Chapter 8
General discussion and future perspectives
General discussion

Preeclampsia (PE) is a pregnancy-induced disease that is characterized by endothelial dysfunction, an antiangiogenic state, an exaggerated inflammatory response and placental oxidative stress. Clinical signs of PE include hypertension and proteinuria. During the last decades, gasotransmitters such as nitric oxide (NO) and carbon monoxide (CO) have been the subject of translational research in PE. Gasotransmitters are gaseous signaling molecules that possess a broad spectrum of, often overlapping, biological functions, such as regulation of vascular tone, immunomodulation, cytoprotection by scavenging of reactive oxygen species (ROS), and angiogenesis. It became clear that NO and CO are involved in the physiology of pregnancy and the pathogenesis of PE. Furthermore, animal and human studies provided evidence that both NO, CO and their related enzymes have therapeutic potential in PE. More recently, a third gasotransmitter, hydrogen sulfide (H$_2$S), was discovered. At the start of this thesis project, the role of H$_2$S in the pathophysiology of PE was unknown.

Despite extensive research, the pathophysiology of PE is still not completely understood. As a result, a (mechanism-based) therapy for the multifactorial syndrome is still lacking nowadays. H$_2$S has been proven to have significant therapeutical potential in various conditions such as cardiovascular disease and ischemia/reperfusion. In those conditions the beneficial aspects of hydrogen sulfide relate to vasodilation, angiogenesis, scavenging of reactive oxygen species and immunomodulation. Therefore, in this thesis, we hypothesized that H$_2$S is a novel factor involved in the pathogenesis of PE. We provide insights into the role of H$_2$S in normal pregnancy and PE, and into the potential of H$_2$S to be a therapeutic compound for PE.

Endogenous H$_2$S production in the human placenta during preeclampsia

The present thesis describes the placental expression of H$_2$S-producing enzymes during healthy pregnancy and PE. We found the H$_2$S-producing enzyme cystathionine-β-synthase (CBS) to be decreased in placentas from patients with early-onset PE (chapter 3). Another research group found decreased placental production of CBS protein during PE as well, though the group of PE patients was not further subdivided into early- and late-onset. In contrast we did not find any decrements in the expression of cystathione-γ-lyase (CSE), as described recently by others. CBS expression (both gene and protein) is also decreased in placentas derived from women with a growth-restricted
fetus. This is intriguing, since PE and fetal growth restriction (FGR) are both associated with impaired placentation, and PE is often accompanied by FGR. In chapter 3, three PE patients with a pregnancy complicated by FGR were included. However, as the data of these patients with respect to mRNA and protein expression fitted well within their study group, the placental expression of CBS in our study seems not to be different between patients with only PE and patients with PE and FGR. The reason for differences in placental CBS and CSE expression during PE between the aforementioned studies are not exactly known. Possibilities include differences in the time interval between partus and sample collection, characterization of the study groups (mode of delivery, maternal characteristics, fetal sex), and/or heterogeneity of the placental tissue.

Generally taken, our group and others showed that the production of H$_2$S-producing enzymes is impaired in placentas during PE. Given that the enzymatic activity of CBS and CSE was shown to produce H$_2$S in the human placenta, the data on H$_2$S-producing enzymes suggests that the endogenous production of H$_2$S is reduced in preeclamptic placentas. Therefore, it could well be that H$_2$S is involved in the pathophysiology of PE like the two other gaseous transmitters NO and CO, either as cause or consequence of the placental syndrome.

**H$_2$S as a vasodilator in the fetoplacental circulation**

With the suggestion that the production of H$_2$S is decreased in preeclamptic placentas, the question arises which functional role H$_2$S could have in placentation and placental function. The most widely studied function of H$_2$S is regulation of vascular tone, and the first identified target in H$_2$S-induced vasorelaxation was the ATP-sensitive potassium (K$_{ATP}$) channel in vascular smooth muscle cells. More recently, H$_2$S has also been recognized as an endothelial derived hyperpolarization factor (EDHF). During pregnancy, the H$_2$S producing enzymes CBS and CSE are expressed by the endothelium of fetal vessels within the placental villi (chapter 3). Indeed, in vitro, H$_2$S can act as a vasodilator of the villous vessels in the human placenta via K$_{ATP}$-channels. The production of H$_2$S or its producing enzymes in the umbilical artery was never investigated. Therefore, it is unknown whether H$_2$S production can be related to the increased resistance in umbilical arteries, which is often observed in PE patients and cases of FGR. However, chapter 6 describes that treatment of healthy pregnant rats with H$_2$S resulted in increased fetal weight, without increase in placental weight. This might be explained by the ability of H$_2$S to improve fetoplacental blood flow and perfusion. Indeed, in animal models of hindlimb ischemia,
it was shown that H$_2$S is able to increase blood flow and increase tissue perfusion.$^{11}$ Nevertheless, to confirm this hypothesis, ultrasound Doppler of the uterine arteries and/or fetal vessels should be performed after H$_2$S treatment, preferably in animal models for FGR. Another explanation could be that if H$_2$S is produced by fetal tissue and/or H$_2$S treatment of the mother reaches the fetus, H$_2$S has direct effects on fetal growth. For example via upregulation of insulin-like growth factor (IGF) which is associated with fetal growth regulation.$^{12,13}$ Whether H$_2$S is bioactive in the fetus is unknown.

H$_2$S at the fetomaternal interface

In chapter 3, villous syncytiotrophoblasts were found to be negative for both CBS and CSE by immunohistochemistry. This in contrast to other reports which actually did show expression of CBS and CSE by syncytiotrophoblasts.$^{4,5}$ The differences between these studies and ours might be explained by the use of different antibodies, possibly recognizing different isotopes of CBS and CSE proteins. Indeed, more recently we used another antibody for CSE (clone 4E1-1B7; Abnova, Tapei City, Taiwan) on the same tissue samples as used in chapter 3, and were able to show CSE expression by the villous syncytiotrophoblasts as well (unpublished data).

The expression of CBS and CSE by syncytiotrophoblasts suggests the production of H$_2$S by these cells, at least at term. Given the ability of H$_2$S to regulate oxidative stress and scavenge reactive oxygen species (ROS), a cytoprotective role of H$_2$S in syncytiotrophoblasts can be hypothesized. Syncytiotrophoblasts are fetal in origin and form the epithelial surface of the placental chorionic villi.$^{14}$ The cells are in direct contact with the maternal blood and facilitate in oxygen and nutrient transport between the mother and the fetus.$^{14}$ Especially during the first trimester, syncytiotrophoblasts are prone to oxidative stress. Therefore, it would be interesting to investigate whether the H$_2$S producing enzymes are already present in syncytiotrophoblasts during the first trimester. We hypothesize that H$_2$S might be potential regulator of oxidative stress at the fetomaternal interface, either by direct scavenging of ROS or by upregulation of other antioxidant enzymes,$^{15,16}$ but this remains to be elucidated.

A role for H$_2$S in impaired placentation?

It is generally believed that poor placentation is a key factor in the development of PE. In healthy pregnancy, deep invasion of interstitial and endovascular cytotrophoblasts results in remodeling of the spiral arteries in the uterine placental bed so that they become dilated...
and low-resistant.\textsuperscript{1,17} However, in the early stages of PE, trophoblast invasion is shallow and spiral arteries remain narrow and high-resistant.\textsuperscript{1,17} The expression of H\textsubscript{2}S producing enzymes in the human placental bed has never been investigated. However, there are some papers that connect H\textsubscript{2}S to trophoblast invasion. Mice with a CBS deficiency show impaired decidualization and lower uterine NK-cells numbers.\textsuperscript{18} The latter cells are known to be crucially involved in trophoblast invasion.\textsuperscript{19} Moreover, the invasion of cultured first trimester trophoblast cells was reduced by inhibition of CSE activity. These data indicated that H\textsubscript{2}S might be involved in trophoblast invasion. Whether H\textsubscript{2}S influences remodeling of the spiral arteries remains to be examined.

**H\textsubscript{2}S can influence the angiogenic balance**

It is generally accepted that before the onset of the clinical symptoms of PE, the diseased placenta secretes multiple factors into the maternal circulation. One of these factors is sFlt1; a potent antagonist of VEGF. Due to high levels of sFlt1, VEGF signaling through its receptor VEGFR2 is impaired in women with PE, subsequently contributing to the onset of endothelial dysfunction and the clinical syndrome. As elaborated in chapter 5 and by others, H\textsubscript{2}S is a profound proangiogenic compound. The majority of its proangiogenic actions are executed through interactions with the VEGF signaling pathway.\textsuperscript{20,21} Interestingly, chapter 5 describes that H\textsubscript{2}S is able to reduce free plasma levels of sFlt1 in non-pregnant rats. This effect is probably due to the induction of VEGF production and/or release of VEGF. The potential of H\textsubscript{2}S to interact with the VEGF signaling pathway makes the gas an interesting factor in PE since other studies on compounds that target angiogenic factors, such as recombinant VEGF or PlGF, have proven to be an effective strategy in reducing sFlt1-induced endothelial damage.\textsuperscript{22-24}

In chapter 6, the therapeutic effect of H\textsubscript{2}S was tested in a pregnant model with high sFlt1. Within our experimental setting, however, pregnant rats did not develop hypertension and proteinuria despite high circulating sFlt1 levels. Moreover, H\textsubscript{2}S was not able to down regulate free plasma sFlt1. There can be several explanations for the differences in the effect of H\textsubscript{2}S on angiogenic factors between chapter 5 and chapter 6. At first, there might be an effect of pregnancy itself. Perhaps the VEGF signaling pathway is less sensitive to H\textsubscript{2}S treatment during pregnancy as compared to non-pregnant conditions. Moreover, the differences in the administered doses of H\textsubscript{2}S in the two experimental settings in chapter 5 and chapter 6 might contribute to the differences in the effect of H\textsubscript{2}S. As the non-pregnant rats in chapter 5 received 50 uM NaHS twice
daily, while the pregnant rats in chapter 6 received 100 uM NaHS once per day, the 
plasma levels of H\textsubscript{2}S were perhaps more constant in the non-pregnant rats. Indeed, in a 
pilot experiment with sFlt1 overexpressing pregnant rats and twice daily administration 
of 50 uM NaHS, we observed a beneficial effect of NaHS on blood pressure, proteinuria 
and on sFlt1 levels (unpublished data). Finally, dynamic physiological alterations that 
occur during pregnancy might have influenced the distribution and elimination of H\textsubscript{2}S as 
well.\textsuperscript{25} Future experiments should be designed to investigate the pharmacokinetics of H\textsubscript{2}S 
related therapies and its efficacy to target the VEGF signaling pathway during pregnancy.

A possible target of H\textsubscript{2}S: the maternal endothelium
Secondary to excessively secreted placental factors, such as sFlt1 and proinflammatory 
cytokines, generalized endothelial dysfunction develops. H\textsubscript{2}S is an important player in 
regulation of endothelial function.\textsuperscript{10} Besides promoting angiogenesis, H\textsubscript{2}S is involved 
in regulation of vascular tone by endothelium-dependent, but also independent 
ways (chapter 7, figure 1). Moreover, H\textsubscript{2}S inhibits vascular inflammation by inhibiting 
proinflammatory factors and scavenging reactive oxygen species, among others.\textsuperscript{10} Mice 
with a deficiency for either the CSE or CBS gene develop hypertension.\textsuperscript{26,27} In women 
with PE, who suffer from generalized endothelial dysfunction, circulating H\textsubscript{2}S levels are 
impaired.\textsuperscript{5} Interestingly, women with a specific single nucleotide polymorphism of the 
CBS gene have a decreased risk to develop early-onset PE (chapter 4). Whether this SNP 
is related to endothelial dysfunction in these patients remains to be explored.

We show in chapter 5 that H\textsubscript{2}S is able to lower blood pressure in non-pregnant 
rats with high plasma sFlt1. Aside from established mechanisms by which H\textsubscript{2}S exerts its 
vasodilatory effect, such as opening of K\textsubscript{ATP} channels in vascular smooth muscle cells 
and acting as an endothelial derived hyperpolarization factor;\textsuperscript{9,10} an additional signaling 
pathway involved in H\textsubscript{2}S mediated vasorelaxation may be proposed. Specifically, chapter 
5 provides evidence that H\textsubscript{2}S vasodilation is partly dependent on VEGF signaling; since 
blockade of VEGFR2 receptor attenuates H\textsubscript{2}S mediated vasodilation in aortic rings. This 
effect could be due to the capability of H\textsubscript{2}S to directly target the VEGFR2.\textsuperscript{21}

Chapter 6 suggests that H\textsubscript{2}S can influence vascular tone by reducing the sensitivity 
to angiotensin II, a potent vasoconstrictor. As the use of renin angiotensin aldosterone 
blockade (RAAS) blockade in pregnancy is contraindicated, the RAAS might be another 
interesting target for H\textsubscript{2}S in PE. The mechanisms by which H\textsubscript{2}S influences angiotensin II 
sensitivity, remains to be elucidated but might include decrement of the binding affinity
of the angiotensin II type I (AT1R) to angiotensin II, and down regulation of AT1R expression. However, the latter was not observed in the rat aorta or kidney in chapter 6. An additional mechanism by which H$_2$S might influence angiotensin II sensitivity is by increasing At2r gene expression, which was observed in kidneys from sFlt1 rats treated with NaHS. Finally, as shown by others, H$_2$S is able to interfere with the RAAS by inhibiting plasma renin activity. However, as renin synthesis is suppressed during PE, this mechanism seems to be less relevant in that situation.

PE is recognized by generalized vascular inflammation. Vascular inflammation usually starts in the endothelium, where proinflammatory cytokines induce up regulation of adhesion molecules, and leukocyte adhesion is initiated. Vascular inflammation can be attenuated by H$_2$S via several pathways, including scavenging local reactive oxygen species and up regulating other antioxidants. Though the anti-inflammatory role of H$_2$S on the systemic vascular endothelium was not investigated in this thesis, we hypothesize that cytoprotection of the endothelium by H$_2$S can be an interesting target for PE.

A renal role for H$_2$S

The kidney is one of the organs that is affected during PE, clinically characterized by proteinuria. Histologically, kidneys from PE patients can be recognized by glomerular endotheliosis, a lesion that is coherent with the generalized endothelial dysfunction in these patients. It is likely, that the antiangiogenic environment in PE contributes to these glomerular lesions. This hypothesis is further confirmed in chapter 6, where endothelial lesions are present in pregnant rats with high sFlt1, but in the absence of hypertension and proteinuria. Renal VEGF is mainly derived from podocytes, the cells that protect the glomerular filtration barrier. Interestingly, mice with a podocyte-specific knockout for VEGF show glomerular endotheliosis. It is suggested that restoring the angiogenic balance systemically and in the kidney would prevent renal damage in PE. Administration of H$_2$S in non-pregnant rats with high sFlt1 resulted in an increased renal VEGF gene expression accompanied by a decrease in proteinuria, and in vitro stimulation of podocytes with H$_2$S induced VEGF release and production (chapter 5). However, no such effects of H$_2$S were observed in pregnant rats (chapter 6). Whether the absence of a renal H$_2$S effect is pregnancy dependent remains to be investigated.

Taken together, this thesis provides insights into several in vivo (endothelial) effects of H$_2$S in non-pregnant and pregnant rats. In non-pregnant rats with sFlt1 overexpression,
treatment with H₂S attenuated hypertension, albuminuria, and glomerular endotheliosis, and reduced free circulating levels of sFlt1. In pregnant rats with sFlt1 overexpression, H₂S decreased vascular sensitivity to angiotensin II. However, H₂S therapy had no effect on the angiogenic balance in pregnant rats. Moreover, as pregnant rats did not develop hypertension and albuminuria despite of high sFlt1 levels, we were not able to investigate the in vivo effect of H₂S on those parameters during pregnancy. Nevertheless, we feel that H₂S has the potential to lower blood pressure and albuminuria in (experimental) PE. This should be confirmed in other animal models with experimental PE.

**Future Perspectives**

This thesis, combined with recent literature, suggests that the gasotransmitter H₂S might be involved in the pathophysiology of PE like the two other gaseous transmitters NO and CO, either as cause or consequence of the placental syndrome. To elucidate the exact functional role of H₂S in pregnancy and PE, more studies are warranted. At first, to understand the importance of H₂S in the pathophysiology of PE, more knowledge should be gathered with regard to the functional role of H₂S in the physiology of pregnancy, and especially during early pregnancy. At the moment, it is unclear which exact functions H₂S exerts in the human placenta throughout gestation. Functional in vitro experiments with placental explants and primary trophoblasts, or in vivo studies with and CBS or CSE knockout mice, might provide more insight in the role of H₂S in placentation, trophoblast invasion and cytoprotection at the fetomaternal interface. Secondly, the bioavailability of H₂S during gestation should be investigated. As pregnancy is featured by major hemodynamic changes, such as increased plasma volume, cardiac output and a decreased peripheral vascular resistance, increased (vascular) H₂S production along with gestation can be expected. For NO, a similar pattern during pregnancy is observed. By understanding H₂S production during healthy pregnancy, the data of systemic H₂S in pregnancy-related diseases such as PE and FGR will become much more meaningful. To achieve this goal, techniques to measure plasma H₂S should be improved. Alternatively, the end metabolites of H₂S, sulfate and thiosulfate, should be determined in urine of pregnant women. These metabolites are thought be a reliable estimate for H₂S bioavailability. Unfortunately, we have not been able to measure these metabolites in during pregnancy or PE. However, preliminary data from previously pregnant women in
a cohort of 173 women from the PREVEND study, showed that excretion of thiosulfate per 24 hours is decreased in women who have had PE or the HELLP syndrome (hemolysis, elevated liver enzymes and low platelets syndrome; n = 25), as compared to women who had a healthy pregnancy (n = 148) (unpublished data, PREVEND study). This suggests that H$_2$S levels are decreased in women who formerly suffered from PE or the HELLP syndrome. This may also suggest that H$_2$S levels in these women were decreased during pregnancy. Moreover, it could be hypothesized that these lower H$_2$S levels contribute to the development of (premature) cardiovascular and renal disease after PE.

Our results suggest that H$_2$S may be promising therapeutic compound in the setting of PE, since we have shown the therapeutic effect of H$_2$S in non-pregnant animals with high levels of sFlt1. We also attempted to study the in vivo therapeutic effect of H$_2$S as a therapy in experimental PE, using pregnant rats with high levels of sFlt1. Unfortunately, at least in our experimental setting, the animal model did not exhibit hypertension and proteinuria. Apparently, high levels of sFlt1 do not per se induce a PE-like phenotype. Future studies should be designed to elucidate which factors trigger the onset of hypertension and albuminuria upon high levels of sFlt1, for example stress factors or microbiological status.

As the model used in chapter 6 was not suitable for evaluating therapeutic effects of H$_2$S on a PE-like phenotype, one of the first steps to take is applying H$_2$S in another model for experimental PE. Such models can for example be based on dysregulation of the immune system (infusion of e.g. low-dose endotoxin, IL-6 or TNF-α), the RAAS (injection of angiotensin receptor autoantibodies, rats transgenic for human renin and angiotensinogen), or on dysregulation of oxygen (reduced uterine perfusion pressure (RUPP) model). The following major challenge for the use of H$_2$S as a therapeutic compound is the development of H$_2$S based drugs that are applicable in the clinic. In view of the current thesis, a major hurdle is to reach acceptance for the use of a compound during gestation, which is initially known for its toxic effects. Developing accurate measurement techniques that provide a reliable reflection of endogenous H$_2$S levels, probably contribute in taking this hurdle. In that way, endogenous H$_2$S levels can be regulated more strictly. Therefore, future studies that develop such techniques are warranted. Moreover, the question remains whether the use of VEGF-inducing compounds is eligible during pregnancy. Induction of vessel growth raises several concerns, particularly in relation to (fetal) hemorrhage and tumor growth. Placental vascular defects were also described in a mouse model with
VEGF overexpression.46 Of course, teratogenicity of H2S should be extensively studied before moving towards clinical studies in pregnancy or PE.

Currently, few therapies that increase endogenous H2S are already clinically available; statins and sodium thiosulfate. Statins are interesting agents since they induce the production of NO, CO and H2S, have anti-inflammatory and -oxidative properties, and are able to inhibit sFlt1 release.47-49 Interestingly, statins have been shown to protect podocytes and decrease albuminuria.50 Furthermore, in a mouse model with increased placenta derived human sFlt1, amelioration of the PE- and FGR-phenotype was observed after statin treatment.22 Additional studies to evaluate whether statins are effective in prevention/treatment of PE and have no adverse effects in pregnancy are currently performed.47 Another promising compound is sodium thiosulfate, one of the H2S metabolites that is also able to increase H2S bioavailability when exogenously administered.51 Sodium thiosulfate is clinically available for the treatment of calciphylaxis.52 Interestingly, sodium thiosulfate has been proven to have vasodilating and renal protective effects by inhibition of oxidative stress.53,54 Studies about the safety of sodium thiosulfate in pregnancy are lacking.

Finally, lifestyle interventions with regard to H2S bioavailability might be an interesting approach in the prevention of PE. It is likely that a high dietary intake of sulfur-containing amino acids contribute to an increase in endogenous H2S synthesis, which might favorably influence the renal and cardiovascular profile.55 In accordance with the importance of dietary intake, it has recently been shown that H2S levels were reduced in germ-free mice.56 This study indicated that dietary intake of sulfate or sulfur-containing amino acids can be a rich source for endogenous H2S production by various species of sulfate-reducing bacteria in the gut. Diets containing high levels of sulfur-containing amino-acids (SAA) might therefore be beneficial in (the prevention of) PE through increments in systemic H2S. Although sulfate, the end-product of SAA may contribute to the metabolic acid load and thereby adversely influence acid-base homeostasis, we previously found no significant association of sulfate with blood pH and HCO3− in renal transplant recipients.55

In conclusion, this thesis provides evidence that H2S may be a novel factor involved in the pathophysiology of PE. H2S therapy has the potential to target the pathogenesis and clinical syndrome of PE on several levels, such as placentation, the antiangiogenic balance and endothelial protection. We believe that gaining more insights in the functional role of H2S during pregnancy and PE would significantly lead to a better understanding of the multifactorial disease PE. Perhaps more important, these insights will contribute to the
development of new mechanism-based therapies for PE, which are currently not available. Other placenta-related diseases, such as fetal growth restriction, might benefit as well.

**Figure 1 - The pathogenesis of preeclampsia**

Effects of H$_2$S are depicted in the blue boxes. Arrow-headed lines represent activation / up regulation, and bar-headed lines inhibition / down regulation. CBS, cystathionine-β-synthase; FGR, fetal growth restriction; ang II, angiotensin II; sFlt1, soluble FMS-like tyrosine kinase 1.
References


