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General discussion and future directions

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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.

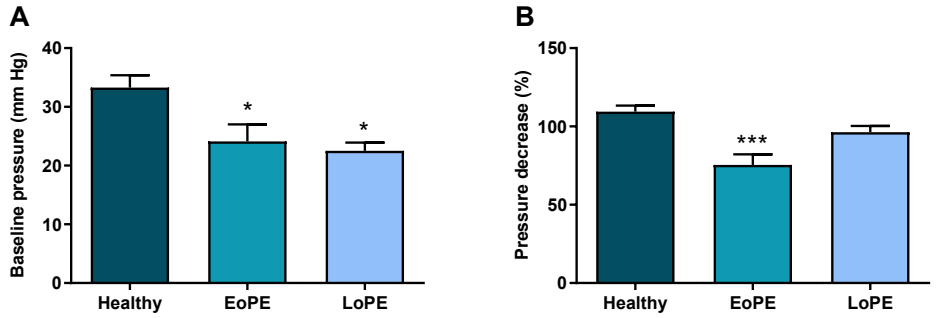

Figure 1. Differences in vascular response between early onset - and late onset preeclampsia. Panel A shows the baseline pressure of healthy, early onset preeclamptic (EoPE) and late onset preeclamptic (LoPE) placentas in the perfusion model. Panel B depicts the pressure decrease (%) in response to the 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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