Summary, discussion and future perspectives
Hydrogen sulfide (H$_2$S), formerly regarded as a toxic gas, is now recognized as a gas also endogenously produced in mammals in low concentrations with beneficial properties. With its pro-angiogenic, vasodilating, anti-inflammatory, and antioxidant functions, H$_2$S plays a considerable role in human (patho-)physiology. In this thesis, we therefore investigated the role of H$_2$S in cardiovascular disease and diabetes-associated vascular disease.

**Therapeutic role of H$_2$S in blood pressure regulation and cardiovascular disease**

The involvement of both exogenous and endogenous H$_2$S in blood pressure regulation is demonstrated in this thesis. In a mouse model of cardiac ischemia/reperfusion (IR) injury, administration of gaseous H$_2$S in a concentration of 100 parts per million (ppm) lowered blood pressure significantly, as shown in chapter 5. Additionally, higher urinary levels of the H$_2$S metabolite sulfate was associated with a lower blood pressure in healthy subjects (chapter 3) and in patients with type 2 diabetes (chapter 7), suggesting that also in humans endogenously produced H$_2$S is involved in blood pressure regulation. These two findings - the association of lower bioavailability of endogenous H$_2$S with higher blood pressure, and the blood pressure lowering effects of exogenous H$_2$S administration - opens up H$_2$S-based treatment possibilities in the course of hypertension.

Although the exact mechanism of H$_2$S in blood pressure regulation is not known, evidence is accumulating on the vasodilatory effects in H$_2$S. H$_2$S is capable of regulating vascular tone in several ways. The involvement of H$_2$S in vasodilation is one of the first described functions of H$_2$S in mammalian physiology. As comprehensively discussed in chapter 2, at least five mechanisms are known by which H$_2$S plays a role in blood pressure regulation. First, H$_2$S facilitates NO-mediated vasodilation through directly influencing eNOS activity by increasing serine 1177 phosphorylation (p-eNOS$^{S1177}$). Second, H$_2$S opens adenosine triphosphate (ATP)-sensitive potassium (K$_{ATP}$) channels. Through cysteine-S-sulhydration of K$_{ATP}$ channels and subsequent hyperpolarization of the cell membrane, L-type Ca$^{2+}$ channels are inactivated, leading to vascular smooth muscle cell relaxation and blood vessel dilation. Third, H$_2$S can have sympatoinhibitory effects in the rostral ventrolateral medulla. Local H$_2$S production stimulates K$_{ATP}$ channels that inhibit renal sympathetic nerve activity, causing vasodilation. Fourth, H$_2$S activates protein kinase
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G1alpha, with vascular relaxation as a consequence. Lastly, it was recently suggested that the soluble guanylyl cyclase/cyclic GMP (sGC/cGMP) pathway is also involved in \( \text{H}_2\text{S} \)-related vasodilation. \( \text{H}_2\text{S} \) acts as endogenous inhibitor of phosphodiesterase-5 (PDE-5), thereby decreasing the breakdown of cGMP. Based on results of our own group, \( K_{\text{ATP}} \) channel-mediated blood pressure effects only accounts for a small proportion of the blood pressure-lowering effects of \( \text{H}_2\text{S} \). Inhibition of \( K_{\text{ATP}} \) channels by glibenclamide resulted in less than 30% reduction of the blood pressure-lowering effects of \( \text{H}_2\text{S} \) in \textit{ex vivo} perfused rat kidneys. This indicates that the other mechanisms account for the major part of \( \text{H}_2\text{S} \)-mediated effects on blood pressure, most likely via NO-dependent vasodilatory mechanisms.

However, although being a major function of \( \text{H}_2\text{S} \), induction of vasodilation can not be held fully accountable for all the cardiovascular protective effects of \( \text{H}_2\text{S} \). In chapter 5, we demonstrated that administration of low dose gaseous \( \text{H}_2\text{S} \) (10 ppm) in mice is - at least on the long term - as effective as a high dose (100 ppm) of \( \text{H}_2\text{S} \), without effects on blood pressure. This interesting finding indicates that even normotensive patients could benefit from \( \text{H}_2\text{S} \)-treatment in the course of cardiovascular disease. It is earlier demonstrated that \( \text{H}_2\text{S} \) is an endogenous antioxidant and inhibitor of inflammatory responses. In line with this, we were able to demonstrate that the antioxidant and anti-inflammatory effects of \( \text{H}_2\text{S} \) could be responsible for these blood pressure-independent beneficial effects. Exogenous treatment with \( \text{H}_2\text{S} \) (both high and low dose) significantly lowered mRNA expression of the ROS-related genes nicotinamide adenine dinucleotide phosphate oxidase 2 and 4 (NOX2 and NOX4). Moreover, we showed that addition of \( \text{H}_2\text{S} \) donor NaHS \textit{in vitro} resulted in reduced antimycin A-induced ROS production over time in H9c2 cardiomyocytes. These findings underline the importance of ROS-related damage in cardiovascular disease, and ROS reduction as a therapeutic strategy.

In chapter 5, we additionally demonstrated that administration of gaseous \( \text{H}_2\text{S} \) results in reduced influx of granulocytes after one day of reperfusion of the ischemic heart, which emphasizes the important role of \( \text{H}_2\text{S} \) in dampening the inflammatory response. In accordance with these anti-inflammatory effects of \( \text{H}_2\text{S} \), we showed in chapter 3 an inverse association between urinary excretion of \( \text{H}_2\text{S} \) metabolite sulfate and serum high sensitive C-reactive protein (hs-CRP) levels at baseline in the PREVEND study, a cohort based on the general population. We additionally demonstrated that excretion of sulfate is inversely associated with all-cause mortality and risk of cardiovascular disease. However, sulfate lost its predictive properties for risk of cardiovascular disease.
after adjustment for hs-CRP, indicating that the inflammatory pathway accounts for the beneficial effects of sulfate. CRP has earlier proven to be an independent risk factor of cardiovascular disease. With this in mind, it could well be that higher endogenous levels of H$_2$S slow down the progress of atherosclerosis by dampening the systemic and/or local inflammatory response, resulting in a reduced risk of cardiovascular events.

Systemic atherosclerosis is often the underlying cause of cardiovascular disease, and the result of a complex interplay between different factors such as high blood pressure, endothelial dysfunction, inflammation within the vessel wall, and the formation of reactive oxygen species (ROS). As discussed in the previous section, the role of H$_2$S in blood pressure regulation, inflammation, and ROS scavenging, is demonstrated in this thesis as well as by others. Moreover, considering the beneficial effects of antihypertensives and antioxidants in cardiovascular disease, H$_2$S could serve well as therapeutic agent in the development of atherosclerosis. In support of this, H$_2$S was indeed shown to protect from endothelial dysfunction and development of atherosclerosis in experimental models.

However, even though H$_2$S exerts various beneficial vasculoprotective effects, the pro-angiogenic effects might be a downside of H$_2$S in cardiovascular disease progression. The formation of newly-formed microvessels within an atherosclerotic plaque is associated with a more vulnerable (i.e. rupture prone) phenotype, with subsequent increased risk of stroke or infarction. In chapter 4, we have demonstrated that H$_2$S is expected to be locally produced in atherosclerotic plaque microvessels, as the H$_2$S-producing enzyme CSE is expressed within intraplaque microvascular endothelial cells. Moreover, CSE inhibition in human microvascular endothelial cells in vitro drastically reduced micro-angiogenesis. These data implicate that H$_2$S-based therapy might be potentially hampered by its pro-angiogenic effects; atherosclerotic plaques could even benefit from local inhibition of H$_2$S production. By allowing stable atherosclerotic plaques to form (more) intraplaque microvessels, H$_2$S-stimulated angiogenesis could increase patient risk of plaque rupture with subsequent stroke or infarction. The proposed association between H$_2$S-induced intraplaque angiogenesis and plaque vulnerability seems in contrast with the earlier described beneficial effects of H$_2$S in atherosclerosis and cardiovascular disease. However, this contradiction can be explained by different stages of development of atherosclerotic plaques. We propose that H$_2$S might be protective in the early phase of atherosclerosis by e.g. attenuating endothelial dysfunction, inhibiting ROS, and reducing inflammation. However during plaque progression H$_2$S might actually promote plaque vulnerability of
advanced plaques due to its pro-angiogenic effects. We should be aware of this potential risk when considering H$_2$S as treatment modality for cardiovascular disease.

**The role of H$_2$S in diabetes and its associated vascular disease**

In this thesis, we measured urinary sulfate levels as a proxy for endogenous H$_2$S-metabolism. We demonstrated lower urinary sulfate levels in patients with type 2 diabetes and nephropathy (Sun-MACRO cohort, chapter 7), compared to non-diabetic subjects from the PREVEND cohort (chapter 3). Urinary sulfate concentrations were ~22% lower in diabetic patients with nephropathy compared to healthy subjects. Additionally, urinary sulfate concentrations were ~19% higher in patients with type 2 diabetes without nephropathy compared to patients with type 2 diabetes with diabetic nephropathy. These sulfate measurements were performed sequentially. For this variable measurement (inter-assay variation of 9.3%), it is important to measure sulfate consecutively to make a reliable comparison. Taken all the beneficial cardiovascular effects of H$_2$S into account, this lower bioavailability of H$_2$S could partly explain the high cardiovascular risk of patients with diabetes and renal disease.

The role of H$_2$S in the development of diabetes is not fully understood, and present literature on this topic contains contradictory results. However, in line with our observations, in different diabetes animal models, lower bioavailability of H$_2$S and H$_2$S-producing enzymes is observed.$^{29-31}$ Moreover, in human studies, lower plasma H$_2$S levels were found in patients with type 2 diabetes compared to healthy individuals.$^{30,32}$ Interestingly, in non-diabetic subjects, obesity was associated with lower levels of plasma H$_2$S. This suggests that lower H$_2$S bioavailability is already present at a pre-diabetic stage and predisposes for the development of diabetes. Additionally, exogenous administration of H$_2$S donor NaHS resulted in improved vascular relaxation and reduced ROS production in STZ-induced diabetic mice.$^{33}$ On the contrary, CSE deficiency in mice delayed the onset of streptozotocin (STZ)-induced type 1 diabetes,$^{34}$ and showed lower plasma glucose levels as a result of reduced gluconeogenesis.$^{35,36}$ Additionally, H$_2$S treatment inhibits insulin secretion from pancreatic beta-cells *in vitro*.$^{37-39}$ However, in a high-fat diet-induced model of type 2 diabetes in 6 months old mice, CSE deficiency is reported to have a deleterious effect on the development of diabetes.$^{40}$ In this study, CSE
deficiency is accompanied by lower insulin sensitivity, higher glucose levels and lower insulin levels. The latter results are more in line with the lower H₂S bioavailability in subjects with obesity or patients with type 2 diabetes, compared to healthy controls. These contradictive results of H₂S in the development of diabetes in the experimental setting seem to be related to the age of the mice and the model of diabetes. The high-fat diet-induced diabetes has more pronounced insulin resistance and STZ-induced diabetes is characterized by beta cell destruction and subsequent insulin deficiency.

In chapter 8, we studied the renal expression of H₂S-producing enzymes after STZ-induced diabetes in mice. In kidneys from these diabetic mice, CSE and especially CBS protein expression were downregulated compared to healthy control mice. CBS expression was decreased with 82% in 6-weeks diabetic mice, compared to healthy controls. To study the progression or reversibility of diabetic renal damage, transplantation of isogenic pancreatic islets of non-diabetic mice was performed 6 weeks after STZ-induced diabetes. Glucose and body weight were normalized after islet transplantation. Diabetic renal damage was not reversed but rather stabilized i.e., it did not progress during the additional 6 weeks follow-up after islet transplantation and restoration of normoglycemia. Interestingly, the change in renal CBS expression was restored to 64% of the CBS expression observed in healthy control animals. This is in line with a previous report, where insulin treatment also attenuated diabetic changes in CSE and CBS expression. These data indicate that expression of CBS is dependent on glucose or insulin levels, and could also partly explain lower H₂S bioavailability in patients with diabetes.

The presence of diabetes is associated with the development of different vascular complications. Diabetic vascular complications are divided into microvascular and macrovascular complications. Microvascular complications comprise of diabetic retinopathy, neuropathy and nephropathy, and macrovascular complications consist of cerebrovascular-, coronary artery- and peripheral arterial disease. The role of H₂S, and the other gasotransmitters NO and CO, in the course of diabetic vascular complications is extensively reviewed in chapter 6. Moreover, the therapeutic potential of H₂S in diabetic vascular disease is discussed. The beneficial effects of H₂S as therapeutic compound in diabetic macrovascular disease has already been shown in experimental models of peripheral vascular disease and myocardial ischemia/reperfusion injury. H₂S therapy in the course of microvascular complications of diabetes results in both attenuating and aggravating effects, dependent on the complication. In STZ-induced diabetic retinopathy, heterozygous CBS deficiency resulted in increased loss of retinal
ganglion cells,\textsuperscript{48} which is a hallmark of diabetic retinopathy. Exogenous treatment with NaHS in STZ-induced diabetic rats prevented diabetes-induced vascular injury in the eye.\textsuperscript{49} In diabetic neuropathy, \( \text{H}_2\text{S} \) treatment with either \( \text{H}_2\text{S} \) donor NaHS or \( \text{H}_2\text{S} \) substrate L-cysteine resulted in increased neuropathic pain symptoms in STZ-induced diabetic rats.\textsuperscript{50} Inhibition of endogenous \( \text{H}_2\text{S} \) formation by CSE or CBS inhibitors resulted in reduced neuropathic pain symptoms in these rats.\textsuperscript{51,52} In STZ-induced diabetic rats, treatment of NaHS improved renal function and diminished renal injury, inflammation, and ROS.\textsuperscript{53,54} Based on these experimental data, \( \text{H}_2\text{S} \)-based therapy is promising in diabetic vascular disease, but with a risk of aggravating diabetic neuropathy.

Lower \( \text{H}_2\text{S} \) bioavailability in diabetic patients is described, and the beneficial effects of \( \text{H}_2\text{S} \) supplementation in experimental renal disease is also known. However, the influence of endogenous \( \text{H}_2\text{S} \) bioavailability on renal disease in patients with type 2 diabetes is unknown. In chapter 7, we determined the predictive properties of urinary concentrations of \( \text{H}_2\text{S} \) metabolite sulfate for progression of diabetic nephropathy. Urinary sulfate concentration was strongly associated with eGFR and albuminuria at baseline in these patients with type 2 diabetes. Moreover, urinary sulfate is inversely correlated with the risk of renal events in patients with diabetic nephropathy, independent of potential confounders (\textit{i.e.} gender, age, eGFR and albuminuria). Our findings are consistent with the results of others, showing that in patients with type 1 diabetes and nephropathy, high levels of urinary sulfate, as a proxy for \( \text{H}_2\text{S} \) levels, are associated with a slower decline in GFR.\textsuperscript{55} Additionally, in patients with diabetic nephropathy who are on dialysis, lower plasma levels of \( \text{H}_2\text{S} \) were measured compared to non-diabetic patients who were on dialysis or compared to healthy control subjects.\textsuperscript{56} Although these association studies do not prove causality, these data suggest that patients with low endogenous \( \text{H}_2\text{S} \) levels and accompanied high risk of renal events, could benefit from \( \text{H}_2\text{S} \)-based therapy.

**Future perspectives**

Based on the work described in this thesis, combined with recent literature, \( \text{H}_2\text{S} \) could serve well as a therapeutic in the course of cardiovascular disease. Endogenous bioavailability seems to be lowered in patients with cardiovascular disease, and probably even before that stage. However, as yet it is unclear whether this lower bioavailability precedes cardiovascular disease, or results from endothelial dysfunction (\textit{i.e.} reduced \( \text{H}_2\text{S} \)-
production from damaged endothelial cells). Functional in vitro and in vivo experiments with induction of endothelial dysfunction and measurement of CSE and CBS might provide more insight into the role of \( \text{H}_2\text{S} \) bioavailability in cardiovascular disease. Additional \( \text{H}_2\text{S} \)-based therapy in in vivo experiments could answer the question whether patients with lower \( \text{H}_2\text{S} \) bioavailability might benefit from restoration of their \( \text{H}_2\text{S} \) levels.

Additionally, the role of \( \text{H}_2\text{S} \) in diabetic vascular complications should be researched more rigorously. It would be worthwhile to study the expression levels of \( \text{H}_2\text{S} \)-producing enzymes in different organs of a diabetic animal showing most of the features of diabetic vascular complications. Moreover, administrating \( \text{H}_2\text{S} \) in these mice would allow us to analyze the effects of \( \text{H}_2\text{S} \)-based therapy on different vascular complications simultaneously, and consider all the pros and cons of \( \text{H}_2\text{S} \) supplementation in a single diabetic animal.

Exogenous treatment with \( \text{H}_2\text{S} \), or \( \text{H}_2\text{S} \) donors, is beneficial in most experimental models of cardiovascular disease. In humans, \( \text{H}_2\text{S} \) treatment is currently not yet available, although lots of effort is invested in the development of \( \text{H}_2\text{S} \)-releasing drugs. Especially in the field of anti-inflammatory medicine, various \( \text{H}_2\text{S} \)-releasing non-steroidal anti-inflammatory drugs (S-NSAIDs) are being developed.\(^{57,58}\) Moreover, according to ClinicalTrials.gov (accessed: January 26, 2016), nine trials related to \( \text{H}_2\text{S} \), both observational and intervention trials, are currently undertaken.

However, the therapeutic properties of \( \text{H}_2\text{S} \) itself are hampered by some serious downsides. On the first place, the therapeutic window is quite small. Overdosage of \( \text{H}_2\text{S} \) would be rather dangerous, since \( \text{H}_2\text{S} \) is toxic in high concentrations. And besides the possible beneficial effects of \( \text{H}_2\text{S} \), it also has some potential side effects other than toxicity. As accounts for many antihypertensive drugs, the vasodilatory effects of \( \text{H}_2\text{S} \) can result in (orthostatic) hypotension. Second, in experimental models, \( \text{H}_2\text{S} \)-based therapy increased neuropathic pain symptoms. Third, the pro-angiogenic function of \( \text{H}_2\text{S} \) can have some detrimental effects in patients with cancer or atherosclerosis.\(^{59}\) It has been shown that short hairpin RNA (shRNA)-mediated gene silencing of CBS on tumor xenograft drastically inhibited tumor volume, tumor growth and tumor neovascularization.\(^{60}\) Additionally, \( \text{H}_2\text{S} \) therapy, with subsequent induction of angiogenesis could backfire as a treatment for cardiovascular disease by stimulating atherosclerotic plaque vulnerability that had initially caused the patients’ stroke or myocardial infarction.

Therefore, it would be better to be on the safe side when considering \( \text{H}_2\text{S} \)-related therapies. Thiosulfate would serve well as \( \text{H}_2\text{S} \) donor, and has already been proven to be safe. Thiosulfate can act as an \( \text{H}_2\text{S} \)-donor via (glutathione-dependent) thiosulfate
reductase activity, where 2 molecules of glutathione (GSH) together with thiosulfate ($S_2O_3^{2-}$) is reduced to glutathione disulfide (GSSG), sulfite ($SO_3^{2-}$) and $H_2S$ (chemical reaction $2GSH + S_2O_3^{2-} \rightarrow GSSG + SO_3^{2-} + H_2S$).\(^{[61]}\) Thiosulfate is in clinical use for cyanide poisoning, where it forms the less harmful thiocyanate in the reaction $CN^- + S_2O_3^{2-} \rightarrow SCN^- + SO_3^{2-}$.
\(^{[62]}\) Additionally, thiosulfate is used for the treatment of calcific uremic arteriolopathy, also known as calciphylaxis.\(^{[63]}\) The exact working mechanism of thiosulfate is unknown, but vasodilation, calcium chelating properties, and induction of acidosis are proposed to contribute to its protective effects in vascular calcification.\(^{[64]}\) Additionally, diet can play a major role in $H_2S$ bioavailability. First, the intake of sulfur-containing amino acids such as cysteine or methionine contributes to formation of $H_2S$.\(^{[65]}\) The endogenous production of $H_2S$ by CSE, CBS and 3MST is dependent on its substrates methionine, homocysteine, and cysteine. Second, several fruits and vegetables have been identified as dietary organopolysulfides, serving as $H_2S$ donors.\(^{[66]}\) Especially broccoli, cauliflower, onions, garlic and leek are vegetables with a high content of organosulfides. The tropical fruit durian (Durio zibethinus) is the only fruit containing lots of organosulfide content. Durian is a highly popular exotic fruit in Southeast Asia due to its distinct taste, texture, and sulfurous odor. Ironically, the latter characteristic is the reason why this fruit is prohibited in public transport and most hotels in Asia. At last, $H_2S$ is also generated by various species of sulfate-reducing bacteria in the gut. Germ-free mice showed significant lower levels of $H_2S$,\(^{[67]}\) indicating that addition of dietary sulfate or sulfur-containing amino acids can act as natural $H_2S$ donors. Moreover, the gut microbiome has earlier shown its important role in blood pressure regulation\(^{[68]}\) and lipid metabolism.\(^{[69]}\)

The current intensive and conventional treatment to reduce cardiovascular risk still leaves space for improvement, indicating that novel avenues for reduction of cardiovascular risk have to be explored. This holds true for especially those patients who are relatively resistant to conventional treatment. As described in this thesis, $H_2S$-based therapies are highly promising, but with still some hurdles to be taken. The combination of clinical (observational) studies and experimental (intervention) studies will speed up to process to future clinical use of $H_2S$-based therapy.
References


Chapter 9


