Gasotransmitters in vascular complications of diabetes

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Abstract
In the last decades three gaseous signaling molecules - so called gasotransmitters - have been identified: nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H\textsubscript{2}S).

These gasotransmitters are endogenously produced by different enzymes in various cell types, and play an important role in physiology and disease. Despite their specific functions, all gasotransmitters share the capacity to reduce oxidative stress, induce angiogenesis, and promote vasorelaxation. In patients with diabetes, a lower bioavailability of the different gasotransmitters is observed when compared to healthy individuals. As yet it is unknown whether this reduction precedes, or results from diabetes. The increased risk for vascular disease in patients with diabetes in combination with the extensive clinical and financial societal burden, calls for action to either prevent or improve the treatment of vascular complications. In this Perspectives in Diabetes, we present a concise overview of the current data on the bioavailability of gasotransmitters in diabetes, and their potential role in the development and progression of diabetes-associated microvascular (retinopathy, neuropathy, nephropathy) and macrovascular (cerebrovascular, coronary artery and peripheral arterial disease) complications. Gasotransmitters appear to have both inhibitory and stimulatory effects in the course of vascular disease development. This Perspective concludes with a paragraph on gasotransmitter-based interventions as therapeutic option.
Diabetes and its complications

Diabetes mellitus is characterized by hyperglycemia and insulin resistance or deficiency. Diabetes is a top ten cause of death worldwide; its prevalence is increasing and currently estimated to be 9% among adults. Both type 1 - and type 2 diabetes are important risk factors for vascular diseases, with a 2- to 4-fold increased risk when compared to non-diabetic individuals. These vascular complications are divided into microvascular (retinopathy, neuropathy, nephropathy) and macrovascular (cerebrovascular-, coronary artery- and peripheral arterial disease) complications with respective clinical symptoms (Figure 1). Although the pathophysiology of type 1 - and type 2 diabetes is different, the proposed underlying mechanism leading to vascular complications seems to be similar and is thought to be related to endothelial dysfunction and the associated formation of reactive oxygen species (ROS). Chronic hyperglycemia promotes multiple biochemical pathways to overproduce ROS, either through mitochondrial overproduction or through enzymatic responses to high glucose.

Diabetic retinopathy is the main cause of blindness in adults. The worldwide prevalence is approximately 35% in patients with diabetes. The essentials of diabetic retinopathy can be best characterized by the combination of increased vessel permeability and progressive vascular occlusion. While the clinical diagnosis of retinopathy is still made by the changes in small (early) and larger (later) vessels, it has become clear that almost every cell type in the retina can be subject to damage by complex metabolic changes, induced by chronic hyperglycemia.

Polyneuropathy is defined as a diffuse and bilateral disturbance of functions or pathological changes in multiple peripheral nerves. Diabetic peripheral polyneuropathy is very frequent in the course of diabetes mellitus and even in pre-diabetes, affecting up to 50% of all diabetes patients. However, although being frequent and severe, it is inadequately treated in most patients – 77% of those with chronic painful peripheral neuropathy report persistent pain over 5 years. Experimental studies suggest the importance of neurovascular vasodilation in diabetic neuropathy, however, the mechanisms remain poorly understood which may explain the current lack of adequate treatment in (diabetic) neuropathic pain.

Diabetic nephropathy (DN) is the leading causes 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 based on US data. At the structural level the glomeruli are often affected as evidenced by basement membrane
thickening, mesangial lesions (Kimmelstein-Wilson lesions) and nodular sclerosis. Clinically, diabetic nephropathy is accompanied by proteinuria and chronic renal failure. In addition, arterioles are often affected as well. Mechanisms leading to renal changes include the metabolic defect, non-enzymatic glycation of proteins, and hemodynamic changes like hypertension leading to glomerular hypertrophy.\(^\text{18}\)

Macrovascular complications are characterized by the development of atherosclerosis in arteries throughout the body. Atherosclerosis results from a pro-inflammatory state starting with endothelial dysfunction and culminating in narrowing of the arterial lumen as a result from atherosclerotic plaque formation. As opposed to stable plaques, vulnerable, non-stable plaques are prone to rupture causing downstream ischemic events such as TIA and stroke (Figure 1).

**Figure 1** - Schematic overview of diabetes-associated microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (cerebrovascular-, coronary artery-, and peripheral arterial disease) complications and their clinical long-term manifestations. Abbreviation: TIA; transient ischemic attack.
Gasotransmitters in vascular complications of diabetes

Nitric oxide and microvascular complications of diabetes

NO was first recognized as an endothelium-derived relaxing factor (EDRF).\(^\text{19}\) It is endogenously formed from its substrate L-arginine by three different nitric oxide synthase (NOS) enzymes. Endothelial nitric oxide synthase (eNOS) is predominantly associated with vascular tone. Inducible nitric oxide synthase (iNOS), although also present in the vascular system, is mainly active in the immune system under conditions of oxidative stress. It functions as a promoter of inflammation. Neuronal nitric oxide synthase (nNOS), present in neurons and skeletal muscle cells, is important for neuronal cell-cell interactions.\(^\text{20}\) NO acts as vasodilator, inhibits platelet aggregation and stabilizes atherosclerotic plaques.\(^\text{21}\) In humans, NO-dependent vasodilatation is impaired in patients with type 2 diabetes, and lower eNOS expression and reduced NO production are the suggested underlying cause.\(^\text{22,23}\) Blockade of nitric oxide synthase causes insulin resistance in a rat model, indicating that in this model loss of NO synthesis precedes type 2 diabetes.\(^\text{24}\) In different animal models for diabetes, lower bioavailability of NO is observed. In spontaneous type 1 diabetic BioBreeding (BB) rats\(^\text{25}\) as well as streptozotocin (STZ)-induced type 1 diabetes in male Sprague–Dawley rats\(^\text{26}\) reduced NO production was found. Also in mouse models of diet-induced obesity and type 2 diabetes, NO bioavailability is reduced, leading to endothelial dysfunction and impaired NO-mediated vasodilatation.\(^\text{27,28}\) In contrast to these protective effects of NO, iNOS-produced NO seems to play an important role in inducing nitrosative stress and inflammation, also in the course of diabetes. Thus, NO seems to play a dual role in the development and progression of diabetes as well as in the development of vascular dysfunction.\(^\text{29}\)

Effects of NO depletion and supplementation on the development of microvascular complications have been primarily studied in experimental models as summarized in Table 1 and discussed below.

Retinopathy. In the retina iNOS is sensitive to hyperglycemia and responsible for overproduction of NO.\(^\text{30,31}\) The resulting surplus of NO is either quenched by advanced glycation end products (AGEs) or leads, through the reaction with superoxide, to the formation of peroxynitrite with subsequent nitrosylation of proteins, lipids, and DNA. NO production is important in inflammatory signaling, and inflammation is thought to be important in incipient diabetic retinopathy.\(^\text{32}\) Increased reactive nitrogen species (RNS)
has been observed in diabetic rat retinae and in vitro, and these changes were corrigible by aminoguanidine, an inhibitor of NO synthases.\textsuperscript{33} Being also an inhibitor of AGES, aminoguanidine reduced vascular cell damage in several animal models.\textsuperscript{33,34} Zheng et al. found that nitrosative stress was reduced in the retinae of iNOS\textsuperscript{-/-} mice, together with an inhibition of vasoregression and retinal thinning\textsuperscript{30}. However, the essential role of iNOS for development of diabetic retinopathy seems not to be the case for other NOS isoforms, since deletion of eNOS exacerbates diabetic retinopathy.\textsuperscript{35} In STZ-induced type 1 diabetes, eNOS\textsuperscript{-/-} mice developed more severe retinopathy compared to wildtype diabetic control mice. The worsened phenotype in these eNOS\textsuperscript{-/-} mice was accompanied by an increased iNOS expression, further suggesting an important role for iNOS in the development of diabetic retinopathy. However, eNOS\textsuperscript{-/-} mice suffer from higher blood pressure, so the worsened retinal phenotype can partly be explained by hypertensive injury. In essence, NO appears to have a dual role (i.e., protective and noxious effects) in the diabetic retina as schematically shown in Fig. 2a.

**Neuropathy.** Until now NO is the best characterized gasotransmitter contributing to nociception and pain. Its downstream targets within the peripheral nervous system (PNS) include cyclic guanosine monophosphate (cGMP) production by activation of soluble guanylyl cyclase (sGC), and phosphorylation of membrane receptors and channels by cGMP-dependent protein kinases;\textsuperscript{36} mechanisms usually associated with increased nociception. Consistently, different members of the large family of transient receptor potential channels, several of which are known as nociceptive sensor molecules like TRPV1 and TRPA1, are activated by NO via cysteine S-nitrosylation.\textsuperscript{37} In contrast, several mechanisms were identified that may induce anti-nociception and analgesia and increase efficacy of analgesic compounds. In the central nervous system (CNS), NO interacts with the descending inhibitory control mechanisms of nociception.\textsuperscript{38} In type 2 diabetes patients suffering from painful diabetic neuropathy, treatment with the NO donors glyceryl trinitrate\textsuperscript{39} and isosorbide dinitrate\textsuperscript{40} significantly improved pain symptoms, indicative of beneficial action of NO in diabetic neuropathy. Similar effects were observed when locally applying a NO-releasing cutaneous patch.\textsuperscript{41} These effects may however, also be indirectly explained by variations in local microcirculation: transient changes in sciatic nerve microcirculation were observed in response to NO in diabetic STZ-animals developing diabetic neuropathy.\textsuperscript{42} In STZ-induced diabetic rats, NOS activity is increased in primary sensory neurons.\textsuperscript{43} The potent oxidant peroxynitrite, a product of superoxide anion radical reaction with NO, was suggested to play a role in the induction of peripheral diabetic neuropathy and neuropathic
pain via induction of reactive nitrogen species (RNS)\(^{44}\) including protein nitrosylation, lipid peroxidation, DNA damage and cell death\(^{29}\). Hyperglycaemia activates iNOS, and therefore generally increased nitrosative stress in the PNS.\(^{44,45}\) Absence of iNOS reduced nitrosative stress in peripheral nerve fibers displaying normal nerve conduction velocities; diabetic neuropathy was also less severe in diabetic iNOS\(^{-/-}\) mice than in diabetic wildtype mice. Thus, diabetic neuropathy depends on nitrosative stress induced in axons and Schwann cells by NO produced from iNOS. In contrast, nNOS is required for maintaining PNS function and nerve fiber density, and contributes to a lesser extent to development of diabetic polyneuropathy.\(^{45}\) In summary, NO may play pivotal direct and indirect roles in the progression of diabetic neuropathy presumably via impairing microcirculation in PNS at pathophysiological levels, and contributing to oxidative stress and inflammation and tissue injury\(^{29}\) as schematically shown in Fig. 2b.

**Nephropathy.** There is still controversy regarding whether generation of NO is enhanced or decreased in DN. In the early stages of DN Chiarelli and coworkers\(^{46}\) found significantly higher concentrations of NO end-products (nitrite/nitrate) in serum of DN patients with microalbuminuria compared to healthy individuals. However, an association as such does not imply causality per se. This excess of NO can indicate an upregulated inflammatory response by iNOS, or a (protective) compensatory response against renal injury, mediated by eNOS. In experimental STZ-induced type 1 diabetes, renal NO production is decreased in the early phase of the disease.\(^{47}\) Deficiency of eNOS results in accelerated nephropathy in diabetic mice,\(^{48,49}\) also supporting a protective role for NO in DN.\(^{50}\) Supplementation of tetrahydrobiopterin (BH\(_4\)), a co-factor of NOS, reduced proteinuria and renal damage in type 2 diabetic rats.\(^{51}\) Taken together, NO production is clearly modulated in DN and decrements in its expression point to a contributing role for this gasotransmitter in DN. Scavenging of ROS positively influences the redox status, and may mechanistically underlie these findings. The modes of action of NO in the development of DN are schematically shown in Fig. 2c.

As discussed above, NO demonstrates both protective and damaging properties in the development of microvascular disease.

The producing enzyme seems to play a major role in the contrasting actions of NO: eNOS- and nNOS-derived NO exerts the vast majority of its positive effects via upregulation of the production of cGMP by activation of sGC. On the other hand, iNOS produced NO is involved in inflammatory signaling and is an important contributor to the development of diabetic angiopathy. Next to that, the presence of ROS is important in the actions of NO.
An excess of NO in the presence of abundant ROS (superoxide) production leads to the formation of peroxynitrite with subsequent nitrosylation of proteins, lipids, and DNA. Next to nitrosylation, there is increasing evidence for harmful effects of NO in protein tyrosine nitration.\textsuperscript{52} Protein nitration is a post-translational modification which takes place in combined presence of oxidative stress and NO, which is the case in disease conditions such as diabetes.\textsuperscript{53} Based on the data available, we conclude that NO plays a dual role in the progression and maintenance of diabetic microvascular complications, and which is mostly driven by the expression of its producing enzymes (NOS) and the presence of ROS.

**Table 1 - Effect of NO in diabetic microvascular disease**

<table>
<thead>
<tr>
<th>Model</th>
<th>Intervention</th>
<th>↑ / ↓</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Retinopathy</strong></td>
<td>Mouse – STZ-induced diabetes</td>
<td></td>
<td>L-NAME, iNOS\textsuperscript{−/−}</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduced diabetic leukostasis and BRB permeability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>eNOS\textsuperscript{−/−}</td>
<td>↓</td>
<td>Increased and accelerated retinopathy features</td>
<td>35</td>
</tr>
<tr>
<td>Rat – STZ-induced diabetes</td>
<td>Molsidomine</td>
<td>↑</td>
<td>Prevented diabetes-induced vascular injury</td>
<td>54</td>
</tr>
<tr>
<td><strong>Neuropathy</strong></td>
<td>Human – type 2 diabetes</td>
<td></td>
<td>NO donors (glyceryl trinitrate, isosorbide dinitrate)</td>
<td>39, 40</td>
</tr>
<tr>
<td></td>
<td>Mouse – STZ-induced diabetes</td>
<td></td>
<td>iNOS\textsuperscript{−/−}</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓</td>
<td>Improved nerve conduction velocities, less neuropathy</td>
<td></td>
</tr>
<tr>
<td><strong>Nephropathy</strong></td>
<td>Mouse – Lepr\textsuperscript{db/db}</td>
<td></td>
<td>eNOS\textsuperscript{−/−}</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Mouse – STZ-induced diabetes</td>
<td></td>
<td>eNOS\textsuperscript{−/−}</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓</td>
<td>Increased glomerular injury, proteinuria and renal insufficiency</td>
<td></td>
</tr>
<tr>
<td>Rat – OLETF</td>
<td>NOS cofactor BH\textsubscript{4}</td>
<td>↑</td>
<td>Reduced glomerular injury and proteinuria</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>L-NAME</td>
<td>↓</td>
<td>Increased glomerular injury, inflammation and proteinuria</td>
<td>50</td>
</tr>
</tbody>
</table>

↑ indicates increased NO; ↓ indicates reduced NO.

**Abbreviations**: BH\textsubscript{4}: tetrahydrobiopterin; BRB: blood-retinal barrier; eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; L-NAME: L-N\textsuperscript{6}-Nitroarginine methyl ester; NO: nitric oxide; OLETF: Otsuka Long Evans Tokushima Fatty; STZ: streptozotocin.
Carbon monoxide and microvascular complications of diabetes

The second gasotransmitter, carbon monoxide (CO) is produced by the different heme oxygenase (HO) enzymes as a product of heme metabolism. Heme is converted to biliverdin, iron and CO by heme oxygenase (HO). Three different isoforms of heme oxygenase exist; the inducible form heme oxygenase-1 (HO-1), and the constitutive isoforms HO-2 and HO-3. HO-1 and HO-2 are physiologically active, whereas the role of HO-3 in human physiology remains unclear. CO has numerous physiological functions, among others vasodilation and inhibition of platelet aggregation. In skeletal muscle biopsies and circulating leukocytes from patients with type 2 diabetes, mRNA expression of HO-1 was dramatically decreased compared to age-matched non-diabetic controls. In STZ-induced type 1 diabetic rats a decreased vasorelaxant function of CO was demonstrated, despite higher HO-1 expression levels. In Zucker diabetic fatty (ZDF) rats CO-production was decreased in aortic tissue compared to non-diabetic controls. Increasing HO-1 activity with cobalt protoporphyrin resulted in higher levels of CO, lower glucose levels and increased insulin sensitivity. These data are in favor of reduced vascular risk in the presence of higher CO levels, which might be mediated via effects on insulin sensitivity. Taken together, reduced bioavailability of CO in the diabetic state is accompanied by insulin resistance and a reduction of endothelial health, indicating a potential role for the HO-1/CO pathway in the development of diabetes and its associated complications.

Effects of CO depletion and supplementation in diabetic mice and rats on the development of microvascular complications are summarized in Table 2 and will be discussed below.

Retinopathy. Oxidative stress in the diabetic retina promotes the activation of heme oxygenases. In the diabetic retina, HO-1 is predominantly found in glial cells, in particular in Müller cells, and to some extent in the microvasculature. In vitro, HO-1 overexpression protects retinal endothelial cells from high glucose and oxidative/nitrosative stress. In a STZ-induced type 1 diabetes model in rats, HO-1 upregulation by hemin resulted in protection against the development of diabetic retinopathy. This protection is reflected by down-regulation of p53, VEGF, and HIF-1α and a reduction of diabetes-induced apoptosis in retinal ganglion cells (RGCs). On the contrary, HO-1-derived CO is pro-angiogenic, and angiogenesis, causing increased retinal blood flow, is a key factor in development of diabetic retinopathy. This implies that the pro-angiogenic effects of CO may actually aggravate diabetic retinopathy. The effects of CO in the diabetic retina are schematically shown in Fig. 2a.
**Neuropathy.** In the case of diabetic neuropathy, CO acts as a pain-modulating second messenger within the nervous system. Activation of HO/CO signaling reduced symptoms of neuropathic pain, presumably by activation of anti-inflammatory and anti-oxidant mechanisms. CO exerts anti-nociceptive effects and increases the anti-allodynic and anti-hyperalgesic efficacy of morphine in chronic inflammation and neuropathic pain - the latter strictly depending on NO produced by nNOS and iNOS. Furthermore, CO relieves neuropathic pain symptoms via reducing expression of iNOS and nNOS as well as by reduced activation of spinal microglia. Interestingly, the constitutive isoform HO-2 is co-expressed with NOS in the PNS and CNS and CO - as NO - is also capable of activating the proalgesic cGMP-protein kinase pathway. In fact, there is a close interaction between the CO and NO systems in the course of neuropathic pain suggesting that they might act as co-transmitters in neuronal signaling transmission. In nociception, the more stable CO may set basal activity by tonic background stimulation and NO may transiently amplify nociceptive signaling. Substances increasing endogenous CO (e.g., CO-releasing molecules, or HO-inducers alone or in combination with analgesics) may thus be useful for the treatment of (diabetic) neuropathic pain. The effects of CO in diabetic neuropathy are depicted in Fig. 2b.

**Nephropathy.** In the kidney, HO-1 and HO-2 are important in cytoprotection and serve as physiologic regulators of heme-dependent protein synthesis during which CO is produced. Inducers of the HO pathway (like hemin) are protective against renal inflammation and ameliorate DN in type 2 diabetic ZDF rats and STZ-induced type 1 diabetic rats. The anti-oxidant effect of HO-1 is believed to play a role in renal protection in diabetic rats. The opposite, deficiency of HO-2 results in higher superoxide anion levels and increased renal dysfunction after STZ-induced diabetes. Enhanced production of CO seems to be beneficial for the kidney in DN suggesting possibilities for therapeutic intervention. The effects of CO in diabetic nephropathy are shown in Fig. 2c.

Besides the beneficial effects of CO, high CO concentrations are toxic because of the high affinity of CO to bind heme-proteins like hemoglobin. Due to high levels of CO bound to hemoglobin (forming carboxyhemoglobin [COHb]), O₂ is not able to bind to that particular hemoglobin molecule and disrupted oxygen transport develops. However, endogenous production of CO by HO enzymes does obviously not result in toxic levels. In contrast to NO, the exact working mechanism and molecular targets of CO are mostly unknown. Nevertheless, one of the known pathways is that CO is able to increase cGMP production by activation of sGC albeit with lower affinity than NO. Moreover, CO is able
to bind to complex IV (cytochrome c oxidase) of the mitochondrial electron transport chain, and thereby regulating ROS production. In summary, CO is mainly protective in diabetic vascular disease via inhibition of ROS formation, via interaction with NO, and via the sGC/cGMP pathway.

Table 2 - Effect of CO in diabetic microvascular disease

<table>
<thead>
<tr>
<th>Model</th>
<th>Intervention</th>
<th>$\uparrow$ / $\downarrow$</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinopathy</td>
<td>Rat – STZ-induced diabetes</td>
<td>HO inhibitor SnPP $\downarrow$</td>
<td>Prevented diabetes-induced vascular injury</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hemin $\uparrow$</td>
<td>Maintained RGCs, reduced ROS in retina</td>
<td>64</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>Mouse</td>
<td>CORM-2, CORM-3, HO-inducer CoPP $\uparrow$</td>
<td>Reduced neuropathic pain</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Hemin, CORM-2 $\uparrow$</td>
<td>Reduced neuropathic pain, inflammation and ROS/RNS</td>
<td>68</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>Mouse – STZ-induced diabetes</td>
<td>HO-2$^{-}$ $\downarrow$</td>
<td>Enhanced renal injury and loss of renal function</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HO inducer CoPP $\uparrow$</td>
<td>Reduced glomerular injury and renal insufficiency</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Rat – STZ-induced diabetes</td>
<td>HO inducers Hemin, CoPP $\uparrow$</td>
<td>Improved renal injury, inflammation, ROS and renal function</td>
<td>72-74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HO inhibitors SnMP, CrMP $\downarrow$</td>
<td>Enhanced renal injury and renal function. Counteracted the protective effects of hemin</td>
<td>72,73</td>
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<tr>
<td></td>
<td>Rat – ZDF</td>
<td>Hemin $\uparrow$</td>
<td>Improved renal injury, inflammation and renal function</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HO inhibitor SnMP $\downarrow$</td>
<td>Enhanced renal injury and renal insufficiency</td>
<td>71</td>
</tr>
</tbody>
</table>

$\uparrow$ indicates increased CO; $\downarrow$ indicates reduced CO.

**Abbreviations:** CO: carbon monoxide; CoPP: cobalt protoporphyrin; CORM: CO-releasing molecule; CrMP: chromium mesoporphyrin; HO: heme oxygenase; RGC: retinal ganglion cell; ROS: reactive oxygen species; STZ: streptozotocin; ZDF: Zucker Diabetic Fatty
Hydrogen sulfide and microvascular complications of diabetes

Next to NO and CO hydrogen sulfide (H$_2$S) is the third gasotransmitter, and recognized as such in the nineties.$^{76,77}$ It is endogenously produced by three different enzymes. The pyridoxal-5’-phosphate (PLP)-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are the two major H$_2$S producing enzymes. The third H$_2$S producing enzyme is 3-mercaptopuruvate sulfurtransferase (3MST). The main substrates for H$_2$S production are homocysteine and cysteine. 3MST produces H$_2$S from 3-mercaptopuruvate (3-MP), which is produced by the enzymes cysteine aminotransferase (CAT) and d-Amino acid oxidase (DAO) from respectively L-cysteine and D-cysteine.$^{78}$ H$_2$S is a physiologically active compound, and is called endothelium-derived hyperpolarizing factor (EDHF)$^{79,80}$ causing vasodilatation but also acts as scavenger of reactive oxygen species (ROS) and stimulating angiogenesis.$^{81}$ Renal CSE and CBS expression and H$_2$S production are markedly lowered in spontaneous diabetic Ins2$^{Akita}$ mice.$^{82}$ In non-obese diabetic mice, another mouse-model of type 1 diabetes, it was also shown that diabetic mice had lower H$_2$S levels compared to non-diabetic mice.$^{83}$ And also in STZ-induced type 1 diabetes in rats, H$_2$S levels were lower compared to aged matched non-diabetic rats.$^{84}$ However, CSE deficiency delayed the onset of STZ-induced type 1 diabetes, and diabetes was accompanied by increased pancreatic H$_2$S production without changes in pancreatic CSE of CBS protein expression.$^{85}$ Another player in the development of vascular complications is insulin resistance. Insulin resistance is affected by H$_2$S in a high fatty diet-induced obesity mouse model. Interestingly, both inhibition of H$_2$S production by D,L-propargylglycine (PPG) and treatment with slow-release H$_2$S donor GYY4137 improved insulin resistance in these mice.$^{86}$ This unexpected beneficial effect of PPG could be explained by an upregulation of HO-1 resulting in higher CO levels, an effect of PPG that was recently described.$^{87}$ Next to that, this contradiction could be explained by the fact that PPG is an unspecific CSE inhibitor (based on its cofactor PLP), thereby potentially inhibiting other PLP-dependent enzymes as well.$^{88}$ In humans, diabetes is associated with lower levels of H$_2$S. In a small group of patients with type 2 diabetes, plasma H$_2$S levels were reduced with 73% compared to healthy (lean) individuals.$^{89}$ Interestingly, also obesity is correlated with lower levels of H$_2$S compared to lean volunteers. Collectively, human and experimental diabetes is associated with reduced H$_2$S bioavailability, which might be related to increased cardiovascular risk as observed in diabetic subjects. Effects of H$_2$S depletion and supplementation in diabetic mice and rats on the development of microvascular complications are summarized in Table 3 and will be discussed below.
**Retinopathy.** \( \text{H}_2\text{S} \) has recently received attention in research on diabetic retinopathy as some \( \text{H}_2\text{S} \)-related changes are compatible with a significant role of \( \text{H}_2\text{S} \) in the development and propagation of diabetic retinopathy. Reduced \( \text{H}_2\text{S} \)-mediated cell protection supposedly plays a role in retinal diseases as CBS expression is found in various eye compartments, including the retina, suggesting that the trans-sulfuration pathway is present in the eye.\(^9^0\) Many CBS deficiency-related eye disorders are associated with increased homocysteine levels, and the retinæ of CBS\(^{-/-} \) mice are characterized by ganglion cell loss, which is mediated by mitochondrial dysfunction.\(^9^1\) It is thus conceivable that \( \text{H}_2\text{S} \) is neuroprotective, and there is indeed experimental proof for protective properties of \( \text{H}_2\text{S} \) in the retina, as evidenced by a decreased retinal ganglion cell loss in \( \text{H}_2\text{S} \) pretreated animals after retinal I/R.\(^9^2\) Si et al. investigated the effect of \( \text{H}_2\text{S} \) in experimental retinopathy of STZ type 1 diabetic rats. They reported beneficial effects on neuronal dysfunction (based on electroretinography) and retinal structure (i.e., inhibition of diabetes-induced retinal thickening and extracellular matrix proteins), while others clearly showed a link to improved endothelial function such as tightened blood-retinal barrier and reduced vasoregression.\(^9^3\) \( \text{H}_2\text{S} \) is a known pro-angiogenic signaling molecule, and can thereby also contribute to enhanced angiogenesis in the diabetic retina. In line with this, increased levels of \( \text{H}_2\text{S} \) were observed in vitreous body of patients with proliferative diabetic retinopathy compared to patients with rhegmatogenous retinal detachment.\(^9^4\) The effects of \( \text{H}_2\text{S} \) in the diabetic retina are schematically shown in Fig. 2a.

**Neuropathy.** \( \text{H}_2\text{S} \) has mainly been reported to increase pain sensitivity via several proposed modes of action.\(^9^5\) These include sensitization of voltage-gated sodium and calcium channels,\(^9^5-9^7\) and/or suppression of potassium channels. Furthermore, the pronociceptive transient receptor potential channels TRPV1 and TRPA1,\(^9^8,9^9\) as well as NMDA receptors, were suggested to be sensitized by \( \text{H}_2\text{S} \). \( \text{H}_2\text{S} \) displayed pro-nociceptive actions in inflammatory pain, both in STZ-induced type 1 diabetes and in non-diabetic control animals. Interestingly, when treated with antagonists of the \( \text{H}_2\text{S} \)-producing enzymes, pain reduction was much more pronounced in diabetic than in non-diabetic animals indicative of an increased \( \text{H}_2\text{S} \) sensitivity of the nociceptive system in rats suffering from diabetes.\(^1^0^0\) *Vice versa*, reduction of \( \text{H}_2\text{S} \) reduced the tactile allodynia developed in course of diabetes. The T-type voltage-gated calcium channel \( \text{Ca}_\text{V} \text{3.2} \) is sensitized by \( \text{H}_2\text{S} \) leading to increased pain sensitivity.\(^1^0^1\) An increased \( \text{H}_2\text{S} \) tissue content and hyperactivity of \( \text{Ca}_\text{V} \text{3.2} \) were observed in chemotherapy-induced neuropathic hyperalgesia and pain that could be reversed by blocking \( \text{H}_2\text{S} \) production.\(^9^6\) Painful peripheral diabetic
neuropathy is accompanied by an enhancement of Ca\(_{v3.2}\) T-type calcium channels and neuronal excitability. Thus, Ca\(_{v3.2}\) T-channels are thought to represent signal amplifiers in peripheral sensory neurons contributing to hyperexcitability that ultimately leads to the development of pain in diabetic neuropathy.\(^{102}\) Conversely, also anti-nociceptive effects have been reported for H\(_2\)S. These effects were antagonized by ATP-sensitive potassium (K\(_{ATP}\)) channel blocker glibenclamide, and by NOS-inhibition.\(^{103}\) Inhalation of H\(_2\)S reduced the development of neuropathic pain by reducing the resulting increase in IL-6 and chemokines – which was attributed to an inhibition of microglia activation in course of neuropathy.\(^{104}\) Furthermore, H\(_2\)S functions as a neuroprotective agent by enhancing the production of glutathione, a major intracellular anti-oxidant that scavenges mitochondrial ROS.\(^{105}\) Collectively, these data indicate that H\(_2\)S displays both pro- and anti-nociceptive actions in diabetic neuropathy. The effects of H\(_2\)S in diabetic neuropathy are depicted in Fig. 2b.

**Nephropathy.** In DN patients with atherosclerosis who are on dialysis, lower plasma levels of H\(_2\)S were measured, which could indicate a loss of the supposed protective effects of H\(_2\)S in these patients.\(^{106}\) This might be caused by endothelial damage or downregulation via other pathways of the enzymes producing hydrogen sulfide. Also, high urinary sulfate, as a proxy for H\(_2\)S, is significantly associated with a slower decline in GFR in type 1 DN patients.\(^{107}\) In the experimental setting exogenous H\(_2\)S reduces blood pressure and prevents the progression of DN in SHR rats.\(^{108}\) Renal protection via blood pressure reduction is also shown in AngII-induced hypertension and proteinuria in rats.\(^{109}\) Other studies also suggest that H\(_2\)S is a key modulator in renal remodeling and that its actions can be affected by the matrix-metalloproteinase MMP-9 which is shown to modulate CBS and CSE.\(^{82}\) In STZ-induced type 1 diabetic mice, the administration of H\(_2\)S attenuated oxidative stress and inflammation, reduced mesangial cell proliferation and inhibited the renin-angiotensin-aldosterone system (RAAS).\(^{110}\) These data indicate that in DN H\(_2\)S has predominantly beneficial effects and is therefore a promising target for intervention. Thiosulfate might be the perfect H\(_2\)S donor since it is already in use in the clinic for patients with calcifylaxis with end-stage renal disease,\(^{111}\) and has shown to be beneficial in hypertensive renal disease in rats.\(^{109}\) The effects of H\(_2\)S on the kidney in diabetic nephropathy are schematically shown in Fig. 2c.
Gasotransmitters in vascular complications of diabetes

Figure 2 - Beneficial and deleterious effects of gasotransmitters in the development of microvascular complications in diabetes: (A) retinopathy, (B) neuropathy, and (C) (glomerular) nephropathy. In these schematic representations of the three target organs, gasotransmitters are depicted in green when having beneficial effects and depicted in red when having deleterious effects on the development of microvascular complications. Gasotransmitters may have different properties as indicated by numbers 1-14 in the cartoons and explanatory text box. As indicated with #8, NO and CO might activate the cGMP-pathway via sGC (e.g., via phosphorylation by protein kinases, release of transmitters, synaptic plasticity).

Knowledge on working mechanisms of H_2S is continuously increasing and summarized below. H_2S regulated vasodilatation acts partly via activation of K_ATP channels, and a rather new hypothesis is the interference of H_2S with the cGMP pathway by inhibition of PDE-5 activity, a mode of action comparable with “natural Sildenafil”. ROS production is decreased by H_2S through direct interference with the mitochondrial respiration chain. It binds to cytochrome c oxidase, thereby directly inhibiting the formation of ROS. Another important effect of H_2S in terms of diabetic angiopathy is angiogenesis.
VEGFR2 is the natural target for H$_2$S to achieve its proangiogenic effect. In diabetic retinopathy, the development of new vessels reflects the severity. However, increased angiogenesis might also have some protective effects; e.g. angiogenesis of vaso nervorum in diabetic neuropathy. The effects of H$_2$S on different ion-channels is mainly important in diabetic neuropathy. Its interference with - for instance - TRPV1, TRPA1 and Ca$_v$3.2 channels contributes to increased nociception. Taken together, H$_2$S exerts dual effects in diabetic angiopathy, positive effects via its vasodilatory actions, and unwanted detrimental effects via different ion-channels and angiogenesis. The effects of H$_2$S in DN are depicted in Fig. 2c.

### Table 3 - Effect of H$_2$S in diabetic microvascular disease

<table>
<thead>
<tr>
<th>Model</th>
<th>Intervention</th>
<th>↑ / ↓</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinopathy</td>
<td>Mouse – STZ-induced diabetes</td>
<td>CBS$^{+/−}$</td>
<td>↓</td>
<td>Increased loss of RGCs</td>
</tr>
<tr>
<td></td>
<td>Rat – STZ-induced diabetes</td>
<td>NaHS</td>
<td>↑</td>
<td>Prevented diabetes-induced vascular injury</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>Rat – STZ-induced diabetes</td>
<td>NaHS, L-Cysteine</td>
<td>↑</td>
<td>Increased neuropathic pain symptoms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSE/CBS inhibitors PPG, BCA and HA</td>
<td>↓</td>
<td>Reduced neuropathic pain</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>Mouse – Ins2$^{Akita}$</td>
<td>H$_2$S donor N-acetyl-cysteine</td>
<td>↑</td>
<td>Reduced ROS</td>
</tr>
<tr>
<td></td>
<td>Rat – STZ-induced diabetes</td>
<td>NaHS</td>
<td>↑</td>
<td>Improved renal injury, inflammation, renal function and reduced ROS</td>
</tr>
</tbody>
</table>

↑ indicates increased H$_2$S; ↓ indicates reduced H$_2$S.

**Abbreviations:** BCA: β-cyanoalanine; CBS: cystathionine β-synthase; CSE: cystathionine γ-lyase; HA: hydroxylamine; H$_2$S: hydrogen sulfide; NaHS: sodium hydrosulfide; PPG: D,L-propargylglycine; RGC: retinal ganglion cell; ROS: reactive oxygen species; STZ: streptozotocin
Gasotransmitters in vascular complications of diabetes

Gasotransmitters have been studied in diabetes-associated macrovascular disease and therapeutically used (NO only, not yet CO and H₂S) in clinical practice. Effects of gasotransmitters depletion and supplementation in human and experimental diabetes on the development of endothelial function and macrovascular disease are summarized in Table 4 and briefly discussed below. Already in 1992 NO-donor sodium nitroprusside (SNP) was used to measure NO-dependent vasorelaxation in patients with type 1 diabetes. SNP-stimulated vasodilatation was decreased in diabetes patients compared to non-diabetic subjects, indicating a lower NO sensitivity. In patients with type 2 diabetes, addition of NOS cofactor BH₄ resulted in improved forearm blood flow, an effect which was nullified by NOS-inhibitor L-NG-monomethylarginine (L-NMMA). In patients with type 2 diabetes and coronary artery disease, treatment with NO substrate L-arginine and NOS cofactor BH₄ protected against I/R endothelial dysfunction in the forearm vasculature. The important role of NO in the macrovasculature is also shown in animal models of diabetes. Leprdb/db eNOS⁻/⁻ double knockout mice showed an aggravated vascular phenotype compared to diabetic leprdb/db or eNOS⁻/⁻ single knockouts, as evidenced by an increased aortic wall thickness and reduced re-endothelialization after arterial injury. In ApoE⁻/⁻ mice and mice with STZ-induced type 1 diabetes, treatment with bone marrow-derived mononuclear cells overexpressing-eNOS resulted in reduced plaque progression and improved post-ischemic neovascularization, an effect which was completely inhibited by NOS-inhibitor N⁵-nitro-L-arginine methyl ester (L-NAME).

Protective properties of CO in diabetes have been mainly investigated in STZ-induced type 1 diabetes in rats or mice. Exposure of the tail artery to CO ex vivo resulted in vasodilatation, an effect that was reduced in arteries of STZ-induced diabetic rats, indicating a reduced sensitivity for CO in diabetic animals. In a myocardial I/R model in HO-1⁻/⁻ diabetic mice, infarct size and mortality were dramatically worsened compared to wildtype (HO-1⁺/+⁻) diabetic mice, without affecting glucose levels. In diabetic rats, CO-releasing molecule 3 (CORM-3) or HO-1 inducer cobalt protoporphyrin (CoPP) preserved endothelial function and vascular relaxation, an effect which was reversed by HO-inhibitors chromium mesoporphyrin (CrMP) and tin mesoporphyrin (SnMP). In a model of myocardial I/R injury, treatment with CO-releasing compound PEGylated carboxyhemoglobin bovine (PEG-COOH), reduced infarct size and troponin levels drastically in STZ-induced diabetic mice. Also in mice receiving PEG-COOH only during
reperfusion, infarct size was reduced, suggesting CO as a potential therapeutic agent for patients after myocardial infarction.\textsuperscript{123} In STZ-induced diabetes in rats, induction of HO-1 with hemin, and treatment with CORM-2 to lesser extent, attenuated vascular damage and oxidative stress, and improved vascular relaxation compared to non-treated rats.\textsuperscript{124}

\( \text{H}_2\text{S} \) as a therapeutic agent in diabetic vascular disease is evaluated in both mice and rats. In STZ-induced diabetic Spraque-Dawley rats, treatment with \( \text{H}_2\text{S} \) donor NaHS improved vascular relaxation and NO bioavailability. This indicates that \( \text{H}_2\text{S} \) is a potential therapeutic agent in diabetic vascular disease via crosstalk with NO.\textsuperscript{26} \textit{Ex vivo} administration of \( \text{H}_2\text{S} \) substrate L-cysteine also resulted in dose-dependent vasorelaxation in rat middle cerebral arteries, which was reduced in diabetic animals.\textsuperscript{125} The vasorelaxant effects of NaHS are reduced with additional \( \text{K}_{\text{ATP}} \) blocker glibenclamide, demonstrating that NaHS-induced vasorelaxation takes place via activation of \( \text{K}_{\text{ATP}} \) channels.\textsuperscript{126} \textit{Ex vivo} overexpression of CSE improved vascular relaxation in hyperglycemic conditions and reduced ROS production, while CSE mRNA knockdown with siRNA resulted in a more pronounced ROS production.\textsuperscript{127} Beneficial properties of \( \text{H}_2\text{S} \) have been shown in models for myocardial injury as well. Addition of NaHS in diabetic rats resulted in preserved cardiac function,\textsuperscript{128} reduced infarct size, reduced ROS and inflammatory parameters such as TNF-\( \alpha \) and IL-10, and inhibited expression of adhesion molecules as ICAM-1.\textsuperscript{129} In a model of myocardial I/R injury in diabetic lepr\textsuperscript{db/db} mice, treatment with \( \text{H}_2\text{S} \) donor \( \text{Na}_2\text{S} \) either before I/R or only during reperfusion, both diminished infarct size, troponin levels and ROS.\textsuperscript{130,131}

Although studies on the role of gasotransmitters in the development of macrovascular disease are limited to endothelial dysfunction rather than atherosclerosis, we propose that gasotransmitters may also modulate atherogenesis via different mechanisms as schematically depicted in Figure 3. Considering the data described above and summarized in Table 4, gasotransmitters seem to be promising targets for intervention in the course of diabetic macrovascular disease.
### Table 4 - Effect of gasotransmitters in diabetic macrovascular disease

<table>
<thead>
<tr>
<th>Model</th>
<th>Intervention</th>
<th>↑ / ↓</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO Mouse – Lepr&lt;sup&gt;db/db&lt;/sup&gt;</td>
<td>eNOS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>↓</td>
<td>Increased arterial injury</td>
<td>117</td>
</tr>
<tr>
<td>NO Mouse – STZ-induced diabetes</td>
<td>eNOS overexpression of BM-MNCs</td>
<td>↑</td>
<td>Reduced atherosclerosis, improved angiogenesis</td>
<td>118</td>
</tr>
<tr>
<td>Human – type 1 diabetes</td>
<td>NO donor SNP</td>
<td>↑</td>
<td>Induced vasodilatation, but SNP induced vasodilatation is reduced in diabetes patients</td>
<td>114</td>
</tr>
<tr>
<td>Human – type 2 diabetes</td>
<td>NOS cofactor BH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>↑</td>
<td>Improved forearm bloodflow</td>
<td>115</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>↓</td>
<td>Reduced forearm bloodflow</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>L-Arginine, BH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>↑</td>
<td>Reduced endothelial dysfunction</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>CO Mouse – STZ-induced diabetes</td>
<td>HO-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>↓</td>
<td>Induced oxidative stress and increased infarct size in myocardial I/R model</td>
<td>119</td>
</tr>
<tr>
<td>CO donor PEG-bHb-CO</td>
<td>↑</td>
<td>Reduced myocardial injury and oxidative stress in myocardial I/R model</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Rat – STZ-induced diabetes</td>
<td>CO gas</td>
<td>↑</td>
<td>Induced vasodilatation, but CO-dependent vasodilatation is reduced in diabetic rats</td>
<td>59</td>
</tr>
<tr>
<td>CORM-2, CORM-3, biliverdin</td>
<td>↑</td>
<td>Improved vascular function, reduced endothelial damage</td>
<td>120-122,124</td>
<td></td>
</tr>
<tr>
<td>HO inducers hemin, CoPP</td>
<td>↑</td>
<td>Improved vascular function, reduced oxidative stress</td>
<td>120,122,124</td>
<td></td>
</tr>
<tr>
<td>HO inhibitor SnMP</td>
<td>↓</td>
<td>Diminished protective effects of CORM-3</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;S Mouse – Lepr&lt;sup&gt;db/db&lt;/sup&gt;</td>
<td>Na&lt;sub&gt;2&lt;/sub&gt;S</td>
<td>↑</td>
<td>Reduced myocardial injury in myocardial I/R model</td>
<td>130,131</td>
</tr>
<tr>
<td>Rat – STZ-induced diabetes</td>
<td>NaHS, L-Cysteine</td>
<td>↑</td>
<td>Improved vascular function and reduced myocardial injury</td>
<td>26,125-129</td>
</tr>
<tr>
<td>CSE overexpression</td>
<td>↑</td>
<td>Improved vascular function ex vivo</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>CSE inhibitor PPG</td>
<td>↓</td>
<td>Increased myocardial injury and inhibited vasorelaxation ex vivo</td>
<td>125,129</td>
<td></td>
</tr>
</tbody>
</table>

↑ indicates increased gasotransmitter availability; ↓ indicates decreased gasotransmitter availability. **Abbreviations:** BH<sub>4</sub>: tetrahydrobiopterin; CO: carbon monoxide; CoPP: cobalt protoporphyrin; CORM: CO-releasing molecule; CSE: cystathionine γ-lyase; HO: heme oxygenase; eNOS: endothelial nitric oxide synthase; L-NMMA: L-N<sup>ω</sup>-monomethyl Arginine citrate; Na<sub>2</sub>S: sodium sulfide; NaHS: sodium hydrosulfide; NO: nitric oxide; PPG: D,L-propargylglycine; ROS: reactive oxygen species; SnMP: tin mesoporphyrin; SNP: sodium nitroprusside; STZ: streptozotocin.
Figure 3 - Beneficial and deleterious effects of gasotransmitters in the development of atherosclerosis in diabetes-associated macrovascular complications. In the cartoon, development of an atherosclerotic plaque (yellow layer) is schematically depicted. Gasotransmitters are depicted in green when having beneficial effects (numbers 1-4) and depicted in red when having deleterious (numbers 5 & 6) effects on the development of atherosclerosis as indicated in the cartoon and explanatory text. Ox LDL: oxidized low-density lipoprotein.

Protective mechanisms and mutual gasotransmitter interactions

Although NO, CO and H₂S have different molecular structures and routes of endogenous production, they do share various physiological properties such as the ability to bind to heme groups, and to promote vasorelaxation by stimulation of the sCG/cGMP pathway. NO and CO stimulate cGMP production by targeting of sCG, and H₂S by inhibiting phosphodiesterase type 5 (PDE-5) activity. NO as well as CO and H₂S are direct ROS scavengers, partly by direct interaction with the mitochondrial respiratory chain. They all engage on the K<sub>ATP</sub> channels to achieve this anti-oxidant effect, both in the vasculature and nervous system. In addition, they share several common intracellular pathways such as NF-κB, nuclear factor-like-2 (Nrf-2), and mitogen-activated protein kinases (MAPK), thereby exerting anti-apoptotic, anti-oxidant and anti-inflammatory effects. NO, CO and H₂S inhibit expression of ICAM-1, VCAM-1, and E-Selectin, thereby promoting endothelial health and integrity. Finally, all three gasotransmitters act as pro-angiogenic substances via the vascular endothelial growth factor (VEGF)-pathway. Based on these functional
similarities, it is likely that gasotransmitters have mutual interactions. Such a relationship between CO and NO has been investigated intensively, and is mainly mediated via the sGC/cGMP pathway. These effects include blood pressure regulation and inflammation. It has already been shown that H$_2$S exerts its effects via NO production, since H$_2$S promotes eNOS production and activity. Additionally, vasorelaxant effects of H$_2$S were diminished when aortic rings were pre-treated with NOS inhibitor N$^G$-nitro-L-arginine methyl ester (L-NAME). Reciprocally, NO-donor SNP stimulates endogenous H$_2$S production via upregulation of CBS. Although CO and H$_2$S share a lot of functional characteristics, the mutual relationship between these two gasotransmitters has barely been studied. In one report, in a myocardial I/R injury mouse model HO-1 expression was upregulated 24 hours after intravenous H$_2$S treatment, which was accompanied by a protection against I/R-induced damage.

Methods to measure gasotransmitters

In order to study the association of gasotransmitters and the development of diabetes-associated vascular complications, reliable and sensitive assays to measure NO, CO and H$_2$S are indispensable. Various methodologies are used to measure the different gasotransmitters and these will be briefly described. First of all, measuring NO is quite a challenge because of its instability. There are different methods of measuring NO. Most commonly used is the relatively simple Griess-method, which does not measure NO directly, but rather its oxidated products nitrite (NO$_2^-$) and nitrate (NO$_3^-$). However, nitrite and nitrate can be detected more precise by HPLC. NO can also be directly measured using gas phase chemiluminescence which involves the reaction of NO with ozone (O$_3$) to form excited NO$_2$. During relaxation to (unexcited) NO$_2$, a photon is released which is then detected by chemiluminescence. Using this method NO release from different body fluids and tissues can be measured. Next to the aforementioned methods, different fluorescent probes and electrodes are currently available, with the possibility to measure NO in fluids, tissues, and intracellularly in cells in vitro. Alike NO, also CO levels can be measured using different methods. The most commonly used and relatively simple way to measure CO is in air using gas chromatography. In vivo, CO is generally measured in red blood cells by determining the percentage of
carboxyhemoglobin (COHb) relative to total hemoglobin concentration. Finally, some studies use $[^{14}\text{C}]$Heme \textit{in vitro} to measure endogenous $^{14}$CO production.$^{146}$

When considering $\text{H}_2\text{S}$ as therapeutic target, reliable methods to determine $\text{H}_2\text{S}$ levels in body fluids and tissues are needed. However, measurements of $\text{H}_2\text{S}$ are difficult because of its volatility. For that reason, stable endproducts like sulfate or thiosulfate can be measured in serum or urine,$^{147}$ although a few methodologies have been described to measure $\text{H}_2\text{S}$ itself. The methylene blue assay is the most commonly used technique. It is based on the oxidative coupling of $\text{H}_2\text{S}$ with two N,N-dimethyl-p-phenylenediamine molecules, forming the methylene blue dye which can be detected spectrophotometrically. However, this technique is extremely pH dependent and not very sensitive and reliable. A more sensitive method is based on monobromobimane, in which two monobromobimane molecules will form the stable sulfide dibimane in presence of $\text{H}_2\text{S}$. Sulfide dibimane can be separated by reverse phase chromatography and detected by a fluorescence detector. Nowadays, fluorescent probes and sulfide selective electrodes are extensively used, however with different sensitivity and reliability.$^{148}$ As yet, $\text{H}_2\text{S}$ measurements are difficult, with variable reliability, thereby complicating studies on the role of $\text{H}_2\text{S}$ and its use as therapeutic target in various diseases including diabetes-associated vascular disease.

**Future perspectives and treatment options**

Patients with diabetes have a 2-4 fold increased risk for cardiovascular disease, and adequate treatment and preventive strategies are still lacking. As discussed in this \textit{Perspective}, the different gasotransmitters appear to be important mediators in development of diabetic angiopathy and therefore potential targets for intervention. As aforementioned, NO-based interventions are already applied in humans and readily available. SNP is clinically used and acts as direct NO-donor by releasing NO from the ferrous ion center without need for enzymatic action. The same counts for nitroglycerin and other organic nitrates, which are well established for their vasodilatory effects during angina. Organic nitrates act as NO-donors by enzymatic or non-enzymatic breakdown of nitrates into nitrite and NO.$^{149}$ Molsidomine and Lisidomine are registered in several European countries as anti-anginal drug, and act as vasodilator by non-enzymatic release of NO. At last, also dietary products with high nitrate content can act as NO-donor. The intake of beetroot juice lowered blood pressure significantly, an effect which was accompanied by higher levels of urinary NO(x).$^{150}$ CO administration or CO-releasing
molecules are not in clinical use yet, albeit that some of the vascular protective effects of acetylsalicylic acid and statins are attributed to induction of HO-1. In human endothelial cells in vitro a dose-dependent increase of HO-1 expression was seen after statin\textsuperscript{151,152} or acetylsalicylic acid\textsuperscript{153} treatment. However, this effect was not reproduced in human subjects since no differences in HO-1 expression were observed between acetylsalicylic acid-, statin- or placebo-treated patients.\textsuperscript{154} The anti-oxidative actions of polyphenol resveratrol are also partly attributed to HO-1 upregulation as shown by increased HO-1 expression levels in STZ-induced type 1 diabetes in Sprague-Dawley rats.\textsuperscript{122} Although the HO-1-inducing effects of resveratrol have not yet been described in humans, this dietary supplement is readily available for human use. Alike CO, H\textsubscript{2}S is also not clinically used in humans yet although intravenous Na\textsubscript{2}S administration has been performed in a phase I safety study.\textsuperscript{155} This study revealed increased H\textsubscript{2}S and thiosulfate levels after Na\textsubscript{2}S administration indicating that circulating H\textsubscript{2}S levels can be achieved following parenteral administration. H\textsubscript{2}S metabolite thiosulfate can also act as H\textsubscript{2}S donor via enzymatic conversion by rhodanese (also known as thiosulfate sulfurtransferase).\textsuperscript{156} Thiosulfate is used as treatment for calcifylaxis in patients with end-stage renal disease\textsuperscript{111} and has been described as a protective agent in hypertensive heart and renal disease in rats.\textsuperscript{109,157} Sulphydrylated ACE inhibitors, such as zofenopril and captopril, show additional beneficial effects in different trials.\textsuperscript{158} Recently, it is demonstrated that the beneficial effects of sulphydrylated ACE inhibitors are explained by H\textsubscript{2}S release.\textsuperscript{159} At last, H\textsubscript{2}S is also generated by various species of sulfate-reducing bacteria in the gut. Germ-free mice showed significant lower levels of H\textsubscript{2}S,\textsuperscript{160} indicating that addition of dietary sulfate or sulfur-containing amino acids can act as natural H\textsubscript{2}S donors.

In conclusion, various gasotransmitter-based strategies are currently being studied as potential strategy to treat vascular dysfunction. So far, these strategies have not been explored in the context of diabetes-associated vascular disease. Because of the toxicity of gasotransmitters when exposed to high concentrations, as well as their potential deleterious effects on the development of vascular disease (as discussed in this Perspective), prudence is called for when considering exogenous administration of gasotransmitters. However, gasotransmitter-based interventions are relatively safe, mainly because these gases are also produced endogenously, and therefore highly promising candidate therapeutics.

**Acknowledgements**

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nase-1 in patients with Type 2 diabetes and micro-


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