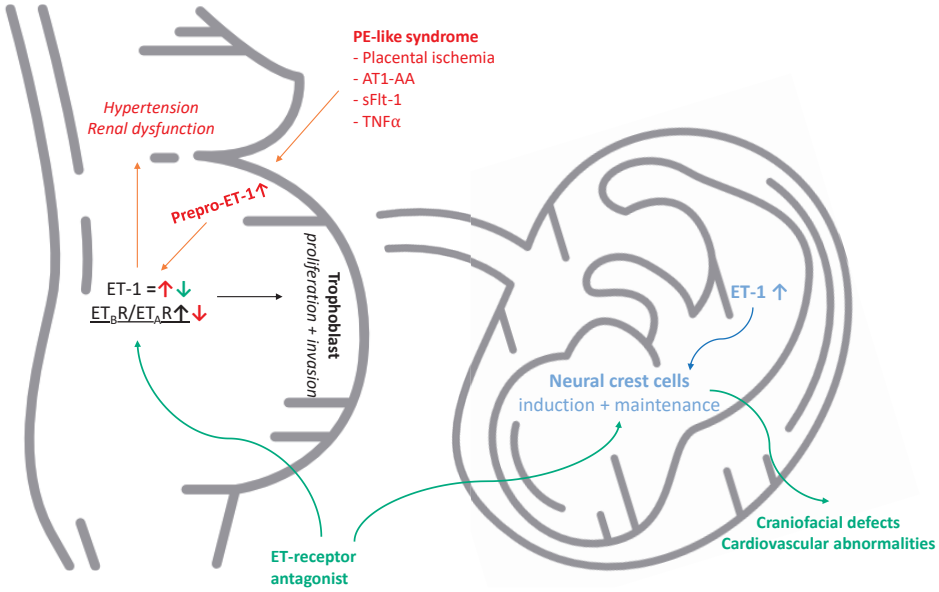

Figure 1. Role of endothelin in healthy pregnancies and preeclampsia-like syndrome. This figure shows the ET-1 levels in healthy pregnancy and non-pregnant women (in black) and the effects of a PE-like syndrome (in red). In blue the effects of increased ET-1 levels in the fetal circulation during healthy pregnancy are shown and in green the effects of ERAs on ET-1 levels.

Table 1. Different endothelin receptor antagonists and their targeted receptor.

ERA	Receptor blockade
A-127722	ET_A
A-182086	ET_A / ET_B
ABT-546	ET_A
ABT-627	ET_A
Ambrisentan	ET_A
Bosentan	ET_A / ET_B
BQ-123	ET_A
BQ-788	ET_B
FR-139317	ET_A
L-753,037	ET_A
SB-209670	ET_A / ET_B
SB-217242	ET_A / ET_B
Sitaxentan	ET_A
TBC-3214	ET_A

Thaete *et al*. examined the effect of selective ET_AR (A-127722 and FR-139317) and dual (A-182086) ERAs on fetal and placental growth in rats, with and without long-term NO synthase (NOS) inhibition with L-NAME. The dual ERA resulted in fetal and placental growth restriction, without attenuating the fetal and placental growth restriction caused by L-NAME infusion. In contrast, the selective ET_AR antagonists improved fetal and placental growth during NOS inhibition, and were without adverse effects in the absence of NOS inhibition.[34] These studies suggest that blocking ET_AR-mediated ET-1 signaling might be more beneficial in PE or FGR then dual ET_AR/ET_BR blockade. Selective ET_BR blockade in the same PE animal model with L-NAME was lethal for all animals.[35]

Nonetheless, excitement about ERAs as a possible treatment for PE has been dampened by severe teratogenic effects following maternal ERA administration as well as by ET-1, ET-3 and ET_AR or ET_BR gene disruption experiments during gestation in animal models, in particular serious craniofacial and cardiovascular malformations, pigmentary abnormalities and aganglionic megacolon.[36] Because of this, clinical trials studying ERA treatment to alleviate or even to prevent PE are lacking.

ENDOTHELIN-1 DURING EMBRYOGENESIS

To explain the occurrence of teratogenic effects during ERA treatment, understanding the role of ET-1 during embryogenesis is crucial. During early embryonal development, the ET-pathway plays an important role in the induction and maintenance of neural crest cells,[37] cells migrating to many different locations and differentiating into a wide variety of cell types including the craniofacial skeleton, cartilage, neurons and glia of the peripheral nervous system, connective tissue, neuroendocrine cells, and melanocytes.[38] The ET-axis is also involved in maintaining the high vascular resistance that is needed in the fetal lung development during pregnancy.[39] In the lung ET_AR is abundantly expressed during both the pre- and (early) postnatal periods, whereas the ET_BR is mildly expressed during early lung development. The lung ET_BR expression increases and stabilizes in the last prenatal stages and after birth, preventing muscularization of pulmonary pre-capillaries by stimulating vasodilation and ET-1 clearance.[40] Alteration of ET-1 or its receptors seems to affect normal embryonal development by impairing neural crest cell migration,[36, 41] as has been demonstrated by studies using murine knockout models.[42, 43] Clouthier *et al*. showed that ET_AR deficient mice have severe craniofacial defects, such as underdeveloped mandibles and abnormal middle ear structures. If left untreated, pups died shortly after birth because of asphyxia due to these structural defects. Furthermore, there was a 100% cumulative penetrance of cardiovascular abnormalities, for example interruption of the aorta (44%), tubular hypoplasia (56%) and ventricular septal defect (92%).[42] ET-1 deficient mice also display cardiovascular malformations including

ventricular septal defect, absent right subclavian artery and interruption of the aorta. Importantly, the frequency and severity of these abnormalities increased when they were additionally treated with BQ-123, a selective ET_AR antagonist (Table S1). This suggests that the lack of ET_AR stimulation by ET-1 is compensated, at least in part, by other ET isoforms (e.g., ET-2, ET-3).[43]

It has also been suggested that ET-1 plays a key role in closure of the ductus arteriosus after birth, although evidence is conflicting. The ductus arteriosus is a shunt connecting the pulmonary artery and aortic arch in utero, this way most of the fetal blood bypasses the pulmonary circulation. At birth, blood oxygenation shifts from the placenta to the lungs, making the ductus arteriosus redundant and initiating its closure.[44] Some studies have indicated that oxygen-triggered ET-1 release regulates closure of the ductus through binding to ET_AR on vascular smooth muscle cells.[45, 46] However, other experimental studies have shown that although blocking the ET_AR indeed counteracts the constricting effects of ET-1, the ductus still closes, both *in vitro* and *in vivo*.[47, 48] Therefore, the exact effect of ET_AR blockade on closure of the ductus arteriosus remains uncertain.

TERATOGENIC EFFECTS OF ENDOTHELIN RECEPTOR ANTAGONISTS

With regard to their teratogenic effects, either selective or dual ERA exposure during animal pregnancy has supplemented the findings of gene disruption studies, revealing the crucial role of ET-1 in embryonal development.

Treatment of wild type 129S6 mice with an ET_AR antagonist (TBC-3214) on gestational day (GD) 8, 9 or 10 showed a significant increase in pups with craniofacial malformations compared to the control group (up to 100% depending on day of treatment). There were no differences in litter size, fetal weight or fetal mortality (Table S1).[49]

The toxicity of Sitaxentan on embryo-fetal development in Sprague-Dawley rats was evaluated by Cross *et al*.[45] Sitaxentan is a highly selective ET_AR antagonist (Table 1) that was withdrawn from therapeutic use in 2010 because of the risk of idiosyncratic liver injury. In this study, rats were divided into 3 groups of different treatment periods (GD 0-6, 6-16 or 16-lactation), and each group was subdivided to test multiple dosages ranging from 20-120 mg/kg/day. There were no differences in fetal weight and fetal mortality between treatment and vehicle groups. Treatment from GD 6-16 with a dosage of 80 mg/kg/day or higher resulted in a significant increase of craniofacial and cardiovascular malformations (Table S1).[50] Similarly, in another study Sprague-Dawley rats were treated with different dosages of another selective ET_AR antagonist (L-753,037) on GD 6-20. There were no differences in litter size and fetal mortality between treated and control groups. However, treatment with the dose of 40 mg/kg/day resulted in a significant reduction in fetal weight, and dosages of 10 mg/kg/day or higher resulted in an

increased incidence of congenital abnormalities (Table S1).[51] To further investigate the function of the ET-system in fetal development, Taniguchi *et al*. exposed pregnant rats to a selective ET_AR antagonist during 5 different gestational periods (GD 7-20, 7-9, 9-11, 11-13 or 7-8/11-20). Unfortunately, the ET_AR antagonist dosage was not specified, nor did the authors report litter size or fetal weight. However, the authors did observe that most congenital malformations were seen in offspring of rats that were treated on GD 7-20 or 9-11 (comparable to GD 13-64 and 17-22 in humans), with a 100% penetrance for craniofacial malformations. In the other treatment groups, no craniofacial malformations were seen (Table S1).[52]

Treinen *et al*. studied the effect of two dual ERAs (SB-217242 and SB-209670) on fetal development in Sprague-Dawley rats on GD 6-17. A significant decrease in litter size and fetal weight was observed at the highest dosages for each antagonist. Moreover, a dose-dependent increase in craniofacial and cardiovascular abnormalities was seen at doses of 50 mg/kg/day (SB-217242) and 10 mg/kg/day and higher (SB-209670). There was no difference in fetal mortality compared to control rats (Table S1).[53]

Lastly, the effect of different dosages of the dual ERAs SB-217242 and SB-209670 (administered on GD 6-20) on fetal development of New Zealand White rabbits has been studied. The SB-217242 treatment group showed a reduction in litter size at 50 mg/kg/day, while no effect was seen for treatment with SB-209670. For SB-217242, a dose-dependent increase in congenital abnormalities (predominantly craniofacial) was seen from 50 mg/kg/day, with 100% of fetuses being affected at 300 mg/kg/day. When rabbits were treated with SB-209670, an increase in congenital cardiovascular malformations was only seen at the highest dosage (Table S1).[53]

ENDOTHELIN RECEPTOR ANTAGONISTS IN HUMAN PREGNANCY

Currently, only the dual ERAs Macitentan, Ambrisentan and Bosentan are approved for clinical use for the treatment of pulmonary arterial hypertension (PAH) and digital ulcers due to systemic sclerosis not responsive to standard therapy. Beneficial effects of these drugs have also been shown in treatment of cancer and renal failure.[54] While ERA use is absolutely contraindicated during pregnancy, there are sporadic cases of women who used ERAs during a certain period of their pregnancy. We conducted a systematic search of the literature (Figure S1, online-only data supplement) up to 18 February 2019 to create an overview of cases reporting the use of ERAs in pregnancy (Table 2).

Thirty-nine cases of ERA use in human pregnancy have been described in 18 articles.[55-72] The study characteristics are provided in Table 2. All women were suffering from PAH, and many of them received combination treatment with e.g. sildenafil or prostacyclin-analogues. In 27 cases, the ERA used for treatment of PAH was the dual

Table 2. Overview of human cases in literature that reported use of ERAs in pregnancy.

Report	Country	N	ERA	Dosage	Treatment stopped (GA)	Outcome	Delivery (GA, weeks)	BW (g)	Perinatal outcome
Alvarez[55]	Argentina	1	bosentan	125 mg/bd	12 weeks	x	x	x	x
Bédard[56]	Multiple	1	bosentan	x	delivery	x	x	x	x
Cotrim[57]	Portugal	2	bosentan	62.5 mg/bd	delivery*	CS	29	x	healthy
			bosentan	125 mg/bd	1st trimester	TOP			
Daimon[58]	Japan	2	bosentan	250 mg/d	1st trimester	CS	29	1356	healthy
			ambrisentan	2.5 mg/d	15 weeks	CS	30	1298	IVH
Duarte[59]	USA	8	bosentan	x	1st trimester	CS	34	x	no CA
			bosentan	x	1st trimester	CS	36	x	no CA
			bosentan	x	1st trimester	CS	32	x	no CA
			bosentan	x	1st trimester	TOP			
			bosentan	x	1st trimester	TOP			
			bosentan	x	1st trimester	TOP			
			bosentan	x	1st trimester	TOP			
			bosentan	x	1st trimester	TOP			
Elliot[60]	UK	1	bosentan	x	6 weeks	CS	25	650	healthy
Jaïs[61]	Multiple	7	x	x	1st trimester	x	x	x	healthy
			x	x	1st trimester	x	x	x	healthy
			x	x	1st trimester	x	x	x	x
			x	x	1st trimester	TOP			
			x	x	1st trimester	TOP			
			x	x	1st trimester	TOP			
			x	x	1st trimester	TOP			

Table 2. Overview of human cases in literature that reported use of ERAs in pregnancy (continued).

Report	Country	N	ERA	Dosage	Treatment stopped (GA)	Outcome	Delivery (GA, weeks)	BW (g)	Perinatal outcome
Kaznica[62]	Poland	3	sitaxentan	x	1st trimester	miscarriage			
			bosentan	x	1st trimester	CS	33	x	no CA
			bosentan	x	1st trimester	CS	37	x	no CA
Kiely[63]	UK	2	bosentan	x	1st trimester	CS	26	650	no CA
			bosentan	x	1st trimester	CS	34	1580	no CA
Molelekwa[64]	Ireland	1	bosentan	x	28 weeks	x	30	1140	no CA
Price[65]	UK	1	bosentan	125 mg/bd	x	CS	28	x	x
Sahni[66]	USA	1	bosentan	x	1st trimester	CS	term	x	x
Smith[67]	USA	1	bosentan	x	4 weeks	CS	36	x	x
Sliwa[68]	Multiple	4	x	x	x	vaginal	term	x	healthy
			x	x	x	CS	preterm	SGA	healthy
			x	x	1st trimester	TOP			
			x	x	1st trimester	TOP			
Streit[69]	Switzerland	1	bosentan	125 mg/bd	5 weeks	CS	37	2760	healthy
Tokgöz[70]	Turkey	1	bosentan	125 mg/bd	delivery	CS	27	x	no CA
Verhaert[71]	USA	1	bosentan	x	1st trimester	miscarriage	x		
Zhang[72]	China	1	bosentan	x	delivery	CS	31	1420	healthy

*treatment started at 28 weeks gestation. Abbreviations: BW = birth weight; CA = congenital abnormalities; CS = cesarean section; ERA = endothelin receptor antagonist; GA=gestational age; IVH = intraventricular hemorrhage; SGA = small for gestational age; TOP = (medical) termination of pregnancy; x = data not available.

ERA Bosentan, while the ET_AR selective blockers Sitaxentan and Ambrisentan were each used in one case; the remaining 11 cases did not specify the type of ERA. ERA treatment was stopped in most patients early in the first trimester. In only 4 cases that continued pregnancy, the ERA was used throughout the entire pregnancy,[56, 70, 72] and in another case up to 28 weeks of gestation.[64] In the case described by Cotrim *et al*., there was only short exposure during the third trimester, as treatment with Bosentan was started at 28 weeks of gestation and the patient delivered one week later because of severe maternal deterioration related to PAH and premature rupture of membranes.[57] Three case reports did not provide information about the period of treatment.[65, 68] Only 8 case reports mentioned the ERA dosages that were used: 62.5-250 mg twice daily for Bosentan and 2.5 mg daily for Ambrisentan, [57, 58, 65, 69, 70] which are standard dosages for PAH treatment.[73] Elective termination of pregnancy was performed in 12 women (31%), all because of maternal condition related to PAH.[57, 59, 61, 68] Two patients had a spontaneous miscarriage (5%).[62, 71] Eighteen women delivered via caesarean section, one had a vaginal delivery and for 6 patients the mode of delivery was not reported. Gestational age at delivery ranged from 25 weeks to term. Most women delivered prematurely on maternal indication (worsening of PAH). In all 5 women that delivered at term treatment has been stopped in the first trimester. No congenital abnormalities were reported (Table 2).

Despite being absolutely contraindicated during pregnancy, the substantial number of reported cases of the use of ERAs during pregnancy is remarkable. Exacerbation of PAH symptoms is likely to occur in pregnancy because of pronounced pregnancy-induced hemodynamic changes, especially the rise in plasma volume and cardiac output.[74] From the reported cases leading to live offspring (n=25) no congenital abnormalities were reported, compared to a prevalence of 4% in the general population.[75] Also the percentage of miscarriages of 5% (2 of 39 reported cases) is lower than the percentage of 10-20% observed in the general population.[76, 77] It should be remarked that medically indicated elective termination of pregnancy was performed in a high number (31%) of women with PAH using ERAs, so it cannot be excluded that the number of miscarriages and congenital abnormalities has been underestimated. In the very limited number of women (n=5) who were exposed to Bosentan in the third trimester of pregnancy no congenital abnormalities were observed.

ENDOTHELIN RECEPTOR ANTAGONISTS AS TREATMENT FOR PREECLAMPSIA

Translating results from animal studies to humans remains difficult, especially since placental morphology differs greatly between species.[78] A method that allows to compare the development of the human embryo with that of for example rats is the Carnegie staging. These 23 stages are based on the morphological development of the vertebrate

embryo and can be applied to all vertebrates, given that the embryonic development of almost all vertebrates, including humans, is identical. As shown in Figure 2, in most of the animal studies that report severe congenital abnormalities ERAs were administered before the end of the Carnegie stages. In pregnant women, these drugs could be administrated later, e.g. at the end of the second trimester when organogenesis is complete and the symptoms of PE typically manifest. Our review of cases using Bosentan as treatment for PAH in women with term and preterm neonates showed no major (congenital) adverse effects as a consequence of treatment. This supports the idea that administration of ERAs later in pregnancy, i.e. from a gestational age of 20 weeks, could be safe. One should keep in mind that earlier administration might be more optimal as it may interfere with the origin of placental insufficiency leading to PE. However, since activation of the ET-axis appears to be involved in the clinical symptoms requiring preterm delivery, administration of ERAs at a later stage might be sufficient to delay delivery, thereby improving neonatal outcome. Another point of concern could be that, although organogenesis has been completed in third trimester of pregnancy, fetal lung maturation has not. Since the ET-axis is involved in fetal lung development[39] and ET_AR are abundantly expressed in the fetal lungs during the pre- and (early) postnatal periods,[40] ET_AR blockade at a later stage of pregnancy might counteract the high pulmonary vascular resistance needed for adequate lung maturation. Here it is important to realize that neonates born to mothers with PE have increased serum ET-1 levels compared to controls,[79] implying that ET_AR blockade might actually normalize this situation. Furthermore, neonates with PAH are often treated with the ERA Bosentan,[80, 81] after which no adverse effects of treatment on lung- and brain development have been described, although no long term follow-up studies are available.[80]

Knowledge about the placental transfer of ERAs is virtually non-existent. In rats, the fetal plasma level of the ERA ABT-546 was only 2% of the maternal plasma levels during maternal administration late in pregnancy, suggesting that only a minute fraction of the drug crosses the placenta.[82] Importantly, this study showed no adverse effect of ERA treatment late in pregnancy on pup weight or survival.[82] However, no third trimester toxicology studies have been performed, nor has any study assessed the transfer of ERAs in human placentas. With the *ex vivo* dual-sided placental perfusion model it is possible to obtain information about the transfer of different ERAs through the human placenta.[83] Although it seems likely that there will be (at least partial) placental transfer, given the low molecular weight and lipophilic properties of these drugs,[84] studying this will provide essential knowledge on placental pharmacokinetics. In addition, it can be examined whether the transfer is different in placentas derived from early onset PE pregnancies as compared to term or preterm uncomplicated pregnancies. Furthermore, methods preventing ERAs from passing the placental barrier (e.g. by linking them to

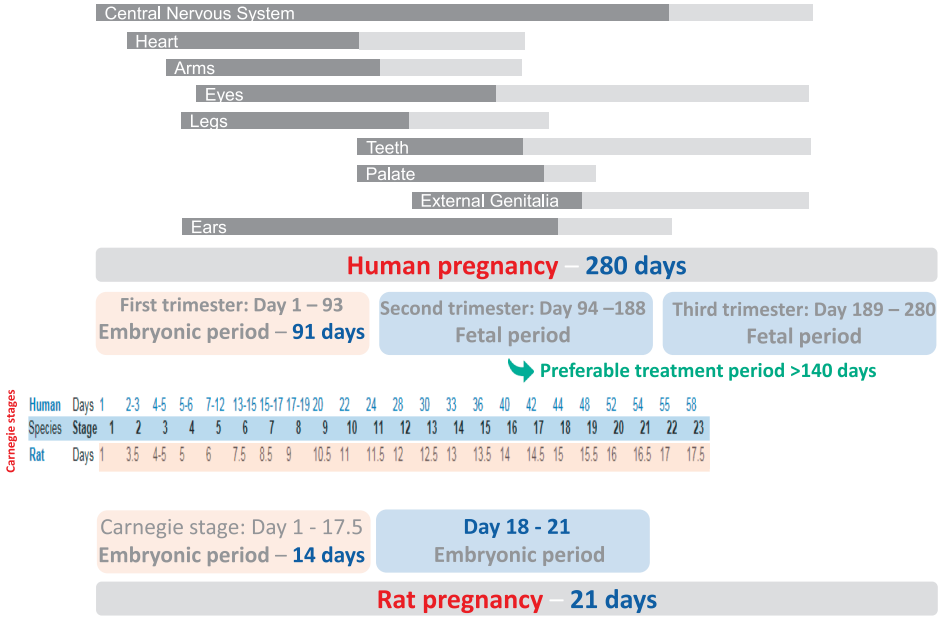

Figure 2. Developmental stages of pregnancy. This figure shows developmental stages of pregnancy in humans and rats, according to Carnegie staging as this can be applied to all vertebrates to compare the timing of development of different organs in different species. To reach the end of embryonic development (Carnegie stage 23) takes 17.5 days in rats and 58 days in humans. In pink highlighted, the period of ERA administration from day 1 – day 17.5, which is during all Carnegie stages in rat pregnancies as discussed in this paper. In green the time point when patients should be treated with ERAs is shown: 82 and 49 days after the Carnegie - and human embryonic stages respectively, much later when compared to the treatment period in animal studies.

elastin-like peptides) can be tested using this model. This approach would allow targeting the maternal ET-system without affecting the fetus.[8]

An important question would be whether a selective ET_A or a dual ERA would be most preferable. In different animal models of PE an ET_AR selective antagonist reverses symptoms without other adverse effects, supporting the use of an ET_AR selective receptor antagonist. In addition, selective inhibition/deletion of the ET_BR in animals is associated with distinct congenital abnormalities and death.[35, 85] On the other hand, based on the limited evidence in human pregnancies indicating that the dual ERA Bosentan, when administered in the third trimester of pregnancy in women with PAH, is not associated with craniofacial abnormalities would perhaps favor the use of this dual receptor blocker, especially because it is already approved for clinical use. A further possibility might be the application of endothelin-converting enzyme-1 (ECE-1) inhibitors, since ECE-1 activity is also increased in women with PE.[86] However, such drugs, by suppressing ET-1 formation, would mimic the effect of dual ERAs. Potential caveats are their selectivity and the possibility that ET-1 is also formed by non-ECE-1 enzymes, like chymase.[87]

Another important point of concern when considering ERAs for treatment of PE are their possible side effects. Some of the reported side effects are similar to PE symptoms, such as edema, headache, and elevated liver enzymes. When these PE symptoms occur during ERA treatment it will be very challenging to distinguish side effects from disease exacerbation. Another common side effect is anemia, and sometimes thrombocytopenia is seen. Therefore, plasma levels of hemoglobin, thrombocytes and liver enzymes should be closely monitored during treatment. Women with elevated liver enzymes, severe anemia or thrombocytopenia should not be started on ERA treatment. Furthermore, in case these side effects occur treatment might have to be discontinued in some cases.

CLINICAL IMPLICATIONS AND PERSPECTIVES

Given the increase in ET-1 during PE, targeting the ET-1 pathway in severe, early onset PE could potentially be a favorable new treatment strategy to improve maternal, fetal and neonatal outcomes when started after the completion of organogenesis. Especially since not all anti-hypertensive drugs can be used during pregnancy, due to either teratogenic effects or the lack of PE prevention. Recently, a randomized controlled trial studying the effects of sildenafil, a phosphodiesterase-5 inhibitor, versus placebo on pregnancy outcome in severe FGR has been halted, since sildenafil did not show beneficial effects and there were more neonatal complications in the treatment group in one of the cohorts.[88] Although no teratogenic effects of ERAs during late pregnancy have been reported in the limited number of human case reports, further research is needed to evaluate the pharmacokinetics and safety of these drugs in pregnancy, preferably by performing third trimester toxicology studies in animals with a longer gestation, like non-human primates. This is relevant because many of the previous experimental studies have used extremely high dosages, sometimes reaching 300 mg/kg/day, while patients with PAH are being treated with a maximum of 250 mg per day, i.e. 100 times less in a patient with an average weight of 70 kg. In addition, something that can be done relatively easy at this moment is to investigate the trans-placental transfer of the two clinically available ERAs that are FDA-approved, making use of the *ex vivo* human dual-sided perfusion model with cotyledons from both uncomplicated and PE pregnancies. Furthermore, the perfusion model might provide information on the passage (or lack thereof) of ERAs bound to peptides preventing them from crossing the placenta. At a later stage, we suggest that proof of principles studies should be performed in patients with very early onset (< 24 weeks of gestation) severe PE, in whom termination of pregnancy is considered, because of the severity of the maternal and/or fetal condition. In this setting, outcome parameters would be maternal blood pressure, proteinuria, and the disappearance of HELLP symptoms. In patients who are untreated we would

start with ERA, while in patients who already use antihypertensive treatment we would continue this medication and add an ERA, guided by the effect on blood pressure. In cases where blood pressure drops too much we would stop or decrease the already used antihypertensive medication.

In conclusion, the findings of this review support the idea that ERA treatment for severe early onset PE might be an option if applied later in pregnancy, when organogenesis is completed to avoid teratogenic risks, with close monitoring of closure of the ductus arteriosus. However, third trimester toxicology studies are warranted to evaluate drug safety and it remains to be established whether ERA treatment is effective for alleviating maternal symptoms, allowing pregnancy prolongation without leading to adverse neonatal outcomes.

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SUPPLEMENTAL INFORMATION

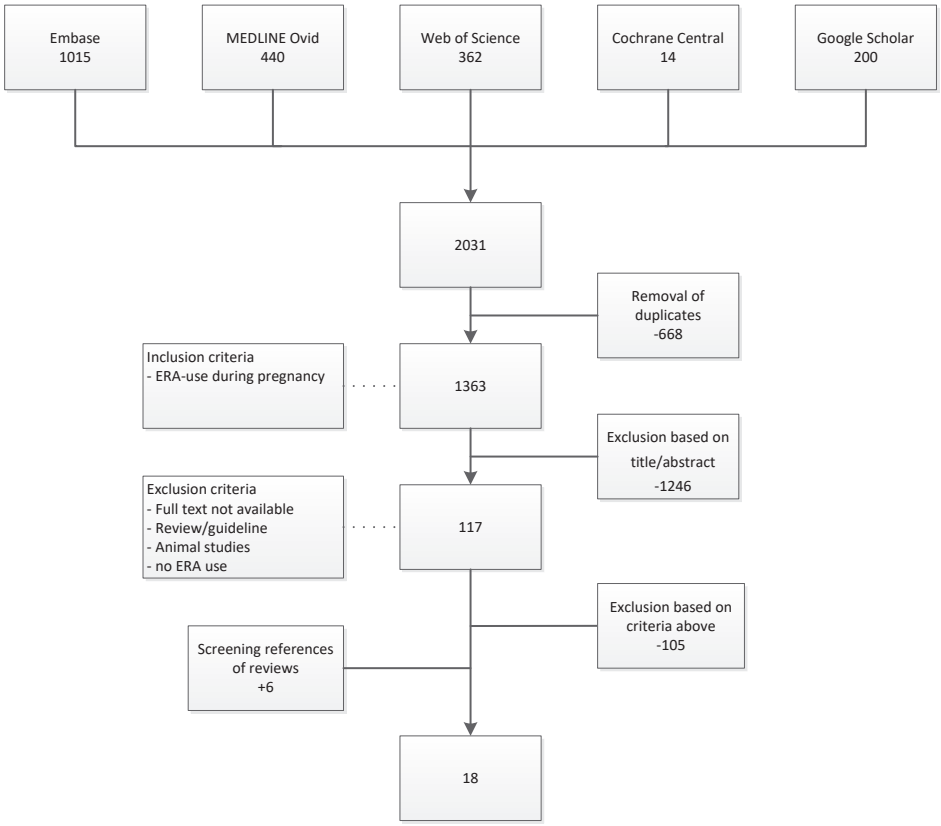

Figure S1. Flowchart of the systematic search in literature. A systematic search in five different databases was performed on 18 February 2019 and yielded 2031 articles. After de-duplication 1363 articles were left for screening. Based on title/abstract relevance 1246 articles were excluded and of the remaining 117 full text was screened. Finally, 18 articles describing 39 cases of ERA use during human pregnancy were found and included in this review.

Table S1. Overview of animal studies reporting administration of ERAs during pregnancy.

Study	Population	N	ERA	Dosage (mg/kg/day)	Treatment period (GD)	Sacrifice / delivery (GD)	Outcome*
				Reproductive toxicity			
Cross[50]	SD rats	24	sitaxentan	20/80/120	0-6	20	No differences in maternal weight gain, litter size, fetal- and placental weight, CA and fetal mortality
				Embryofetal developmental toxicity			
Cross[50]	SD rats	20	sitaxentan	20 bd/40 bd/80 bd/120	6-16	20	↓ Maternal weight gain at ≥ 40 mg ↑ CA (CF and CD) at ≥ 80 mg ND in litter size, fetal weight and mortality
Spence[51]	SD rats	25	L-753,037	2.5/5/10/20/40	6-20	21	↓ Maternal weight gain and fetal weight at 40 mg ↑ CA at ≥ 10 mg ND in litter size and fetal mortality
Taniguchi[52]	Rats	29	x	x	7-20	x	↑ CA (100% CF, 50% CD)
		18	x	x	7-9	x	no CA
		33	x	x	9-11	x	↑ CA (100% CF, no CD)
		33	x	x	11-13	x	↑ CA (no CF, 15% CD)
		20	x	x	7-8, 12-20	x	↑ CA (no CF, 25% CD), 100% mortality[†]
Treinen[53]	SD rats	10	SB-217242	0.01/1/10/50/300	6-17	21	↓ Maternal weight gain, litter size and fetal weight at 300 mg ↑ CA at ≥ 50 mg ND in fetal mortality
		10	SB-209670	0.01/1/10/50	6-17	21	↓ Litter size and fetal weight at 50 mg ↑ CA at ≥ 10 mg ND in maternal weight gain and fetal mortality

Table S1. Overview of animal studies reporting administration of ERAs during pregnancy (continued).

Study	Population	N	ERA	Dosage (mg/kg/day)	Treatment period (GD)	Sacrifice / delivery (GD)	Outcome*
	NZ white rabbits	8	SB-217242	0.01/1/10/50	6-20	29	↓ Litter size and fetal weight at 50 mg ↑ CA at ≥ 10 mg ND in maternal weight gain and fetal mortality
		8	SB-209670	0.01/1/10/25	6-20	29	↑ CA at 25 mg ND in maternal weight gain, litter size, fetal weight and fetal mortality
				Late gestation and lactation toxicity			
Cross[50]	SD rats	24	sitaxentan	20 bd/40 bd/60 bd	16-lactation	x	↑ CA at ≥ 40 mg bd (enlarged liver and lower testes weight) ND in maternal weight gain, litter size, fetal weight and fetal mortality
Spence[51]	SD rats	22	L-753,037	5/20/40	13-lactation	21-24	ND in maternal weight gain, litter size, fetal weight, CA and fetal mortality
				PE/FGR models			
Morris[32]	SD rats	x	ABT-627	5	12-19	x	ND in fetal- and placental weight and fetal mortality
Thaete[33]	SD rats	12	FR-139317	6	17-21	21	Treatment normalized hypoxia-induced maternal-, fetal- and placental weight reduction ND in litter size and fetal mortality
Thaete[34]	SD rats	6	A-127722	0.01/0.1/1/5/10	14-21	21	Treatment partly attenuated L-NAME induced FGR ND in litter size and fetal mortality
		6	FR-139317	6/12/18	14-21	21	Treatment partly attenuated L-NAME induced FGR ND in litter size and fetal mortality
		6	A-182086	0.3/10	14-21	21	↓ fetal- and placental weight at 10 mg ND in litter size and fetal mortality
				Knockout models			
Clouthier[42]	ET_AR knockout mice	x	NA	NA	NA	18.5	↑ CA (100% CF and CD) 100% fetal mortality

Table S1. Overview of animal studies reporting administration of ERAs during pregnancy (continued).

Study	Population	N	ERA	Dosage (mg/kg/day)	Treatment period (GD)	Sacrifice / delivery (GD)	Outcome*
Kurihara[43]	ET-1 knockout mice	x	BQ-123	0.96 mg/day	7 days‡	x	↑ CA (CD)
					Other		
Ruest[49]	129S6 mice	x	TBC-3214	100	8	18.5	↑ CA (10% minor CF)
					8-8.5		↑ CA (83% CF)
					8.5		↑ CA (48% minor CF)
					8.5-9		↑ CA (100% CF)
					9		↑ CA (100% CF)
					9-9.5		↑ CA (97% CF)
					9.5		↑ CA (9% minor CF)
					9.5-10		↑ CA (37% minor CF)
					10		↑ CA (4% minor CF)
Thaete[82]	SD rats	x	ABT-546	20	14-21	21	ND in litter size, fetal weight and fetal mortality
		x	FR-139317	12	12-21	21	ND in litter size, fetal weight and fetal mortality

*treated group vs. control group; †all pups died due to patent ductus arteriosus; ‡started at gestational day 5-8; ↑ = significantly increased; ↓ = significantly decreased; x = not described. Abbreviations: bd = twice daily; CA = congenital abnormalities; CD = cardiac; CF = craniofacial; ERA = endothelin receptor antagonist; ET_AR = endothelin type A receptor; ET-1 = endothelin-1; FGR = fetal growth restriction; GD = gestational day; NA = not applicable; ND = no difference; NZ = New Zealand; SD = Sprague Dawley

Chapter 6

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.

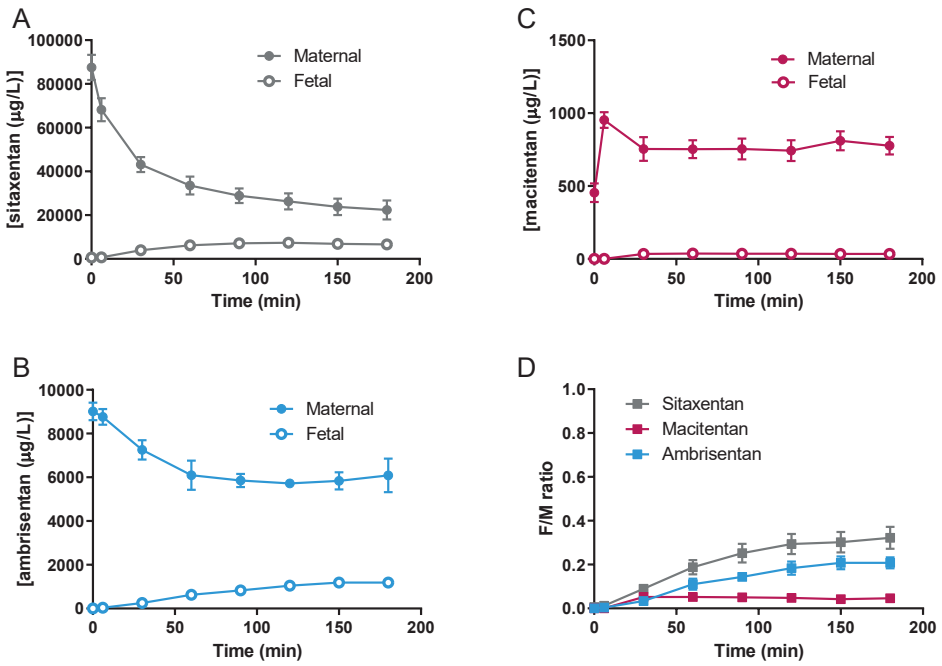

Figure 1. Placental transfer of sitaxentan (A), ambrisentan (B) and macitentan (C). Measured concentrations in the maternal (closed circles) and fetal (open circles) circulations are expressed as µg/L, n=5 per group. Panel D shows the fetal-to-maternal (F/M) transfer ratios over time.

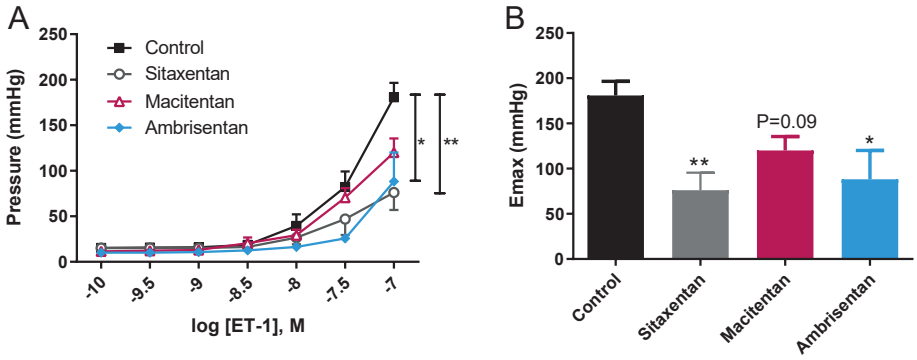

Figure 2. Panel A shows the concentration response curves for fetally applied endothelin-1 (ET-1) in the perfused cotyledon without (control, squares) or with prior exposure of the maternal circulation to the endothelin receptor antagonists sitaxentan (circles), macitentan (triangles) or ambrisentan (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.

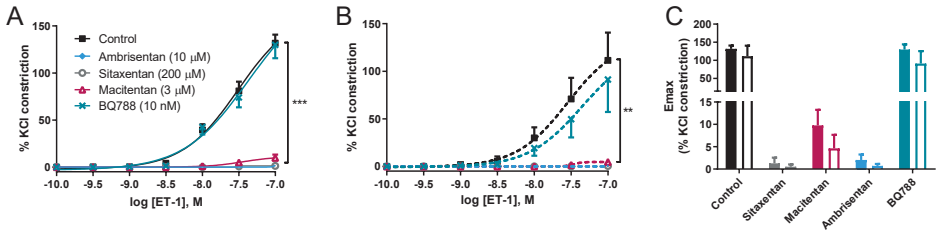

Figure 3. Vascular responses of isolated chorionic plate arteries of healthy (A) and preeclamptic (B) placentas to endothelin-1 (ET-1) in the absence (control, squares) or presence of sitaxentan (circles), macitentan (triangles), ambrisentan (diamonds) or BQ-788 (cross marks). Panel C shows the response 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

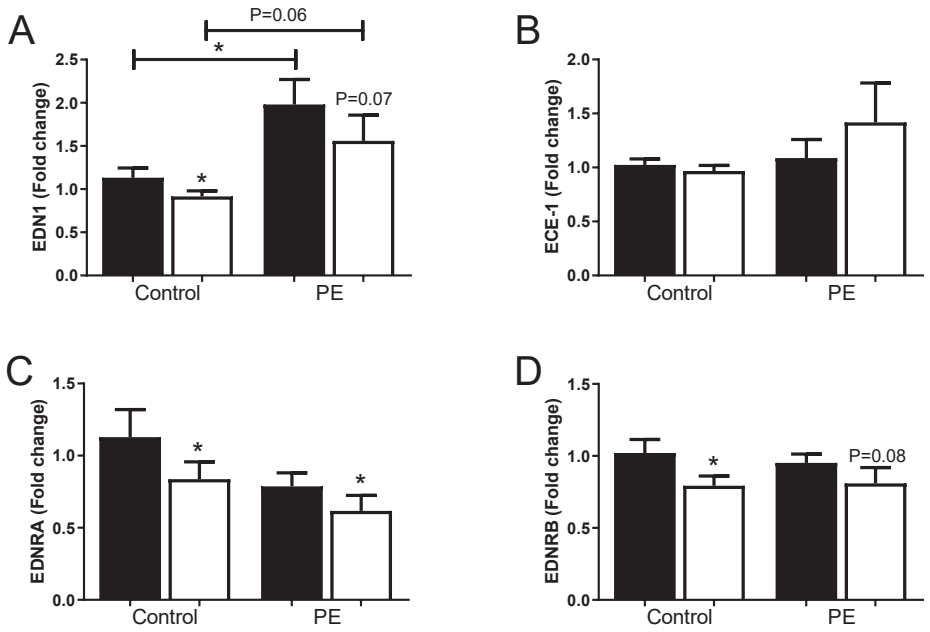

Figure 4. Gene expression of endothelin-1 (A), endothelin-converting enzyme-1 (B), endothelin-receptor type A (C) and endothelin-receptor type B (D) in healthy and preeclamptic placentas (n=12 per group). Expression was measured on both the decidual side (black bars) and amniotic side (white bars) of 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 % unbound_F and the % 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.

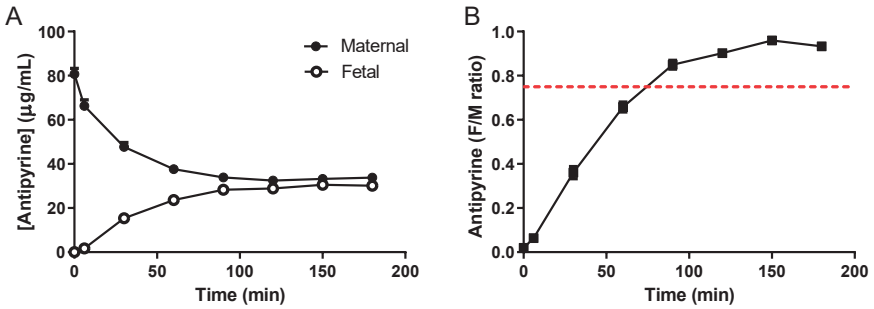

Figure S1. Antipyrine transfer. Panel A shows the mean maternal (closed circles) and fetal (open circles) concentrations of antipyrine, proving good overlap between both circulations. Panel B shows the mean fetal-to-maternal transfer ratio, with the cutoff point of 0.75 (red dashed line).

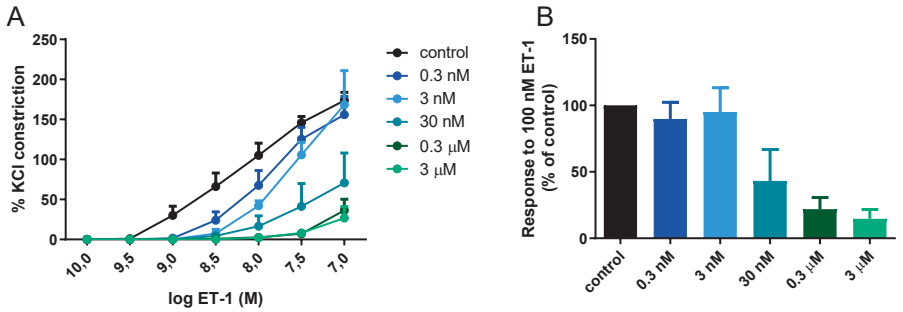

Figure S2. Blocking effect of different macitentan concentrations in chorionic plate arteries. Concentration-response curves to endothelin (ET)-1 are shown in the absence (control) or presence of different macitentan concentrations (A). Panel B shows the blocking effect of those different concentrations 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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Chapter 7

Larger first-trimester placental volumetric parameters are associated with lower pressure and more flow-mediated vasodilation after delivery

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ABSTRACT

Objective: To explore the correlation between *in vivo* placental volumetric parameters in the first trimester of pregnancy and *ex vivo* parameters of fetoplacental vascular function after delivery. Methods: In ten singleton physiological pregnancies, placental volume (PV) and uteroplacental vascular volume (uPVV) were measured offline in three-dimensional ultrasound volumes at 7, 9 and 11 weeks gestational age (GA) using Virtual Organ AnaLysis and Virtual Reality. Directly postpartum, term placentas were *ex vivo* dually perfused and pressure in the fetoplacental vasculature was measured to calculate baseline pressure (pressure after a washout period), pressure increase (pressure after a stepwise fetal flow rate increase of 1 mL/min up to 6 mL/min) and flow-mediated vasodilation (FMVD; reduction in inflow hydrostatic pressure on the fetal side at 6mL/min flow rate). Correlations between *in vivo* and *ex vivo* parameters were assessed by Spearman's correlation coefficients (R). Results: Throughout the first trimester, PV was negatively correlated with pressure increase (R_{growth}=-0.84) and, at 11 weeks GA, also positively correlated with FMVD (R=0.89). At 7 weeks GA, uPVV and uPVV/PV ratio were negatively correlated with pressure increase (R=-0.58 and R=-0.81, respectively) and positively correlated with FMVD (R=0.62 and R=0.90, respectively). Discussion: Mainly in the early first trimester, larger placental volumetric parameters are associated with lower pressure and more FMVD in the fetoplacental vasculature after delivery. This may suggest that larger and/or more vascularized placentas in early pregnancy have better adaptive mechanisms and possibly lead to better pregnancy outcomes.

INTRODUCTION

Placenta-related pregnancy complications, such as preeclampsia (PE) and fetal growth restriction (FGR), are highly prevalent and not only affect fetal development and pregnancy outcome, but also future maternal and offspring health.[1-3] Most of these pregnancy complications originate already in the first trimester of pregnancy.[4] Within this time window, development of the placental bed takes place, which is characterized by remodelling of the uterine spiral arteries, thereby creating a low-resistance circulation. Adequate remodelling is crucial to placental development, subsequently affecting embryonic and fetal health.[5, 6] After the placental vascular network has been formed in early pregnancy, capillary growth continues until delivery, mediated by various growth factors. From mid-gestation onwards, there is an exponential growth in vascular volume of fetoplacental vessels to accommodate the needs of the growing fetus.[7]

Non-invasive assessment of *in vivo* placental development remains challenging, since the value of available markers of placental function and development is limited. Most commonly, placental function is assessed by the use of derivatives of the placental circulation. For example, abnormal uterine artery Doppler waveforms have been related to pregnancy complications, such as pregnancy-induced hypertension and PE.[8, 9] An innovative method to determine placental development resulted from the introduction of Virtual Organ Computer-aided AnaLysis (VOCAL), which enables the assessment of three-dimensional (3D) placental volume measurements and uteroplacental vascularisation indices (i.e. vascularisation indices, flow indices and vascularisation-flow indices). These parameters have all been associated with adverse outcomes, such as miscarriage, PE and FGR.[10-12] A newly developed technique is Virtual Reality (VR) which can be combined with measurements of 3D power Doppler ultrasound volumes and visualizes the placental circulation from early pregnancy onwards, in three dimensions with depth perception. As previously demonstrated by Reijnders *et al.* this technique is feasible and reliable for use in the first trimester of pregnancy to measure placental parameters, that reflect placental volume and uteroplacental vascularisation of the uterine/maternal side (i.e. the placental bed).[13]

Ex vivo assessment of the fetoplacental vasculature can be performed using dual-sided placental perfusion, an experimental model to study fetal vascular reactivity of a single cotyledon directly after birth. Unlike most other vascular systems, the fetoplacental vasculature is not innervated. Local vascular tone and fetal cardiac output are the main determinants of blood flow through these vessels, regulated by circulating and locally produced hormones and vasoactive compounds.[14] Therefore, flow-mediated pressure changes in the *ex vivo* dual-sided perfused cotyledon are a measure of vascular resistance in the placenta. Jones *et al.* have already shown that there is a significant correlation between vascular resistance measured *in vivo* (i.e. umbilical artery Doppler

velocimetry at term) and *ex vivo* placental perfusion.[15] However, no study has yet assessed the relation between *in vivo* parameters of early placental vascular development and *ex vivo* placental vascular perfusion.

Since early non-invasive assessment of placental development is challenging and it is unknown whether available markers actually represent placental function later in pregnancy, the aim of this study was to explore whether correlations exist between *in vivo* ultrasound parameters of first-trimester placental (vascular) development and *ex vivo* parameters of fetoplacental vascular reactivity at delivery. Not only will this provide better insight in the pathophysiology of placental disorders, it could also demonstrate the need for earlier evaluation of the placental circulation.

METHODS

This explorative study was conducted within the Virtual placenta study, embedded in the Rotterdam Periconception Cohort (Predict Study), which is an ongoing prospective cohort study performed at the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands.[16] Women who participated in the Predict study before 10 weeks gestational age (GA), were also invited to participate in the Virtual Placenta study that was carried out between January 2017 and March 2018. Pregnancies conceived either spontaneously or through assisted reproduction techniques (ART) were eligible. The study protocol has been approved by the Erasmus MC Institutional Review Board (MEC 2015-494). All participating women and their partners signed written informed consent at enrolment, also on behalf of their unborn child. Women were asked for consent to use their placenta for research purposes after delivery. Women with multiple pregnancies, (gestational) diabetes, viral infections (e.g. HIV) or placental anomalies were not eligible for inclusion in this subset.

Study Parameters

Maternal characteristics were obtained from self-reported questionnaires filled out upon enrolment. First-trimester body-mass index (BMI) and blood pressure were also measured at intake. After delivery, participating women again filled out a questionnaire on pregnancy and birth outcomes. The retrieved information was checked with data from medical records and delivery reports where available.

Ultrasound

Participants underwent serial 3D ultrasound examinations at 7, 9 and 11 weeks GA to obtain volumes encompassing the whole embryo and placenta. Ultrasound examinations were performed by experienced sonographers only, using a Voluson E8 or E10 system

(GE Medical Systems, Zipf, Austria). In the first trimester, 3D ultrasound examinations were performed using a transvaginal 6-12 MHz transducer. Vasculature of the complete placenta and embryo was imaged using Power Doppler (PD) US with standardized setting (PD gain '-8.0', pulse repetition frequency (PRF) '0.6 kHz', wall motion filter (WMF) 'low1', quality 'high'). To minimize artefacts and measurement errors by movement, participants were asked to hold their breath for approximately 30 seconds during image acquisition. All ultrasound examinations were performed according to international guidelines on safe use of Doppler ultrasound in the first trimester of pregnancy (ALARA-principle) and, as such, total scanning time was kept as low as possible with a maximal duration of 30 minutes and a maximal thermal index of 1.3.[13, 17-19]

Offline 3D measurements

Placental volume (PV) measurements were performed with offline specialized VOCAL software (4D View, GE Medical System) according to standardized methods, to reconstruct the trophoblast (Reus *et al*., 2013). Uteroplacental vascular volume (uPVV) was measured using a VR desktop system (Figure 1). VR enables visualization of a 3D volume as a true hologram, which allows for depth perception and thus more optimal assessment of the uteroplacental vascularization. The VR desktop is a validated system composed of a personal computer using the V-Scope volume rendering application, a two-dimensional (2D) monitor which displays the user interface, a 3D monitor to display the volume, a tracking system allowing for interaction of the observer with the 3D volume, a pair of stereoscopic glasses to enable depth perception and a six-degrees of freedom mouse for 3D volume manipulation.[20] By thresholding the 8-bit (range 0-255) Doppler magnitude data, semi-automatic volume measurements of the uPVV were obtained. To enable the most optimal visualization of the uteroplacental vasculature, the lower-Doppler threshold level was set at a value of 100, which means that semi-automatic calculations only color and count by voxels with a Doppler value of 100 or higher. First, embryonic structures were removed from the segmentation. Then, the difference in echogenicity between the myometrium and placenta was used to erase the vessels up to the myometrial-placental border, thereby leaving the maternal vascularization of the uteroplacental bed for volume assessment. Currently it is not possible to distinct between the maternal blood space and embryonic vasculature within the uPVV. A more detailed description and validation of the methods for uPVV measurements has previously been published.[13] After VOCAL and VR measurements, uPVV was divided by PV to calculate a placental vascular volume ratio (uPVV/PV ratio). Due to limited image quality or study inclusion after 7 weeks GA, measurements of PV and uPVV could not be performed for all included pregnancies and/or study visits.

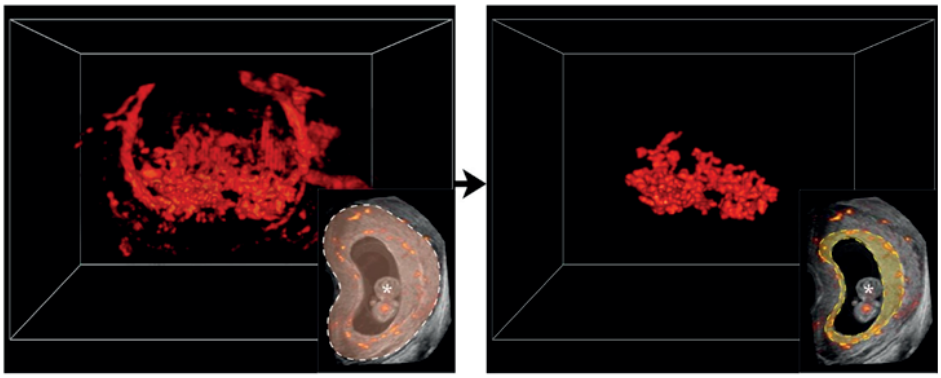

Figure 1. Visualization of a three-dimensional power Doppler utero-placental vascular volume in Virtual Reality. On the left; complete three-dimensional power Doppler vascular volume at 9 weeks of gestation. On the right; Using grey values of the utero-placental tissue, a virtual brush allows to erase vascular voxels up to the myometrial-placental tissue interface margin, leaving uteroplacental vascular volume (uPVV) to be measured by threshold-based segmentation. Lower inserts; two-dimensional power Doppler image with complete vasculature in color delineated by dashed white line (left), placental vascularization delineated by dashed yellow line (right).

Placental perfusion

The perfusion model used in our study has been previously described in detail by Hitzerd *et al*.[21] In short, term placentas were collected immediately after delivery and fetal circulation was established by cannulating a corresponding chorionic artery and vein of an intact cotyledon. Fetal flow rate was started at 1 mL/min. When cannulation was successful, the cotyledon was cut from the placenta and placed inside the perfusion chamber. Maternal circulation (constant flow rate of 12 mL/min) was created by placing four blunt cannulas in the intervillous space. Venous outflow was collected in a reservoir underneath the cotyledon and run back to the maternal reservoir. Perfusion media consisted of Krebs-Henseleit buffer, supplemented with heparin (5000 IU, 0.5 mg/L) and aerated with 95% O_2 – 5% CO_2. A placental washout period of approximately 30 minutes was performed before starting an experiment. Changes in pressure on the fetal side of the placenta were measured by pressure transducers and recorded throughout the experiment using acquisition software (Biopac, Goleta, CA USA). When a stable baseline pressure was reached after the washout period, the fetal flow rate was increased stepwise with 1 mL/min, until a flow rate of 6 mL/min was reached. After each step a new steady state in pressure was awaited before continuing with the next step (Figure 2). The parameters baseline pressure, total pressure increase and flow-mediated vasodilation (FMVD) were used for analysis. Total pressure increase was defined as the difference between baseline at pressure at start of the experiment and the new steady state at a flow rate of 6 mL/min. As previously described by Jones *et al*., FMVD is the percentage of reduction in hydrostatic pressure on the fetal side as a result of increased flow rate, measured at a flow rate of 6 mL/min.[15]

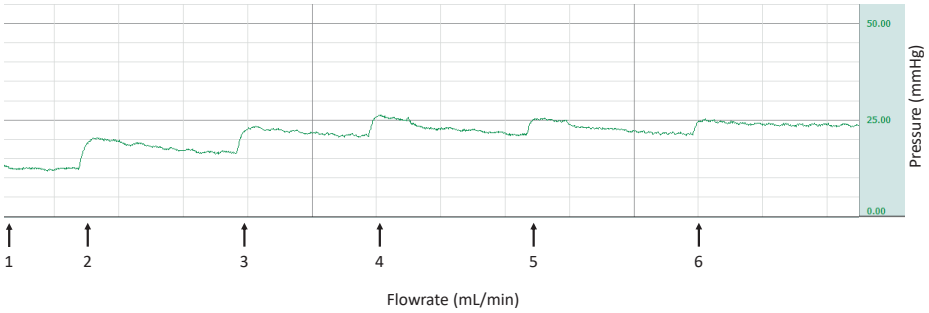

Figure 2. Stepwise increase in fetal flow rate leading to increase in pressure (representative).

Statistical analysis

Because of skewed distributions of most parameters, data are presented as medians (interquartile range). To identify correlations between *in vivo* and *ex vivo* measurements, Spearman's rank correlation coefficients (R-values) were used and correlations were plotted in scatterplots. To further evaluate these correlations, linear mixed models were used to calculate individual slopes for each participant to establish placental growth trajectories throughout the first trimester (at 7, 9 and 11 weeks GA). In these models, uPVV, PV and the uPVV/PV ratio were transformed using a cubic root. The individual slopes were then also correlated with *ex vivo* parameters using Spearman's correlation coefficients. All analyses were performed using SPSS software (version 21.0; SPSS Inc., Chicago, IL, USA) and RStudio Statistics (version 3.5.0, 2018). Correlations >0.5 were considered relevant and *P*-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics

In this explorative study, twelve women were included, of whom ten placentas were successfully perfused. Baseline characteristics of these ten women are provided in Table 1. Women had a median age of 31.9 years (29.7-37.1), 40% were nulliparous and 90% conceived spontaneously. None of the women smoked or used alcohol during pregnancy. In 60% of the women, the mode of delivery was an elective caesarean section (two nulliparous – and four multiparous women), all because a of breech position and/or previous caesarean section. Two women delivered spontaneously and another two women underwent an emergency caesarean because of failure to progress. Median birth weight was 3365 grams (2835-3425) and 70% of the offspring was male. None of the pregnancies were complicated by PE or any other pregnancy complication. All infants were born at term (i.e. >37 weeks GA), and one infant was small-for-gestational-age (birth weight below the 10th percentile).[22] Median placental weight was 395 grams (322-451), and of two placentas, weight was below the 10th percentile.

Table 1. Baseline characteristics (*n*=10).

Characteristic	
Maternal	
Age at intake (years)	31.9 (29.7-37.1)
Nulliparous	4 (40%)
Mode of conception	
Spontaneous	9 (90%)
IVF/ICSI	1 (10%)
Geographic origin	
Dutch	7 (70%)
Western	1 (10%)
Non-western	2 (20%)
Educational level	
Low	0 (0%)
Intermediate	3 (30%)
High	7 (70%)
BMI, first-trimester (measured)	22.8 (21.7-32.5)
Periconceptional folic acid supplement use	10 (100%)
Median first trimester RR (intake)	
Systolic	108.0 (99.0-110.0)
Diastolic	65.0 (60.0-70.0)
Periconceptional smoking	1 (10%)
Periconceptional alcohol use	0 (0%)
At delivery	
Gestational age at delivery	38^{+5} (37^{+4}-39^{+1})
Mode of delivery	
Vaginal	2 (20%)
Elective caesarean	6 (60%)
Emergency caesarean	2 (20%)
Placental weight	395 (322-451)
Placental weight <p10 at birth	2 (20%)
Histology: distal villous hypoplasia	1 (10%)
Neonatal	
Birth weight	3365 (2835-3425)
Small-for-gestational-age	1 (10%)
Sex	
Male	7 (70%)
Female	3 (30%)

Data are expressed as median (interquartile range) or number (percentage).

Placental vascular measurements

For the ten included pregnancies, six women had multiple measurements available for PV and eight women had multiple measurements available for uPVV. Three women had measurements available at all weeks for PV, four women had available measurements at all weeks for uPVV. A total of five measurements of PV and uPVV were available at 7 weeks GA, eight measurements were available of PV and nine of uPVV at 9 weeks GA, and five measurements were available of PV and seven of uPVV at 11 weeks GA. Median values for *in vivo* placental measurements per week GA are displayed in Table 2 and increased from a median of 3.26 cm^3 (0.96-6.40) at 7 weeks GA to 13.36 cm^3 (6.27-30.08) at 11 weeks GA for uPVV, a median of 20.15 cm^3 (12.95-23.90) at 7 weeks GA to 92.76 cm^3 (69.18-125.36) at 11 weeks GA for PV, and a median of 0.16 (0.08-0.23) at 7 weeks GA to 0.17 (0.10-0.35) at 11 weeks GA for the uPVV/PV ratio.

Table 2. Median and ranges of measurable *in-vivo* placental volumetric parameters per week GA.

	7 weeks GA (n=5)		**9 weeks GA (n=9)**		**11 weeks GA (n=7)**	
	Median	***IQR***	***Median***	***IQR***	***Median***	***IQR***
PV (cm^3)	20.15	12.95 - 23.90	44.69	25.57 - 57.25	92.76	69.18 - 125.36
uPVV (cm^3)	3.26	0.96 - 6.40	8.80	3.97 - 10.61	13.36	6.27 - 30.08
uPVV/PV ratio	0.16	0.08 - 0.23	0.16	0.14 - 0.33	0.17	0.10 - 0.35

uPVV = uteroplacental vascular volume (in cm^3); PV = placental volume (in cm^3); uPVV/PV ratio = ratio between uPVV and PV; IQR = interquartile range.

Placental perfusion

Median gestational age at delivery was 38^{+5} (37^{+4}-39^{+1}) weeks. Median values for *ex vivo* placental measurements are displayed in Table 3. Median baseline pressure at the starting flow rate of 1 mL/min was 21 mmHg (19-25) mmHg, which increased to 32.5 mmHg (23.8-36.0) at 6 mL/min, leading to a total pressure increase of 10.5 mmHg (3.0-13.0) (Figure 3a and 3b). Median FMVD at 6 mL/min was 50% (50-100) (Figure 3c).

Table 3. Median and ranges of *ex-vivo* placental measurements at term.

n=10	**Median**	**IQR**
Pressure at baseline	21.0	19.0 - 25.0
Total pressure increase (mmHg)	10.5	3.0 - 13.0
End pressure (mmHg)	32.5	23.8 - 36.0
FMVD at 6 mL/min	50.0	50.0 - 100.0

FMVD = flow-mediated vasodilation (% pressure reduction from peak to new steady state); IQR = interquartile range.

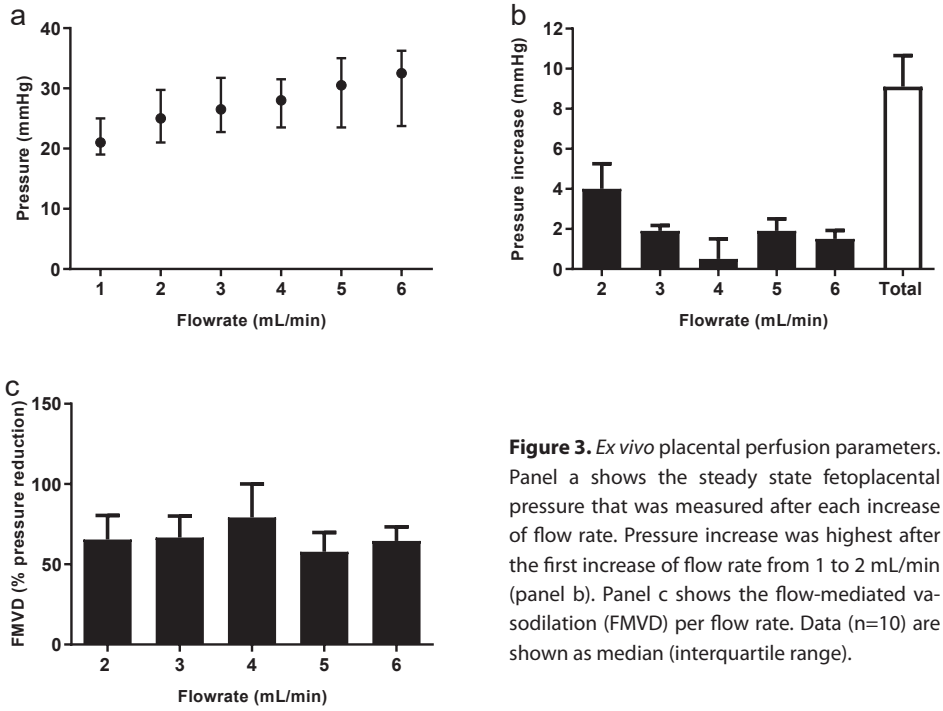

Figure 3. *Ex vivo* placental perfusion parameters. Panel a shows the steady state fetoplacental pressure that was measured after each increase of flow rate. Pressure increase was highest after the first increase of flow rate from 1 to 2 mL/min (panel b). Panel c shows the flow-mediated vasodilation (FMVD) per flow rate. Data (n=10) are shown as median (interquartile range).

Correlations

Correlations between *in vivo* and *ex vivo* measurements are depicted in Table 4 and Figure 4. PV was negatively correlated with pressure increase at 7 weeks GA (R=-0.53), 9 weeks GA (R=0.74) and 11 weeks GA (R=-0.98). At 11 weeks GA, PV was positively correlated with baseline pressure (R=0.62) and FMVD (R=0.89). uPVV was negatively correlated with pressure increase (R=-0.58) and positively correlated with FMVD (R=0.62) at 7 weeks GA. No correlations between uPVV and *ex vivo* parameters were observed at 9 and 11 weeks GA. The uPVV/PV ratio was negatively correlated with pressure increase (R=-0.81) and positively correlated with FMVD (R=0.90) at 7 weeks GA. At 11 weeks GA, a negative correlation was observed between the uPVV/PV ratio and FMVD (R=-0.67), although this correlation was not statistically significant. When studying the correlations between *in vivo* placental growth throughout the first trimester (i.e. slopes of placental parameters) and cross-sectional *ex vivo* placental parameters, a significantly negative correlation was observed between first-trimester PV growth and FMVD (R_{growth}=-0.84) and between uPVV/PV ratio and FMVD (R_{growth}=-0.90). Also, relevant positive correlations, although not statistically significant, were observed between PV growth and FMVD (R_{growth}=0.50) and between uPVV/PV ratio growth and pressure increase (R_{growth}=0.51).

Table 4. Correlations between *in-vivo* and *ex-vivo* placental parameters per week GA.

Placental parameter	R_{7weeks}	R_{9weeks}	$R_{11weeks}$	R_{growth}
PV - pressure at baseline	-0.05	-0.05	0.62	0.25
- total pressure increase	-0.53	-0.74*	-0.98*	-0.84*
- FMVD at 6mL/min	0.05	-0.04	0.89*	0.50
uPVV - pressure at baseline	0.15	-0.40	0.42	0.03
- total pressure increase	-0.58	0.14	0.22	-0.25
- FMVD at 6mL/min	0.62	-0.25	-0.21	0.15
uPVV/PV ratio - pressure at baseline	-0.24	-0.01	0.05	-0.15
- total pressure increase	-0.81	0.12	0.36	0.51
- FMVD at 6mL/min	0.90*	0.21	-0.67	-0.90*

R= Spearman's correlation coefficient. Relevant correlations in bold. *Significant at level <0.05.
FMVD = flow-mediated vasodilation (% pressure reduction from peak to new steady state); uPVV = utero-placental vascular volume (in cm^3); PV = placental volume (in cm^3); uPVV/PV ratio = ratio between uPVV and PV; R = correlation coefficient.

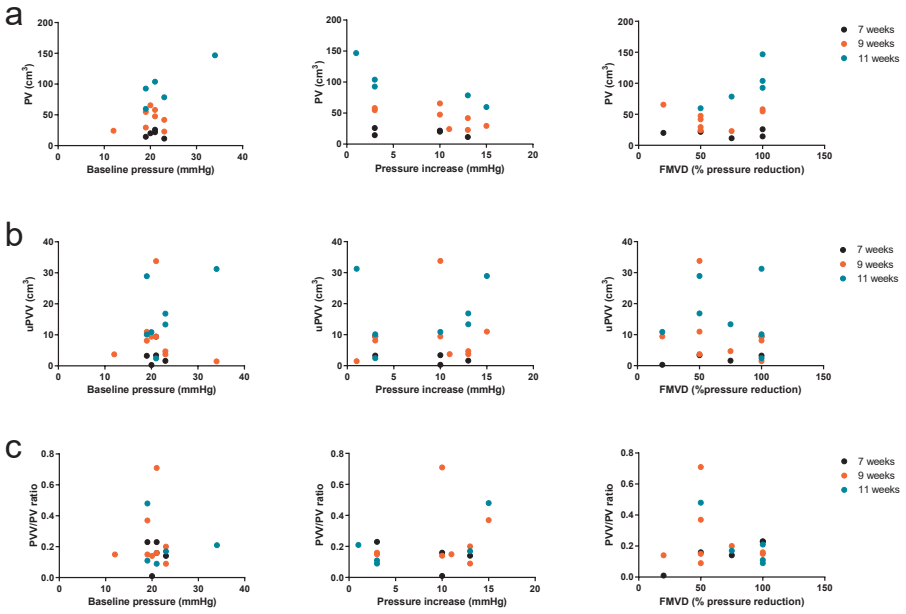

Figure 4. Scatterplots depicting correlations between *in vivo* and *ex vivo* placental parameters. This figure shows the correlations of the *ex vivo* parameters measured postpartum and PV (panel a), uPVV (panel b) and the uPVV/PV ratio (panel c) at 7 weeks GA (black circles), 9 weeks GA (orange circles) and 11 weeks GA (blue circles). FMVD = flow-mediated vasodilation (% reduction in pressure from peak to new steady state); PI = pressure increase; PV = placental volume (in cm3); uPVV = uteroplacental vascular volume (in cm3); uPVV/PV ratio = ratio between uPVV and PV.

DISCUSSION

The results of this study suggest that, mainly in the early first trimester, larger placental volumetric parameters, measured by 3D ultrasound and VR technique, are associated with lower pressure and more FMVD in the fetoplacental vasculature after delivery. Correlations between *in vivo* placental growth throughout the first trimester and *ex vivo* placental parameters were negative for the growth of PV and pressure increase (R=-0.84), but positive for FMVD (R=0.50) although not statistically significant. In contrast, a negative correlation existed between the growth of first-trimester uPVV/PV ratio and FMVD (R= -0.90).

To our knowledge, the current study is the first to investigate associations between *in vivo* first-trimester ultrasound parameters of the maternal uteroplacental circulation and third-trimester *ex vivo* perfusion parameters of the fetoplacental circulation. Previously, it has been shown by Jones *et al.* that at term there is a positive correlation between *in vivo* umbilical artery Doppler velocimetry and *ex vivo* fetoplacental vascular resistance in placentas from uncomplicated pregnancies.[15] However, one should keep in mind that umbilical artery Doppler velocimetry and *ex vivo* vascular resistance both represent the fetal side of the placenta, while our placental parameters reflect the volume and vascularization of the uterine/maternal side (i.e. the uteroplacental bed). In line with the results of Jones *et al.*, we found a negative correlation between pressure increase at *ex vivo* perfusion and PV (throughout the first trimester) and uPVV (at 7 weeks GA only). Furthermore, this corresponds with the positive correlation that was observed between PV, uPVV and FMVD. These findings suggest a greater ability of larger placentas to adjust to higher pressure by vasodilation, due to a greater compensatory capacity in the form of vasodilation. This is contrasted by the finding that the growth trajectory of uPVV/PV ratio was positively correlated with pressure increase and negatively with FMVD, which suggests that placentas with more vascular development (i.e. more increase of uPVV compared to PV throughout the first trimester) demonstrate higher pressure and less vasodilation in response to flow. Since PV and uPVV only reflect the maternal part of the placental circulation, a possible explanation for this correlation could be that less vascularized placentas in the first trimester have been exposed to higher pressure in utero and therefore show more pressure increase and less vasodilation *ex vivo*.

A larger PV at 11 weeks GA was associated with higher baseline pressure in this group of uncomplicated pregnancies. In line with this, previous research by our group showed that baseline pressure during *ex vivo* perfusion in placentas of pregnancies complicated by early onset PE was significantly lower compared to healthy placentas.[21] These placentas were significantly smaller, exposed to higher blood pressure *in vivo,* and displayed altered vascular responsiveness. However, the direct response to flow rate increase was not studied. Interestingly, Jones *et al.* did not find the same positive correlation between

in vivo umbilical artery Doppler velocimetry and *ex vivo* fetoplacental vascular resistance in placentas of pregnancies complicated by FGR as in healthy placentas.[15] Since smaller first-trimester placental volume is associated with the occurrence of FGR and PE,[23] it would be interesting to study whether the correlations seen in the current study also exist in placentas of pregnancies complicated by placental insufficiency (e.g. FGR or PE). Only one patient in the current study delivered a small-for-gestational-age infant, therefore it was not possible to show a clear correlation with fetal growth, however values of this case were not outliers. Comparing histology of the included placentas did not provide additional explanations for our results (data not shown). Histological analysis was performed according to the Amsterdam criteria[24] and included maternal stromal-vascular lesions, fetal stromal vascular lesions, infectious inflammatory lesions, immune/idiopathic inflammatory lesions, massive perivillous fibrin(oid) deposition, abnormal placental shape or umbilical insertion site, morbidly adherent placentas (accreta), meconium-associated changes and increased circulating nucleated red blood cells.

The differences in findings across the increasing gestational ages could be attributed to the unplugging of the spiral arteries around 9 weeks gestation. In early gestation, cytotrophoblast plugs occlude the spiral arteries, preventing perfusion of the intervillous space to safeguard a low-oxygen environment,[25] which is needed for vasculogenesis and cytotrophoblast proliferation.[26] Later in the first trimester, extravillous cytotrophoblast cells invade around the spiral arteries, initiating their remodelling and unplugging.[27] This leads to a low-resistance circulation with an increased perfusion capacity and reduced blood flow velocity into the intervillous space.[28, 29] We hypothesize that these vascular modifications impact PV and uPVV measurements and, especially after 9 weeks GA, could result in less pressure increase and more FMVD after delivery for larger PV and uPVV in the late first trimester. We did not demonstrate such an impact for uPVV in this study, but we did observe a negative correlation between PV and pressure increase and a positive correlation between PV and FMVD, in particular after 9 weeks GA.

This study is strengthened by the longitudinal data collection, creating a unique data set combining patient characteristics with *in vivo* and *ex vivo* measurements of the placenta. On the other hand, there is a large time gap between our measurements by first-trimester ultrasound and perfusion postpartum. Alterations in placental development during second- and third trimesters could have impacted our results, since capillary growth continues until delivery to accommodate the growing fetus, resulting in an exponential increase in volume of placental vessels in the third trimester.[7] Still, it is known that failure of the maternal spiral arteries to properly remodel in early pregnancy is already associated with higher fetoplacental vascular resistance later in pregnancy.[15, 30] Despite ongoing alterations, the foundation for placental vascular development is established in the first trimester, and this knowledge supports the correlations found in this study. Further, it remains uncertain whether mode of delivery could have affected

vascular resistance. Most placentas in our study were obtained after elective caesarean section (70%) and have not been subjected to labour. Only two placentas were delivered vaginally and one after emergency caesarean section. There is much debate in literature whether mode of delivery affects placental perfusion experiments. On the one hand it has been demonstrated that placentas after vaginal delivery show increased oxidative stress and inflammatory cytokines on both gene- and protein levels.[31] On the other hand, multiple studies showed no difference in placental barrier function during *ex vivo* perfusion for delivery mode.[32, 33] Lastly, identified correlations should be cautiously interpreted due to the small sample size of the study, which also hampered correction for multiple testing. Furthermore, such small sample size could lead to bias. However, values of patients with characteristics that stood out from the rest (e.g. IVF pregnancy, periconceptional smoking, spontaneous delivery), were not outliers. Also, male/female differences could introduce bias. Unfortunately only 3 female neonates were included in this study which made verification of bias impossible, although they were not outliers. A larger sample size would have probably strengthened the identified correlations, but since this was an explorative study and *ex vivo* placental perfusion is a difficult, expanding the group size within a reasonable time frame was not feasible.

In conclusion, we showed that *in vivo* larger first-trimester PV and uPVV are associated with less pressure increase and higher FMVD of the *ex vivo* fetoplacental vasculature at term, suggesting that enhanced adaptive mechanisms after delivery relate to a more optimal development of the placenta early in pregnancy. First-trimester evaluation of placental volume and vascularization could therefore be of value to predict placental function in later pregnancy, thereby providing future opportunities for early prevention as well as treatment of placenta-related pregnancy complications. As a next step towards this, future research should focus on validation of these measurements in the general population and in placentas from complicated pregnancies (FGR and/or PE).

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Chapter 8

General discussion and future directions

The placenta is the most important organ in pregnancy, connecting the fetus to the mother. Placenta-related pregnancy complications can affect the health of mother and child, both during pregnancy and in later life. Optimal pregnancy outcome for both mother and child thus requires close collaboration between obstetrical and neonatal care givers. The research described in this thesis is the result of the idea to establish a placenta lab and connects these departments.

PLACENTA RESEARCH

The importance of studying the placenta is driven by the fact that approximately 10% of pregnancies is complicated by placenta-related disorders.[1] Furthermore, up to 85% of women will be prescribed medication at one point during pregnancy, that possibly crosses the placental barrier and thereby exposes the fetus to exogenous substances.[2] The importance has recently been emphasized even further by the outcome of the STRIDER study. In this large international consortium of randomized controlled trials, the effect of sildenafil versus placebo on pregnancy outcome in severe fetal growth restriction (FGR) was evaluated.[3] Due to an overall lack of beneficial effects and increased neonatal morbidity in one of the cohorts, the study was halted prematurely.[4-6] Clinical trials were started after sildenafil had shown promising results in animal studies, although knowledge on the use of this drug in pregnancy was lacking. For example, there was no pharmacokinetic model developed for pregnant women, nor were there studies available on the transfer of sildenafil across the human placenta. Now it seems that placental transfer of sildenafil could be higher, and the beneficial effects less evident in preterm preeclamptic placentas (**Chapter 3**). This could at least partially explain the futility and neonatal toxicity. Therefore, when considering drugs for the treatment of pregnancy complications, its placental transfer and effect should be investigated first, preferably per trimester of gestation.

The only way to study placental transfer of novel drugs in humans without exposing the mother, and thus potentially the fetus, to the risk of toxicity, is the *ex vivo* placental perfusion model. It has not only been proven to be a reliable method to estimate fetal drug exposure, but it can also be used for studying transfer and release of hormones, amino acids, electrolytes and viruses.[7] Since a whole cotyledon is perfused, structural integrity and cell-cell organization are maintained, making it the closest resemblance of the *in vivo* situation. Furthermore, it is possible to measure transfer over time and tissue accumulation can be assessed. Although it has been shown that this model can accurately predict *in vivo* placental transfer at steady state at term,[7] there are some limitations. As discussed in **Chapter 3**, perfusion of preterm placentas remains very challenging, and determining transfer in the first trimester of pregnancy is not possible.

Second, since this is a very specialized technique with intricate equipment, there are many inter-laboratory differences. To optimize future placental perfusion studies, protocols need to be standardized between laboratories.

TREATMENT OF PREECLAMPSIA

Currently, the only effective treatment of preeclampsia (PE) is termination of pregnancy, often leading to severe preterm birth of the baby. Different antihypertensive drugs, e.g. methyldopa and calcium antagonists, have been tested in randomized controlled trials. However, they only temporarily stabilize the maternal symptoms of PE, and do not improve hard clinical outcomes such as mortality.[8-10] Targeting the generalized endothelial dysfunction seems a promising strategy in developing new treatment options for this disease (**Chapter 2**).

As discussed in **Chapter 5**, (over)activation of the endothelin (ET) system has emerged as an important factor in the pathophysiology of PE. Plasma levels of ET-1 are significantly increased in women with PE,[11] contributing to the development of hypertension and proteinuria.[12, 13] These findings led to preclinical studies with endothelin receptor antagonists (ERAs) and indeed, in PE animal models, blockade of the ET type A receptor (ET_AR) attenuated hypertension and proteinuria, and prevented FGR.[14-16] Unfortunately, developmental toxicity studies showed severe teratogenic effects of both selective ET_AR and dual ET_AR/ET_BR blockade, mainly craniofacial and cardiovascular malformations, arguing against the start of clinical trials.[17, 18] However, the placenta is the most species-specific organ, making translation of results from animal studies to humans complicated.[19] Furthermore, in most animal studies ERAs were given before the end of the Carnegie stages (i.e. 23 stages of the morphological development of the vertebrate embryo). In contrast, in women with PE these drugs could be given after the completion of embryogenesis. The fact that evaluation of human cases of ERA exposure during pregnancy did not show an increased incidence of fetal congenital malformations (**Chapter 5**), opens the door for the possibility of treating severe PE with ERAs. Future research should focus on evaluation of pharmacokinetics and safety during pregnancy, first by performing second/third trimester toxicology studies in animals with a longer gestation, for example non-human primates, and with clinically relevant dosages. In **Chapter 6** we showed that only a very small fraction of macitentan passes the placental barrier, favoring this dual ERA for further research. Another option to prevent ERAs from passing the placenta, would be to bind them to peptides that do not cross the placental barrier. At a later stage, 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.

Another suggested therapeutic intervention is the non-selective phosphodiesterase (PDE) inhibitor pentoxifylline (PTX), because of its supposed anti-inflammatory properties as well as the ability to improve endothelial function.[20] It has been shown that PTX reduces inflammation in placental explants and that it has a beneficial effect on the fetoplacental circulation *in vivo*.[21, 22] In **Chapter 4** we showed that PTX induces vasodilation in placental vasculature, especially in that of preeclamptic placentas. As a next step, we would like to investigate its placental transfer, both in healthy and PE placentas. Next we suggest to expand knowledge on the anti-inflammatory effects of PTX in the PE placenta, using placental explants and/or trophoblast cell culture. Furthermore, a pharmacokinetic model of PTX in pregnant women should be made before starting a clinical trial. No teratogenic effects of PTX are described in animal studies, and currently in Poland pregnant women are already treated with PTX for imminent preterm labor.[21] Blood samples of these women (both maternal plasma and umbilical cord) in combination with the transfer data from our placental perfusion experiments could be used to make such a model.

Although PDE5 inhibition with sildenafil to restore the decreased activity of the NO pathway was not effective in PE, this does not necessarily mean that targeting this pathway is the wrong approach. It could be that stimulation of NO on other levels does improve endothelial function. Clinical studies using compounds that (in)directly increase NO concentrations did reduce hypertension, but did not change maternal or fetal outcome.[23-25] However, stimulating the NO pathway in an NO-independent manner with soluble guanylate cyclase stimulators or activators, has been shown to improve endothelial function in PE tissue, and to inhibit placental production of soluble FMS-like tyrosine kinase-1 (sFlt-1).[26] sFlt-1 is produced in villous trophoblast cells and binds with high affinity to vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), preventing them from promoting angiogenesis and maintaining vascular endothelial function.[27, 28] It has been shown that sFlt-1 infusion in pregnant animals induces a PE-like syndrome.[29, 30] Indeed, plasma levels of sFlt-1 are elevated in women with PE, and these levels are positively correlated with ET-1 levels.[11, 31, 32] Furthermore, PE placentas have an increased gene expression of sFlt-1.[29] Decreasing sFlt-1 levels (e.g. through extracorporeal removal using dextrane sulphate apheresis) has resulted in significant improvement of PE symptoms.[33] Therefore, targeting the excessive production of sFlt-1 could be beneficial in PE treatment. Future placental perfusion studies and clinical trials remain to confirm safety and efficacy of these drugs.

EARLY IDENTIFICATION OF PLACENTAL INSUFFICIENCY

One of the complicating factors of successful PE treatment is the fact that early detection of placental insufficiency remains difficult. In the current clinical setting the standard method for evaluation of the placenta is ultrasound biometry in combination with Doppler parameters.[34, 35] However, although ultrasound examination provides accurate information on the location, size and anatomy of the placenta, it has limited value for the assessment of placental function. Only when placenta-related complications have been already established, placenta function is suspected to be abnormal on ultrasound.[36] Placental insufficiency is characterized by impaired trophoblast invasion and aberrant remodeling of the spiral arteries, causing increased vascular resistance and placental hypoperfusion.[37, 38] Indeed, this can result in increased pulsatility indices (PI) of the maternal uterine arteries and fetal umbilical artery, and a decrease in PI of the fetal medial cerebral artery.[34, 39] However, measurements of the uteroplacental circulation assess blood flow and are thus an indirect estimate of placental function. Moreover, the predictive value of uteroplacental circulation assessment for fetal outcome in an unselected population is low.[35]

Novel parameters for the early identification of patients at risk for developing placental-related pregnancy complications are placental volumetric parameters, measured offline in three-dimensional ultrasound volumes from the first trimester.[40] As described in **Chapter 7**, mainly early in the first trimester, larger placental volumetric parameters are associated with lower pressure and more flow-mediated vasodilation in the feto-placental vasculature of healthy placentas after delivery. This may suggest that larger and/or more vascularized placentas have better adaptive mechanisms and possibly lead to better pregnancy outcomes. Therefore, routine first-trimester evaluation of placental volumetric parameters could help to predict placental function in later pregnancy, thereby providing opportunities for early detection of placenta-related pregnancy complications. Future research should focus on repeating these measurements in placentas from complicated pregnancies (FGR and/or PE).

Another promising, non-invasive technique for a more direct assessment of placental function is Magnetic Resonance Imaging (MRI). MRI with magnetic fields up to 3 Tesla has been safely used in pregnancy for over 30 years.[41, 42] MRI of the placenta was primarily used for the assessment of an abnormally invasive placenta,[43] and in recent years it has been increasingly applied to evaluate fetal structural anomalies, especially at advanced gestational age or in obese women.[44] Experience with functional MRI (fMRI) has shown promising insight into the fetal brain and placental function *in vivo*.[45, 46] Placental fMRI allows assessment of functional tissue aspects, and could be used to examine placental functions related to vascularization, oxygenation, and metabolism.[47] Blood Oxygen

Level-Dependent (BOLD) MRI is an fMRI technique that measures changes in tissue oxygenation during hyperoxia or hypoxia, using hemoglobin (Hb) as an endogenous contrast agent. It is mainly used in clinical neuroimaging, linking brain anatomy and cognitive function.[48, 49] The BOLD effect is based on the fact that paramagnetic properties of Hb and deoxyhemoglobin (dHb) are different. The paramagnetic properties of dHb affect the spin of neighboring protons, thereby creating magnetic field in-homogeneities, which decrease T2-T2* weighted signal intensity. Thus, reduced tissue oxygenation leads to a decreased BOLD signal. During hyperoxia, there is a decrease in dHb, and an increase in BOLD signal is expected.[50, 51] This process has been previously proven in animal placentas and fetal organs.[52-55] In normal pregnancies, creating a state of maternal hyperoxia will give an increase in BOLD signal intensity in the placenta and fetal organs.[51, 55-57] It is thought that in pregnancies complicated by placental insufficiency there will be less of an increase.[56, 58] As a first step towards using fMRI as a diagnostic tool to identify high risk pregnancies we have started a feasibility study for performing BOLD MRI in the Erasmus MC. Women with uncomplicated singleton pregnancies between 28 and 34 weeks of gestation are eligible for inclusion. Placenta function is assessed using the BOLD technique. When this is successful, a next step would be to repeat these measurements in women with placental insufficiency.

TOWARDS PERSONALIZED MEDICINE

More and more, medicine is moving towards individualized treatment instead of population-based care. Evidence is accumulating that often different entities exist within one disease. Unlike previously thought, with the classical diagnosis of hypertension and proteinuria, we now recognize that PE is a spectrum disorder that can involve many organ systems, with a widely varying clinical manifestation. For example, it is now the general assumption that early onset PE (manifestation before 34 weeks of gestation) and late onset PE (manifestation from 34 weeks of gestation onwards) have different pathophysiological mechanisms, as clear histopathological differences have been shown.[59, 60] Late onset PE seems more of a maternal, rather than a placental syndrome. That there is a difference between these two sub-classifications is also apparent in our vascular research. We found that, although both early – and late onset PE placentas have a lower baseline tension in the perfusion model (Figure 1A), they do not display the same decreased responsiveness of the NO pathway (Figure 1B). These differences need to be taken into account in further research, as well as other sub-classifications such as PE with and without FGR, or FGR alone.

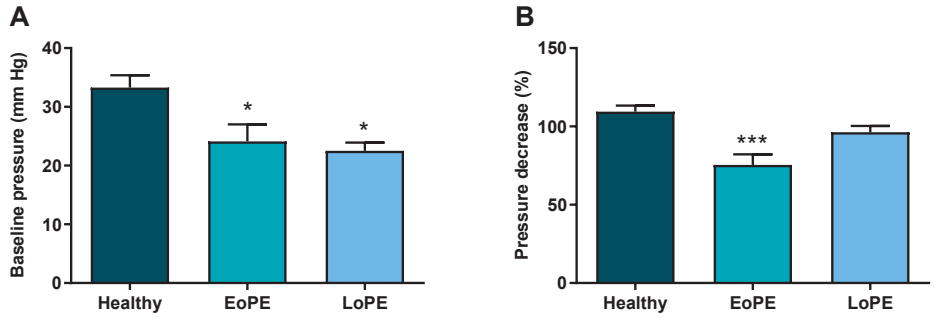

Figure 1. Differences in vascular response between early onset - and late onset preeclampsia. Panel A shows the baseline pressure of healthy, early onset preeclamptic (EoPE) and late onset preeclamptic (LoPE) placentas in the perfusion model. Panel B depicts the pressure decrease (%) in response to the NO-donor sodium nitroprusside. *p<0.05, ***p<0.001 (Kruskal-Wallis test with Dunn's correction for multiple testing).

As described in this thesis many pathways are involved in the pathogenesis of PE. Possibly, it could differ per patient - even within a subgroup - which alterations are most dominant, and would therefore require different diagnostic and/or therapeutic approaches. For example, in one patient the rise in ET-1 could be more prominent, and in another the inflammatory imbalance. Something that could help to better understand these different parts of the spectrum would be extensive phenotyping of the placenta. However, in-depth fundamental knowledge regarding human placental development in health and disease is still lacking. Novel techniques to study this, such as single-cell RNA sequencing and organoids, are currently still in its infancy, but could provide more answers in the future. To establish this, there is a need for expert centers, specialized in both placental research and clinical care for women and their fetuses/neonates that are affected by placenta-related pregnancy complications.

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Chapter 9

Summary
Samenvatting

SUMMARY

Preeclampsia (PE) is a common but potentially severe placenta-related complication of pregnancy, that accounts for a substantial part of perinatal and maternal morbidity and mortality worldwide. To date, no cure is available for PE, except for termination of pregnancy, often leading to preterm birth.

Many pathophysiological pathways have been proposed, such as oxidative stress, immunological imbalance, and environmental and genetic factors, all contributing to inadequate development of the placenta. Instead of the low-resistance circulation that is seen in healthy placentas, PE is characterized by increased placental vascular resistance and hypoperfusion, leading to generalized endothelial dysfunction and endovascular inflammation. In **Chapter 1** we presented (historical) background on PE and explained the experimental model that was used in this thesis.

In **Chapter 2**, the most important vascular reactivity pathways of the placenta were summarized, which are the nitric oxide (NO) pathway and its NO-dependent vasodilators, vascular endothelial growth factor and the endothelin (ET) system, the renin-angiotensin system, prostaglandins, serotonin and tryptophan, and calcitonin gene-related peptide. Many of these pathways show alterations in PE, that are a consequence of, or could have attributed to the pathogenesis of this disease. Targeting these disturbances could be a promising strategy in developing novel treatments for PE.

Chapter 3 focused on the NO pathway. One of the novel therapeutic strategies that had emerged over the last years is the phosphodiesterase (PDE)5 inhibitor sildenafil. Sildenafil enhances systemic vasodilation through the NO pathway, and because of its promising effects in PE animal studies, a large randomized controlled clinical trial was started (the STRIDER study). Unfortunately, this trial was halted due to futility to show beneficial effects and increased neonatal morbidity. Beforehand, not much was known about the transfer and effect of sildenafil in the human placenta, especially in the case of PE, as studied in this chapter. We found that although sildenafil improved NO-mediated vasodilation in isolated chorionic plate arteries of healthy placentas, this was not the case in arteries of PE placentas. The lack of this NO-potentiation by sildenafil in PE placentas, and the fact that there was no PDE5 upregulation on gene – or protein level in PE, made us question sildenafil as a suited therapy for this disease. Furthermore, placental transfer of sildenafil was highest in the PE placentas, which could (partially) explain the increased neonatal morbidity as seen in the STRIDER study.

In **Chapter 4** we described the effect of another potential therapeutic for PE, the non-selective PDE inhibitor pentoxifylline on porcine coronary arteries and human chorionic plate arteries. We found that its vasodilator effect is mainly cGMP-mediated, and that it is more pronounced in vasculature of preeclamptic placentas.

In **Chapters 5 & 6** we focused on targeting the ET system. Increased activity of the ET system plays a role in the pathogenesis of PE, and blocking this system with endothelin receptor antagonists (ERAs) would be a logical step. However, although ERAs indeed attenuated hypertension and proteinuria in PE animal studies, they also caused severe teratogenic effects on offspring (mainly craniofacial malformations) in developmental toxicity studies. Therefore, no clinical trials have been performed with these drugs. In **Chapter 5** we discussed the involvement of the ET system in normal pregnancy, the pathophysiology of PE, and embryogenesis, to evaluate if possibly a therapeutic window exists in which ERAs could be safely administered during human pregnancy. Through a systematic review of literature we found 39 reported cases of women who were exposed to ERAs during pregnancy. All these women were treated with ERAs because of its registered indication (pulmonary arterial hypertension) and there was no increased incidence of congenital malformations. Although in most cases exposure was of short duration and the rate of elective termination of pregnancy was high (31%), this supported the theory that ERA treatment could be safe, when started after organogenesis is completed to avoid teratogenic risks. To further study this possibility, we investigated the placental transfer and effects of three ERAs (sitaxentan, ambrisentan and macitentan) in **Chapter 6**. We found that sitaxentan and ambrisentan both substantially passed the placental barrier, however the transfer of macitentan was very limited. All three blockers were capable of blocking the vasoconstrictive effect of ET-1 in the chorionic plate arteries, which seems to be solely mediated by the ET type A receptor. Furthermore, we confirmed that gene expression of ET-1 is increased in PE placentas, but that there is no difference in receptor expression.

In **Chapter 7** we explored the correlation between placental volumetric parameters, measured by ultrasound in the first trimester of pregnancy, and parameters of fetoplacental vascular function, measured using placental perfusion after delivery. We found that, mainly early in the first trimester, larger placental volumetric parameters are associated with better vascular function (lower pressure and more vasodilation in response to flow increase) at placental perfusion. This suggests that larger and/or more vascularized placentas in early pregnancy have better adaptive mechanisms, which could possibly lead to better pregnancy outcomes.

Finally, in **Chapter 8** we discussed the clinical implications and addressed the strengths and limitations of the studies in this thesis. Furthermore, we have elaborated on what our research has added and made suggestions for future research.

SAMENVATTING

Pre-eclampsie (PE) is een veelvoorkomende, maar potentieel ernstige, placenta-gerelateerde zwangerschapscomplicatie, die wereldwijd verantwoordelijk is voor een substantieel deel van de perinatale en maternale morbiditeit en mortaliteit. Tot op heden is er geen adequate behandeling voor PE, behalve beëindiging van de zwangerschap, wat vaak leidt tot vroeggeboorte.

Er zijn vele oorzakelijke factoren die bijdragen aan het ontstaan van PE, zoals oxidatieve stress, immunologische onbalans, omgevingsfactoren en genetische factoren. Al deze factoren samen leiden tot een inadequate ontwikkeling van de placenta. In plaats van de lage weerstandscirculatie die normaliter ontstaat in gezonde placenta's, wordt PE juist gekenmerkt door verhoogde vaatweerstand en verminderde perfusie, hetgeen leidt tot generaliseerde endotheel dysfunctie en endovasculaire ontsteking. In **Hoofdstuk 1** presenteerden we de (historische) achtergrond van PE en hebben we het experimentele model besproken dat is gebruikt voor een groot deel van het onderzoek in dit proefschrift.

In **Hoofdstuk 2** zijn de belangrijkste factoren die betrokken zijn bij de vasculaire reactiviteit van de placenta samengevat, namelijk stikstof en stikstof-afhankelijke vaatverwijders, 'vascular endothelial growth factor' en het endotheline (ET)-systeem, het renine-angiotensine-systeem, prostaglandines, serotonine en tryptofaan en 'calcitonin gene-related peptide'. Veel van deze factoren vertonen veranderingen bij PE, als gevolg van, of die juist kunnen hebben bijgedragen aan het ontstaan van deze ziekte. Het proberen te herstellen van de verstoringen in deze factoren zou een veelbelovende strategie kunnen zijn in de ontwikkeling van nieuwe behandelmogelijkheden voor PE.

In **Hoofdstuk 3** lag de focus op stikstof. Een van de nieuwe behandelstrategieën die de laatste jaren in opkomst was, is behandelen met de fosfodiësterase (PDE)5 remmer sildenafil. Sildenafil stimuleert de systemische vaatverwijding door stikstof en vanwege veelbelovende effecten in dierstudies was een grote gerandomiseerde klinische studie gestart (de STRIDER studie). Helaas is deze studie voortijdig gestaakt, omdat er geen gunstige effecten werden gezien en zelfs een toename van neonatale complicaties in één van de cohorten. Voorafgaand aan deze studie was er weinig bekend over de effecten van sildenafil op de placenta en de passage van moeder naar kind, vooral in het geval van PE, zoals we in dit hoofdstuk hebben bestudeerd. In onze studie vonden we dat sildenafil de vaatverwijding niet stimuleert in bloedvaten van PE placenta's, in tegenstelling tot in vaten van gezonde placenta's. Dit in combinatie met het gebrek aan PDE5 opregulatie op gen- of eiwitniveau, suggereert dat sildenafil wellicht geen geschikte behandeling is voor PE. Bovendien was de placentapassage van sildenafil het hoogste in de PE placenta's, wat deels een verklaring zou kunnen zijn voor de toename aan neonatale complicaties in de STRIDER studie.

Hoofdstuk 4 beschrijft de effecten van de non-selectieve PDE-remmer pentoxifylline op coronairvaten van varkens en humane placentavaten. We hebben in dit hoofdstuk aangetoond dat de vaatverwijding die wordt veroorzaakt door pentoxifylline met name cGMP-gemedieerd is, en versterkt is in vaten van PE placenta's.

In **Hoofdstuk 5 & 6** hebben we ons gericht op het ET-systeem. Verhoogde activiteit van het ET-systeem speelt een rol in het ontstaan van PE, dus behandeling middels blokkade van dit systeem met endotheline receptorantagonisten (ERA's) zou een logische stap zijn. Echter, hoewel ERA's inderdaad de symptomen van PE verbeterden in dierstudies, gingen ze ook gepaard met ernstige aangeboren afwijkingen bij het nageslacht (voornamelijk afwijkingen aan de botten van het gezicht). Vanwege deze teratogene effecten zijn er geen klinische studies uitgevoerd met deze medicijnen. Om te evalueren of er tijdens humane zwangerschappen een window bestaat waarin ERA's veilig gegeven kunnen worden, werd in **Hoofdstuk 5** de rol van het ET-systeem in normale zwangerschappen, in het ontstaan van PE en in de ontwikkeling van het embryo bediscussieerd. Door middel van een systematisch review van de literatuur vonden we 39 gerapporteerde casussen van vrouwen die zijn blootgesteld aan ERA's tijdens de zwangerschap. Al deze vrouwen werden behandeld met ERA's vanwege pulmonale arteriële hypertensie, waarvoor deze medicijnen ook geregistreerd zijn. Er werd geen verhoogde incidentie gezien van aangeboren afwijkingen. Hoewel er in de meeste casussen slechts kortdurig blootstelling was aan ERA's en er een hoog percentage (31%) van de zwangerschappen werd afgebroken op medische indicatie, ondersteunt het gebrek aan aangeboren afwijkingen de theorie dat behandeling met ERA's veilig kan zijn wanneer ze gegeven worden nadat de foetale organen zijn aangelegd. Om dit verder te onderzoeken hebben we in **Hoofdstuk 6** de placentaire passage en effecten van drie ERA's (sitaxentan, ambrisentan en macitentan) onderzocht. We vonden dat substantiële hoeveelheden van sitaxentan en ambrisentan de placenta barrière passeren, maar dat de passage van macitentan zeer beperkt is. Alle drie de ERA's konden het effect van ET-1 nagenoeg compleet blokkeren in placentavaten, wat volledig afhankelijk lijkt van de ET type A receptor. Ook hebben we bevestigd dat de genexpressie van ET-1 verhoogd is in weefsel van PE placenta's, maar vonden we geen verschil in receptorexpressie.

Hoofdstuk 7 exploreerde de correlatie tussen volume parameters van de placenta, gemeten middels echografie in het eerste trimester van de zwangerschap, en placentaire vaatfunctie, gemeten met placenta perfusie na de geboorte. Het blijkt dat grotere placenta (vasculaire) volumes, met name vroeg in het eerste trimester, geassocieerd zijn met betere vaatfunctie (lagere druk en meer vaatverwijding in reactie op verhoging van de stroomsnelheid) bij placenta perfusie. Dit suggereert dat grotere en/of meer gevascularisseerde placenta's mogelijk betere aanpassingsmechanismes hebben, wat zou kunnen leiden tot een betere zwangerschapsuitkomst.

Tenslotte hebben we in **Hoofdstuk 8** de klinische implicaties van dit proefschrift bediscussieerd, evenals de sterke en zwakke punten van de beschreven studies. We hebben besproken wat het onderzoek in dit proefschrift heeft toegevoegd aan de huidige kennis en hebben suggesties gedaan voor toekomstig onderzoek.