General introduction and aim of the thesis
Cardiovascular disease

Cardiovascular disease is the leading global cause of death, and its prevalence is still increasing.\(^1\) Hypertension is an important contributor to the induction of cardiovascular events. Additionally, patients with metabolic syndrome (i.e. obesity, hypertension, type 2 diabetes and high cholesterol),\(^2\,3\) type 1 diabetes, or patients with (diabetic) chronic kidney disease\(^4\,5\) have an increased risk for developing cardiovascular diseases.

The underlying cause of cardiovascular disease is often atherosclerosis, a systemic vascular disease. Development of atherosclerosis is a process still not fully understood. However, arterial stiffness and calcification, neo-intima formation, smooth muscle cell proliferation and migration, and inflammation are important features.\(^6\) Atherosclerotic plaques are classically divided into stable or vulnerable (also referred to as non-stable). Stable plaques contain a lot of connective tissue and smooth muscle cells, collectively responsible for the formation of the so called fibrous cap. Stable plaques mainly cause luminal narrowing, whereas vulnerable plaques are prone to rupture with risk of stroke or infarction.\(^7\) A large lipid/necrotic core, a thin fibrous cap, a high density of microvessels, a large amount of inflammatory cells, and intraplaque hemorrhage are associated with increased atherosclerotic plaque vulnerability (Figure 1).\(^8\,9\)

**Figure 1** - Schematic overview of a stable and a vulnerable plaque.
Diabetes

Diabetes mellitus is characterized by insulin resistance (type 2) or insulin deficiency (type 1) both resulting in high blood glucose levels (hyperglycaemia). Type 1 diabetes is an autoimmune disease in which destruction of pancreatic beta cells has taken place. Beta cells are responsible for insulin production, hence type 1 diabetes results in insulin deficiency with concomitant hyperglycaemia. Insulin therapy is the cornerstone of treatment for type 1 diabetic patients. Type 1 diabetes accounts for up to 10% of all diabetes cases;\(^\text{10}\) the vast majority (90%) of diabetes patients suffer from type 2 diabetes. Type 2 diabetes is a metabolic disorder and lacks the autoimmune component. Its pathogenesis is related to sedentary lifestyle (overweight and obesity) and other lifestyle factors such as smoking. In type 2 diabetes, there is a relative insulin deficiency which is the result of insulin resistance. Increasing insulin sensitivity with dietary and lifestyle interventions and oral medication (e.g. metformin, sulfonylureas, gliptins [dipeptidyl peptidase 4 inhibitors]) forms the main goal of therapy for type 2 diabetes. When oral antidiabetic drugs are not sufficiently effective in lowering blood glucose levels, subcutaneous insulin treatment is considered.\(^\text{11}\)

Diabetes is within the top 10 of global causes of death; its prevalence has reached epidemic proportions and is currently estimated to be 9% among adults.\(^\text{1,12}\) Both type 1 - and type 2 diabetes are important risk factors for cardiovascular diseases, with a 2- to 4-fold increased risk when compared to non-diabetic individuals.\(^\text{13}\) Although the pathophysiology of type 1 - and type 2 diabetes is different, the underlying mechanism leading to vascular complications seems to be similar, and is thought to be related to and incited by endothelial dysfunction\(^\text{14,15}\) and the formation of reactive oxygen species (ROS).\(^\text{16}\)

**Diabetes-associated vascular disease**

Diabetes-associated vascular complications are divided into microvascular and macrovascular complications (cerebrovascular-, coronary artery- and peripheral arterial disease). Microvascular complications comprise of retinopathy, neuropathy and nephropathy. Diabetic retinopathy is the main cause of blindness in adults. The prevalence among diabetic patients worldwide is approximately 35%.\(^\text{17}\) Diabetic neuropathy is defined as a disturbance of function in peripheral nerves. Diabetic peripheral polyneuropathy affects up to 50% of all diabetes patients.\(^\text{18}\) Diabetic nephropathy is the major cause of end stage renal disease in the Western world, occurring in ~30% of both type 1 and type 2 diabetes patients, and accounts for about 40% of new cases of end stage renal disease.\(^\text{19}\)
Gasotransmitters

Gasotransmitters are small signaling molecules which are endogenously produced. Until now, three gasotransmitters have been identified, i.e. nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H\textsubscript{2}S).

NO was recognized as an endothelium-derived relaxing factor more than 25 years ago.\textsuperscript{20} It is endogenously produced by different nitric oxide synthase enzymes (nNOS, eNOS, and iNOS) using L-arginine as substrate. NO acts as vasodilator, stabilizes atherosclerotic plaques, and inhibits platelet aggregation.\textsuperscript{21} Interventions, based on NO-release, are already applied in humans. Anti-anginal organic nitrates like nitroglycerin or isosorbide dinitrate function as NO-donors,\textsuperscript{22} and non enzymatic NO-donors Molsidomine and Linsidomine are registered in several European countries and used as maintenance therapy in case of angina pectoris.

The second identified gasotransmitter is CO which is produced by the different heme oxygenase (HO) enzymes as a product of heme metabolism.\textsuperscript{23,24} CO has numerous physiological functions, among others vasodilatation and inhibition of platelet aggregation. CO-releasing molecules (CORMs) are not clinically used. However, some preliminary data suggest that the vasculoprotective effects of acetylsalicylic acid and statins are associated with an induction of endogenous CO production (i.e. induction of HO-1).\textsuperscript{25,26}

Besides NO and CO, a third gasotransmitter was discovered by Abe and Kimura in the nineties; hydrogen sulfide (H\textsubscript{2}S).\textsuperscript{27,28} Because of its beneficial effects in experimental cardiovascular disease (see below) and the possibilities for interventions, our interest goes to this most recently discovered gasotransmitter.

Hydrogen sulfide

H\textsubscript{2}S is known for a long time because of its pungent smell and toxicity. However, H\textsubscript{2}S was recently discovered as an endogenously produced gaseous signaling molecule. H\textsubscript{2}S production takes place via three different enzymes. The pyridoxal-5\textsuperscript{-}-phosphate dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are the major H\textsubscript{2}S producing enzymes. 3-mercaptopyruvate sulfurtransferase (3MST) is the third H\textsubscript{2}S producing enzyme which uses 3-mercaptopyruvate (3MP) as substrate. 3MP is derived from L-cysteine by cysteine aminotransferase (CAT), or D-cysteine by D-amino acid oxidase.\textsuperscript{29} H\textsubscript{2}S is oxidized into thiosulfate, which is further metabolized into sulfite and
eventually oxidized by sulfite oxidase (SO) into the metabolic end product sulfate (Figure 2). Thiosulfate itself can be reused for the formation of H$_2$S through enzymatic reduction by thiosulfate reductase (TSR, also referred to as thiosulfate:thiol sulfurtransferase).\textsuperscript{30}

H$_2$S is physiologically very active. It is a regulator of blood pressure by causing vasodilatation via several mechanisms.\textsuperscript{31,32} By direct interference with the mitochondrial respiratory chain, H$_2$S is able to inhibit the formation of reactive oxygen species (ROS). H$_2$S furthermore exerts anti-inflammatory properties,\textsuperscript{33} and is an endogenous stimulator of angiogenesis.\textsuperscript{34}

![Figure 2 - Schematic representation of the H$_2$S production of oxidation pathway 3MST, 3-mercaptopyruvate sulfurtransferase; CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DAO, D-amino acid oxidase; SO, sulfite oxidase; TSR, thiosulfate reductase.](image-url)
Hydrogen sulfide in the course of (diabetes-associated) cardiovascular disease

In mice, CSE deficiency with subsequently lowered H$_2$S levels is accompanied by hypertension, hyperhomocysteinemia, and impaired vasorelaxation.\textsuperscript{35} Exogenous administration of H$_2$S, or H$_2$S donors, has shown protective properties in cardiac injury,\textsuperscript{36-38} stroke,\textsuperscript{39} and atherosclerosis.\textsuperscript{40-43} H$_2$S has shown to be an important factor in blood pressure regulation, and parallel to its blood pressure lowering effects, H$_2$S attenuates organ damage. In humans, the role of H$_2$S in cardiovascular disease is barely investigated and therefore not fully understood. The expression of H$_2$S producing enzymes has been evaluated in various human diseases, however, not in human atherosclerosis. Moreover, as an endogenous pro-angiogenic substance, the production of H$_2$S within an atherosclerotic plaque could contribute to plaque vulnerability by promotion of intraplaque angiogenesis. Additionally, the long-term effects of H$_2$S in cardiovascular disease have yet to be explored. Only in a few human cohorts, H$_2$S, or H$_2$S metabolites, are measured without follow-up on cardiovascular outcomes.

In various animal models of diabetes, lower bioavailability of H$_2$S and H$_2$S-producing enzymes is observed.\textsuperscript{44-46} This finding was confirmed in human studies, where lower plasma H$_2$S levels were measured in patients with type 2 diabetes compared to healthy individuals.\textsuperscript{46,47} On the contrary, CSE deficiency in mice delayed the onset of streptozotocin (STZ)-induced type 1 diabetes,\textsuperscript{48} and showed lower plasma glucose levels as a result of reduced gluconeogenesis.\textsuperscript{49} In various models of diabetic nephropathy\textsuperscript{44,50,51} and retinopathy\textsuperscript{52}, exogenous administration of H$_2$S seems to be protective. However, in experimental diabetic neuropathy, H$_2$S treatment is associated with an increase of neuropathic pain symptoms.\textsuperscript{53,54} Human data is scarce in the field of H$_2$S-related diabetes research, especially longitudinal studies are lacking.

All in all, the role of H$_2$S in cardiovascular disease is interesting and promising, but human data are scarce and a lot needs to be investigated. Expression levels of H$_2$S producing enzymes in atherosclerotic plaques, with concomitant plaque vulnerability risk, is yet unknown. Additionally, whether lower H$_2$S bioavailability predisposes for cardiovascular events, or results from is a reduced cardiovascular health status is unclear either. The latter is cardinal in determination whether the prevention of cardiovascular disease could benefit H$_2$S therapy.
Aim of the thesis

The aims of the thesis are to investigate the role of hydrogen sulfide ($H_2S$) in the development and progression of cardiovascular disease (part 1), and the role of $H_2S$ in the course of diabetes-associated microvascular complications (part 2). To elucidate this, we studied $H_2S$ metabolites and $H_2S$-producing enzymes in different (patient) cohorts with cardiovascular diseases. Experimental animal studies were performed to further explore the therapeutic effect of exogenously administered $H_2S$ in cardiovascular diseases, and to determine the effects of hyperglycaemia on endogenous renal $H_2S$ production in a model of STZ-induced diabetes.

Part 1: The role of $H_2S$ in the development of cardiovascular disease

The role of blood pressure regulation in cardiovascular risk management is evident.\textsuperscript{55} Chapter 2 describes the role of $H_2S$ in blood pressure regulation and subsequent effects on cardiovascular disease. This comprehensive review article provides an outline of $H_2S$ research, especially experimental studies performed in the context of hypertension and cardiovascular disease.

Ideally, we want to measure $H_2S$ levels in subjects to determine its predictive properties for all-cause mortality, cardiovascular and renal diseases. However, $H_2S$ is very volatile and therefore difficult to measure accurately. The currently available methods to measure $H_2S$ directly\textsuperscript{56} are not without controversy. $H_2S$ is metabolized into thiosulfate, sulfite and eventually sulfate,\textsuperscript{57-59} which are excreted via the urine. A previous study performed in renal transplant recipients showed that higher urinary sulfur metabolites are associated with a more beneficial cardiovascular risk profile and a reduced risk of all-cause mortality.\textsuperscript{60} However, association studies between urinary $H_2S$ metabolites and risk of cardiovascular disease and all-cause mortality in the general population have never been performed. Therefore, as described in chapter 3, we measured excretion of $H_2S$ metabolites sulfate and thiosulfate in a large cohort based on the general population, containing 6,855 subjects. In this study, we wanted to determine the predictive properties of $H_2S$ metabolites on survival and cardiovascular events in healthy individuals.

Exogenous $H_2S$ administration showed major beneficial effects in experimental models of cardiovascular disease.\textsuperscript{35,40} The stimulation of angiogenesis is one of the physiological effects of $H_2S$. However, this pro-angiogenic effect contributes to the possible downside of $H_2S$. Earlier, it has been shown that $H_2S$ could promote intra-
tumor angiogenesis and promote tumor survival.\textsuperscript{61} Within an atherosclerotic plaque, the formation of microvessels is associated with a more vulnerable phenotype. However, the expression of H\textsubscript{2}S producing enzymes in atherosclerotic plaques, and its role in micro-angiogenesis has not been elucidated. Therefore, to investigate the role of intraplaque endogenous H\textsubscript{2}S production in atherosclerosis, we obtained atherosclerotic plaques from 19 patients who underwent carotid endarterectomy in the UMCG. In \textit{chapter 4}, we investigated the expression pattern of CSE in these plaques. Next to that, we studied the role of CSE-derived H\textsubscript{2}S in micro-angiogenesis \textit{in vitro}, as a proxy for intra-plaque micro-angiogenesis.

The beneficial properties of exogenous H\textsubscript{2}S as a therapeutic agent in myocardial infarction were investigated in \textit{chapter 5}. Mice who underwent cardiac ischemia/reperfusion injury as a model for myocardial infarction, received gaseous H\textsubscript{2}S or normal breathing air. High concentrations of H\textsubscript{2}S are able to extremely decrease the metabolism of mice.\textsuperscript{62} To investigate whether the potential protective effects of H\textsubscript{2}S were determined by hypometabolism, H\textsubscript{2}S was tested at two concentrations: at hypometabolic (100 ppm) and non-hypometabolic (10 ppm) levels.

\textbf{Part 2: The role of H\textsubscript{2}S in diabetes and its vascular complications}

In \textit{chapter 6}, we review the role of the three different gasotransmitters (NO, CO and H\textsubscript{2}S) in the development of vascular complications in experimental diabetes models as well as in diabetic subjects. In this chapter, a comprehensive overview is given on gasotransmitters, their functions, their producing enzymes, and metabolites in the course of type 1 and type 2 diabetes. The role of the different gasotransmitters on the onset and progression of diabetes-related vascular complications is also extensively described.

As mentioned above, as a reflexion of H\textsubscript{2}S metabolism, urinary sulfur metabolites can be measured. The role of H\textsubscript{2}S in diabetic kidney disease is not fully understood, and especially in human patients with diabetes, large epidemiological studies are lacking. Urinary sulfate excretion has been associated with a reduction in renal failure in a small cohort of type 1 diabetes patients.\textsuperscript{63} However, since the vast majority of patients with diabetic nephropathy suffers from type 2 diabetes, studies on urinary sulfate excretion in type 2 diabetes are warranted. In \textit{chapter 7}, we therefore measured sulfate \textit{i.e.} the metabolic end product of H\textsubscript{2}S-metabolism) concentration in the urine of patients with type 2 diabetes, and investigated its predictive properties for renal events in patients with diabetic nephropathy.
The role of $\text{H}_2\text{S}$ in the development of diabetes and its vascular complications is not fully understood, and conflicting data have been reported. On one hand, exogenous $\text{H}_2\text{S}$ therapy is protective in diabetic vascular disease and diabetic renal disease. On the other hand, CSE deficient mice seem to be relatively resistant to STZ-induced diabetes, and showed lower glucose levels through reduced gluconeogenesis. To study the role and mechanisms of $\text{H}_2\text{S}$ production in kidneys of diabetic animals, we investigated expression of the $\text{H}_2\text{S}$-producing enzymes in streptozotocin (STZ)-induced diabetic mice in chapter 8. Next to that, we tested whether CSE, CBS or 3MST play a role in hyperglycemic memory. To this end, hyperglycaemia was reversed by transplantation of isogenic pancreatic islets of non-diabetic mice.

In chapter 9, the work described in this thesis is summarized and discussed. Additionally, future perspectives on $\text{H}_2\text{S}$-related research and the possibilities of $\text{H}_2\text{S}$-based interventions are provided.
References


PART 1

The role of $\text{H}_2\text{S}$ in the development of cardiovascular disease