Janine Ganahl, MSc

Detection of hydrogen sulfide in blood plasma and implications for vascular health

MASTER'S THESIS

to achieve the university degree of

Master of Science

Master's degree programme: Biochemistry and Molecular Biomedical Sciences

submitted to

Graz University of Technology

Supervisor

Univ.-Prof. Dr. Kai-Uwe Fröhlich
Institute of Molecular Biosciences, University of Graz

Ass.-Prof. Dr. James R Mitchell
Harvard School of Public Health

Graz, October 2017

AFFIDAVIT

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly indicated all material which has been quoted either literally or by content from the sources used. The text document uploaded to TUGRAZonline is identical to the present master‘s thesis.

Date

Signature

For my family

Acknowledgements

I want to thank Dr. Kai-Uwe Fröhlich und Dr. Frank Madeo for their support in planning my research stay abroad and for making this possible. My particular gratitude goes to Dr. James Mitchell for giving me the opportunity to conduct my research in his laboratory, guiding me through the whole process and always having an open ear. Without each one of these three mentors my stay wouldn't have been possible.

My thanks also go to Michael MacArthur, especially for helping me getting started in the laboratory and later for his great input and ideas. Additionally, I want to thank the other members of the Mitchell Laboratory Humberto Treviño, Justin Reynolds and Kaspar Trocha for the warm welcome and their help and assistance whenever I needed it. To the other visiting students Vera Reitsema and Gustavo Kajitani I'm especially thankful - not only for their assistance in the laboratory, but also for the amazing time we spent together discovering Boston and surroundings, the lunch and coffee breaks.

I want to thank the Office for International Relations and Mobility Programs at the TU Graz and the Harvard International Office for contributing their knowledge on visa application and funding opportunities and for assisting this whole process from the beginning to the end.

Finally, I want to thank my family and my partner for all their support through my entire university career and also through my stay abroad.

Abstract

Hydrogen sulfide (H_2S) is an endogenously produced gasotransmitter that shows protective effects on multiple organs and whose decrease is considered a risk factor for cardiovascular disease. Reliable, quantitative techniques for measuring H_2S levels under physiological conditions in vivo are still under development, presenting a challenge to the field. However, the activity of the H_2S producing enzymes upon addition of substrate cysteine and cofactor pyridoxal 5'-phosphate (PLP) can be reliably assessed and used as a surrogate marker of H_2S levels.

Measuring H_2S production capacity in extracts of organs with abundant levels of H_2S-producing enzymes, including liver and kidney, is straightforward using the lead acetate overlay method in which H_2S gas from the sample forms a lead sulfide precipitate. Using this assay, we found differences in the effects of different lifespan extending dietary restriction regimens on hepatic H_2S production capacity. While in caloric restriction it has previously been reported that an increase in endogenous H_2S production is essential for the benefits of reduced food intake, we found that upon protein restriction H_2S production was not altered. Restriction in methionine on the other hand, leads to an increase in H_2S production capacity, but likely through a different mechanism than the restriction of total calories.

For measurement of H_2S production capacity in humans, we turned to blood serum/plasma as a more readily accessible source of material than liver biopsy material. Following optimization of the lead acetate overlay method, we were able to detect H_2S production in serum or plasma in the presence of added substrate and cofactor. Unexpectedly, a line of experiments revealed that the H_2S signal from serum, unlike liver, was not enzymatic in nature. H_2S production from serum was resistant to heat denaturation and proteolytic digestion and H_2S production in serum was largely independent of the cofactor PLP. Additionally, DTT could replace cysteine for H_2S generation. Although DTT cannot be used as a substrate for H_2S generation like cysteine, both can serve as reducing agents, suggesting that the H_2S released from serum is originally present in the form of bound sulfur.

Using this assay, we measured H_2S release in human plasma samples of patients with peripheral artery disease (PAD) requiring surgery. We observed significantly reduced H_2S release in plasma of PAD patients compared to age, sex and hypertension-matched controls. Furthermore, among the patients undergoing surgery, 2 year survival was significantly higher in the group with bound sulfur levels above the mean. This finding correlated with reduced free sulfide levels in PAD patients and controls as measured by HPLC-MS.

In conclusion, using a new assay, we found correlations between H_2S levels and vascular disease, underlining the potential importance of H_2S on health and disease in humans. The experiments also shed light on potential similarities and differences between dietary interventions that impact endogenous H_2S production in mice, with the potential to translate to humans.

Kurzzusammenfassung

Schwefelwasserstoff (H_2S) ist ein endogen erzeugter Gasotransmitter mit protektiven Wirkungen auf eine Vielzahl von Organen und dessen Abnahme als Risikofaktor für Herz-Kreislauf-Erkrankungen gilt. Zuverlässige, quantitative Techniken zur Messung von H_2S-Konzentrationen unter physiologischen Bedingungen befinden sich noch in der Entwicklung und stellen eine Herausforderung für das Forschungsgebiet dar. Die Aktivität der H_2S produzierenden Enzyme bei Zugabe des Substrats Cystein und Cofaktor Pyridoxal 5‘-Phosphat (PLP) kann jedoch als Marker für H_2S Spiegel verwendet werden.

Die Messung dieser H_2S Produktionskapazität in Geweben mit reichlich vorhandenen H_2S produzierenden Enzymen wie Leber und Niere ist mit der Bleiacetat-Methode möglich, bei der produziertes H_2S Gas einen Bleisulfidniederschlag bildet, der nachgewiesen werden kann. Mit diesem Assay entdeckten wir Unterschiede in den Auswirkungen verschiedener lebensverlängernder diätetischer Restriktionsregime auf die Produktionskapazität von hepatischem H_2S. Während bei kalorischer Restriktion bereits bekannt war, dass ein Anstieg der endogenen H_2S Produktion für die Vorteile einer Nahrungsrestriktion essentiell ist, konnten wir zeigen, dass diese bei Proteinrestriktion nicht verändert wurde. Eine Restriktion der Aminosäure Methionin dagegen führt zu einer Erhöhung der H_2S Produktionskapazität, jedoch liegt dieser voraussichtlich ein anderer Mechanismus zugrunde als die der Beschränkung der Gesamtkalorien.

Für die Messung der H_2S Produktionskapazität beim Menschen griffen wir auf Blutserum/-plasma als einfache zugängliche Materialquelle zurück. Nach Optimierung der Bleiacetat-Methode konnten wir in Gegenwart von Substrat und Cofaktor eine H_2S Produktion in Serum und Plasma nachweisen. Unerwarteterweise zeigten verschiedene Experimente, dass das H_2S Signal aus Serum im Gegensatz zur Leber nicht enzymatisch war. Die H_2S Produktion aus Serum war nicht nur resistent gegen Hitzedenaturierung und proteolytischen Verdau, sondern auch unabhängig vom Cofaktor PLP und Cystein konnte durch DTT ersetzt werden. Obwohl DTT nicht wie Cystein als Substrat für die enzymatische H_2S Produktion verwendet werden kann, können beide als Reduktionsmittel dienen. Dies legt nahe, dass das aus Serum freigesetzte H_2S ursprünglich in Form von gebundenem Schwefel vorliegt.

Mit diesem Assay haben wir die Freisetzung von H_2S in humanen Plasmaproben von Patienten mit peripherer arterieller Verschlusskrankheit gemessen, welche eine Operation benötigten. Wir beobachteten eine signifikant reduzierte H_2S Freisetzung im Plasma von Patienten im Vergleich zu Kontrollpersonen. Darüber hinaus war unter den Patienten das 2-Jahre-Überleben nach der Operation signifikant höher, wenn diese Levels an gebundenem Schwefel über dem Mittelwert zeigten. Dies korrelierte auch mit freien Sulfidspiegeln, welche mit HPLC-MS gemessen wurden.

Zusammenfassend haben wir mit einem neuen Assay Korrelationen zwischen Plasma H_2S Spiegeln und vaskulären Erkrankungen gefunden, was auf die potentielle Bedeutung von H_2S für Gesundheit und Krankheit beim Menschen hindeutet. Weitere Experimente beleuchteten Ähnlichkeiten und Unterschiede zwischen diätetischen Interventionen, die die endogene H_2S Produktion bei Mäusen beeinflussen und potentiell auf den Menschen übertragbar sein könnten.

1. Introduction

1.1. Hydrogen sulfide – poisonous gas or important signaling molecule?

H_2S is a colorless and flammable gas, that is naturally produced through reduction of sulfur compounds by bacteria or by non-specific reactions (Hill 1973). It can be found naturally in volcanic gases, salt mines, natural gas or thermal baths.

H_2S is mostly known for its unpleasant smell and is easily identified by its strong, distinctive odor of rotten eggs. Its toxicity has been known for a long time and has led to numerous deaths, especially in the petroleum and agriculture industry (Doujaiji und Al-Tawfiq 2010). On the other hand it has been used in alternative medicine for a long time and hot sulfur baths were taken as a cure-all (Forster 1994). Through the centuries H_2S has alternately changed from being seen as toxic and therapeutic and in recent years it has been regaining recognition for leading to numerous beneficial biological effects. It acts as a typical hormetic substance: toxic at high concentrations and with beneficial effects at low concentrations.

1.1.1. Toxicity of H_2S

Currently H_2S is considered by the world health organization as hazardous (WHO, 2003). H_2S poisoning usually occurs through inhalation and exposure levels and duration are the important factors. Starting at concentrations of about 5 parts per million (ppm), H_2S can lead to nausea or headaches upon long exposure. Acute exposure of 50 - 100 ppm can cause eye irritations, neurological disorders like dizziness and headaches, itching and dryness of skin and respiratory symptoms like coughing. Other symptoms can be problems with the gastrointestinal tract, loss of appetite, nausea or libido decrease (Wang et al. 2015).

Between 100 and 500 ppm, severe lung and nose irritation and even unconsciousness can appear. With ambient H_2S concentration this high, the olfactory nerve is paralyzed and we can't smell H_2S odor after a few inhalations. Exposures from 500 ppm of H_2S lead to stimulation of the central nervous system and hyperventilation, which then results in respiratory paralysis with the possibility of death (Beauchamp et al. 1984; Guidotti 1996).

800 ppm of H_2S exposed for five minutes is lethal for 50% of humans (LC_{50}) and 1000 ppm always leads to death after only a single inhalation with immediate respiration arrest.

After an acute intoxication at moderate concentration, the symptoms usually fade very fast and patients recover quickly and completely. Multiple and prolonged exposures can lead to irreversible adverse health effects (Wang et al. 2015).

H_2S is a mitochondrial poison and works through inhibition of the complex IV of the electron transport chain. The complex IV is the cytochrome c oxidase and H_2S builds a complex with the iron in the enzyme. By its inhibition, the oxidative phosphorylation is uncoupled and no ATP can be built. This subsequently leads to an arrest of cellular respiration, similarly to cyanide toxicity (Petersen 1977; Guidotti 2015). Due to this, H_2S can affect all organs, but particularly affected are usually the central nervous system and the pulmonary tract (Guidotti 2015).

Human deaths are mostly associated with accidents in industry, due to sudden release of huge amounts of H_2S in oil refineries. These lead to the instant loss of consciousness, called the “knock-down” phenomenon, which is a consequence of respiratory failure (Doujaiji und Al-Tawfiq 2010).

1.1.2. The gasotransmitter H_2S

Besides its toxicity upon exogenous exposure, H_2S is produced endogenously by enzymes in our body and was recently defined as a third gasotransmitter, next to carbon monoxide (CO) and nitric oxide (NO). To be characterized as a gasotransmitter, the gas has to be membrane permeable, it has to be generated endogenously and enzymatically in a regulated manner, it has specific functions at physiologically relevant concentrations and these effects can be mimicked when exogenously applied (Wang 2002; Vandiver und Snyder 2012). Similarly to H_2S, also NO and CO are toxic in higher concentrations, however, the toxicity of H_2S is five times higher than of CO (Lloyd 2006).

NO was the first gas identified to act as a signaling molecule. Initially it was discovered in the cardiovascular system where it acts as a vasodilator, what was rewarded with a Nobel Prize in 1998. Subsequently its functions in the brain and nervous system were revealed, which characterized it as a neurotransmitter (Bredt und Snyder 1989). Soon after, the

signaling role of CO was discovered and it could be connected with regulation of physiological functions (Verma et al. 1993).

The enzymatic synthesis of both gases was already well characterized when their signaling function was discovered. The three isoforms of nitric oxide synthase exist and the deletion in mice lead to the loss of the NO generation capacity in the relevant organs (Huang et al. 1993; Gadalla und Snyder 2010). CO can be produced by the two isoforms of heme oxygenase (HO) and their function can be stimulated by stressors (Verma et al. 1993).

H_2S levels in our gut could always been explained by bacteria, which can form H_2S by reduction of sulfate or by decomposition of the sulfur amino acids methionine and cysteine or sulfur containing lipids or polysaccharides (Gadalla und Snyder 2010). However, H_2S got more attention when pathways were found on how H_2S could be generated by human and mammalian enzymes. After the discovery of the H_2S producing enzymes cystathionine beta-synthase (CBS) and cystathionine γ-lyase (CGL), it still took a while until it could be proven that endogenous levels of H_2S could have physiological functions. In the late 1990th it could be shown for the first time that the H_2S donor NaHS could induce the hippocampal long-term potentiation at physiologically relevant concentration in the low micromolar range (Abe und Kimura 1996). To definitely proof the physiological importance of endogenous H_2S, gene knock-outs had to be created. However, homozygous knock-outs of CBS in mice were shown to be fatal and heterozygous knock-outs led to hyperhomocysteinemia (Watanabe et al. 1995). In addition, the H_2S production was still significant due to CGL activity. In contrast, when the CGL knock-out could be developed in 2008, it led to major advancement. The deletion of CGL depleted most of the endogenous H_2S levels, leading to physiological changes in the vascular system. KO mice develop hypertension and this phenotype could be rescued by injection of exogenous H_2S (Yang et al. 2008). Ever since, evidence showing the physiological role of H_2S is increasing and H_2S now fulfills all the criteria for being characterized as a gasotransmitter (Wang 2002).

1.1.3. Physiological functions of H_2S

During the years, effects of H_2S have been shown on multiple different organs and tissues. The following are only some examples.

Cardiovascular System:

Similarly to NO, the primary action of H_2S on the vascular system is vasorelaxation, seen in numerous types of blood vessels (Kanagy et al. 2017). H_2S has been argued to be an endothelium-derived hyperpolarizing factor (EDHF), which is a substance that is generated in the endothelium and hyperpolarizes vascular smooth muscle cells, leading to vasodilation and subsequently lower blood pressure (Matoba et al. 2000). Exogenous and endogenous H_2S have also been showed to promote angiogenesis in endothelial cells (EC) in vitro and in vivo, stimulating growth, motility and organization of EC. One of the mechanisms are crosstalk with VEGF, the prototype angiogenic growth factor (reviewed in Kanagy et al. 2017).

Low H_2S has been reported in hypertension (Meng et al. 2015), atherosclerosis (Mani et al. 2013) or diabetes (Jain et al. 2012; Jain et al. 2010). Loss of H_2S production is at least partly responsible for the vascular dysfunction in cardiovascular diseases (Coletta und Szabo 2013; Bucci und Cirino 2011).

Nervous system:

H_2S has been shown to have a neuroprotective effect and could be connected to neurodegenerative diseases like Huntington's disease, Alzheimer's disease (Liu et al. 2008) or Parkinson's disease (Hu et al. 2010). In recent years H_2S has gained recognition for acting as a neurotransmitter and being involved in neuromodulation. It can modify the neurotransmitter receptors for glutamate and GABA (Wang 2012; Panthi et al. 2016). Another effect H_2S has on the brain through its anti-oxidative properties, suppressing oxidative stress (Whiteman et al. 2005).

Immune system and inflammation:

H_2S has been described as both pro-inflammatory and anti-inflammatory. The following example explains the difficulties in this area: LPS induces upregulation of CGL and an increase in H_2S. This could be viewed as pro-inflammatory because H_2S was generated in the process of an inflammation reaction, but it could also be anti-inflammatory if the

increase is a compensatory response and a protective mechanism (Li et al. 2005; Nagai et al. 2004).

There are also opposing outcomes in studies using sulfide salts like NaHS as H_2S donor and slow-releasing donors. The latter mostly act anti-inflammatory, decreasing inflammation in sepsis, pancreatitis, edema or in the GI tract. The concentration and administration routes are also important factors that can determine the outcome. Very few studies were focusing on endogenous H_2S, but also the use inhibitors led to controversial results (Whiteman und Winyard 2011).

Other areas where H_2S was shown to play an important role are in tissue repair (Dalgliesh et al. 2010), the digestive system (Linden 2014), the endocrine system (Zhu et al. 2011), respiration and mitochondria (Olson et al. 2010), aging and apoptosis (Wu et al. 2015; Chen et al. 2007) and the reproductive system (Srilatha et al. 2007; Zhu et al. 2011).

1.2. Endogenous H_2S production

Three enzymes are currently known to be able to produce H_2S in mammalian cells. Additionally there are non-enzymatic pathways which account for a small portion of produced H_2S.

CBS and CGL are enzymes involved in the transsulfuration pathway and they both need pyridoxal 5' phosphate (PLP), the active form of vitamin B_6 to produce H_2S. In recent years a third H_2S producing enzyme was discovered: 3-mercaptopyruvate sulfurtransferase (3MST). There is a lot of research ongoing to examine the regulation of these enzymes under physiological and pathological conditions (Wang et al. 2015).

1.2.1. CBS - Cystathionine beta synthase

CBS was first isolated in 1969 (Braunstein et al. 1969) and in humans it is present as a homotetramer. It is highly homologous between species, catalyzing the same reactions with similar kinetics (Singh et al. 2009).

CBS holds binding sites for PLP and heme at the N-terminus. The PLP binding domain is the catalytic domain, which makes the enzyme dependent on vitamin B_6. By a schiff base the PLP domain is linked to the heme domain (Kery et al. 1994).

At the C-terminus there is an autoinhibitory regulatory domain. Only when the activator S-adenosyl-L-methionine (SAM) binds at that region, does CBS undergo a conformational change and gets activated. If the regulatory domain is deleted, CBS stays active constitutively. Additionally, there is a calmodulin binding domain, which opens the catalytic domain when calmodulin, which has to be activated by calcium, binds to this consensus sequence (Stipanuk 2004).

The canonical reaction of CBS is the condensation of homocysteine and serine to produce cystathionine (*Figure* 1A). This is the first step in the reverse transsulfuration pathway where cysteine is synthesized from methionine. In alternative reactions CBS can produce H_2S, in this case it uses the substrates homocysteine and cysteine (*Figure* 1B) (Jhee und Kruger 2005; Singh et al. 2009). Additionally, Singh et al could show that CBS is also capable of generating H_2S from cysteine alone. However, the k_{cat} values of this reaction are lower than from cysteine and homocysteine, which is the main H_2S producing reaction under physiological conditions, accounting for 96% of the CBS derived H_2S (Singh et al. 2009).

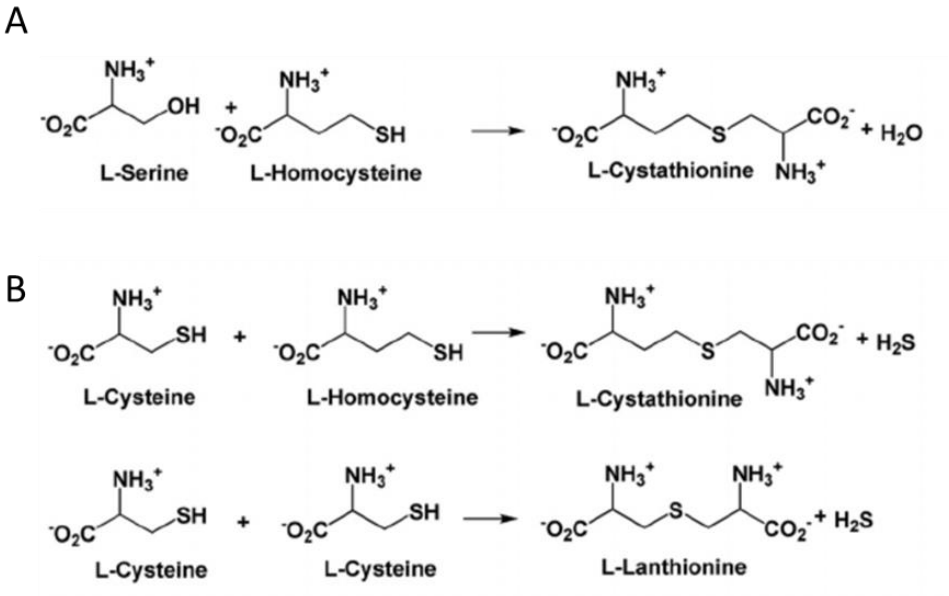

Figure 1 Reactions catalyzed by CBS (A) Canonical reaction of CBS in reverse transsulfuration pathway, using serine and homocysteine to produce cystathionine (B) Reactions catalyzed by CBS which produce H_2S. Modified from (Singh et al. 2009)

Expression of CBS is mainly found in the brain. Mostly in the hippocampus and cerebellum and not so much in the brain stem and cortex (Robert et al. 2003; Abe und Kimura 1996). It could also be shown that CBS is mainly expressed in astrocytes, less in neurons and not in microglia (Enokido et al. 2005).
Other tissues, like the respiratory system, cardiovascular system, testes and spleen show very low to no expression of CBS in mice, rats and humans.

Without CBS, cysteine gets an essential amino acid and extracellular supply is needed, since cysteine can't be produced from methionine via the transsulfuration pathway. It also leads to very high levels of homocysteine since CBS doesn't catabolize the homocysteine. This accumulation of homocysteine also occurs in humans with mutations in CBS. These patients suffer from the hereditary disease homocysteineuria and show high homocysteine levels in serum and urine (Jhee und Kruger 2005). More than 150 mutations in the human CBS gene have been found in homocysteinuric patients (Kruger et al. 2000).

1.2.2. CGL - Cystathionine gamma lyase

The second H_2S producing enzyme CGL occurs as a tetramer and two isoforms are known in humans, one showing an internal deletion, which is probably due to alternative splicing (Levonen et al. 2000). The enzyme is highly conserved between different organisms and even some plants express CGL. When 26 mammalian sequences were compared, 80% sequence similarities were found with especially homologous regions at the active site where PLP binds (Sun et al. 2009)

It has many similarities with CBS: they both are involved in the transsulfuration pathway, both need PLP as a cofactor and both are primarily localized in the cytosol (Ogasawara et al. 1994).

Each CGL monomer contains a large PLP binding domain and a calmodulin binding domain. PLP has to be bound to the enzyme to bring it into a closed form in order to produce H_2S (Sun et al. 2009). The substrate binds most to PLP, which is a common first step for PLP-dependent enzymes (Karsten und Cook 2002). Similarly to NO and CO, and like CBS, H_2S generation through CGL is activated by calcium-calmodulin. Calcium

activated calmodulin binds directly to the enzyme and increases its catalytic activity. (Yang et al. 2008)

CGL is expressed in different tissues like the cardiovascular and respiratory system, highly in liver, kidney, uterus, placenta and pancreatic islets (Hosoki et al. 1997; Zhao et al. 2001). Only little CGL was detected in the brain, suggesting that CBS and CGL are accountable for the H_2S production in different organs (Abe und Kimura 1996).

CGL is responsible for the second step of the reverse transsulfuration pathway to convert cystathionine to cysteine, α-ketobutyrate, and ammonia (CAVALLINI et al. 1962). In various other reactions CGL can catalyze the formation of H_2S from cysteine or homocysteine (*Figure* 2). Also cystine is thought to be a substrate for CGL to form thiocysteine and subsequently H_2S in an non-enzymatic reaction (CAVALLINI et al. 1962; Stipanuk 2004).

The substrates cystathionine, cysteine and homocysteine all bind to the same binding pocket of CGL, forming a schiff base with PLP (Kabil und Banerjee 2014).

Under physiological conditions, the elimination reaction from cysteine accounts for ~70 % of the produced H_2S from CGL and from homocysteine for ~29 % (Chiku et al. 2009). In patients with homocysteinemia, the homocysteine dependent reaction increases proportionally. However, the canonical reaction of the transsulfuration pathway, the cystathionine cleavage, is still the preferred reaction of CGL (*Figure* 2A). The reaction from cystine is probably not relevant under physiological conditions since cystine is not present in the reducing environment of the cell (Chiku et al. 2009).

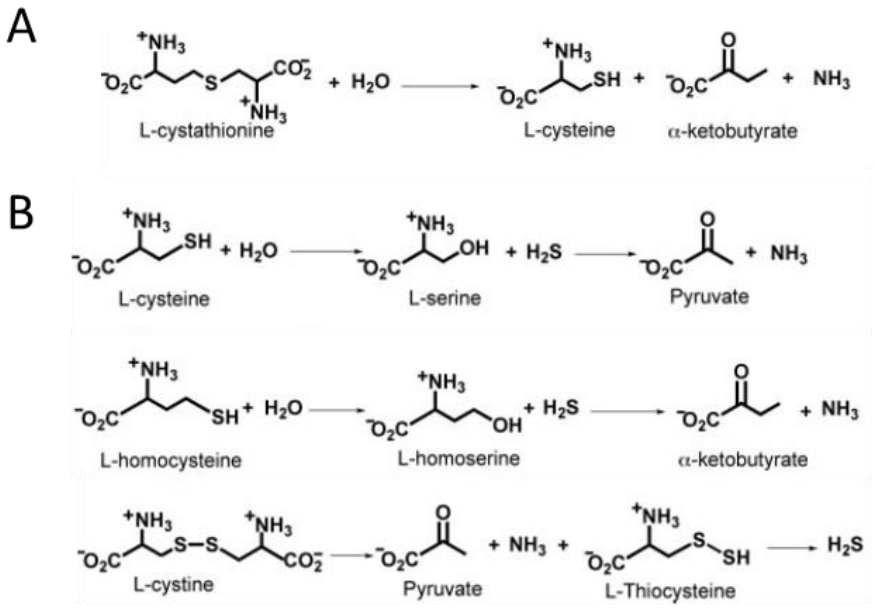

Figure 2 Reactions catalyzed by CGL (A) Canonical reaction of CGL in the second step of the reverse transsulfuration pathway, producing cysteine from cystathionine (B) H_2S producing reactions catalyzed by CGL, using cysteine, homocysteine or cysteine as substrates.
Modified from (Chiku et al. 2009)

CGL deficiency leads to cystathioninuria, which is like the homocysteinuria a hereditary disease, leading to high levels of cysthationine in the urine (Renga 2011). The two CGL mutations that have been further characterized, leading to cystathioninuria, both show a weakened affinity for PLP (Zhu et al. 2008).

In 2008 the first CGL knock-out mouse was generated and both the homozygous and the heterozygous mutants were viable, fertile and showed no difference in appearance. In both of them, endogenous H_2S levels in heart, aorta and also to a miner form in serum were reduced. The brain H_2S levels showed no differences in the mutants. CGL knock-out mice develop an age-dependent hypertension, which can be rescued with injections of exogenous H_2S.

1.2.3.3MST - 3-mercaptopyruvate sulfurtransferase

The most recently discovered H_2S producing enzyme is 3MST, which has to work together with cysteine aminotransferase (CAT) or D-amino acid oxidase (DAO) in order to produce H_2S.

3MST is expressed in kidney, liver, heart and brain (Nagahara et al. 1998). While CBS and CGL are present in the cytosol, 3MST is predominantly localized in the mitochondria but as well found in the cytosol (Yadav et al. 2013). CAT is also found in the mitochondria, while DAO is localized in peroxisomes. However, peroxisomes and mitochondria often have physical contact or are at least in very close proximity and they show high vesicular trafficking and exchange enzymes through this way (Shibuya et al. 2013).

CAT and DAO are able to produce 3-mercaptopyruvate (3MP) from cysteine and 3MST can take the sulfur from 3MP, binding it to its active-site cysteine residue forming an enzyme-bound persulfide and leaving behind a pyruvate anion (*Figure* 3). The persulfide can release H_2S in the presence of a reducing agent like dithiothreitol (DTT) (Shibuya et al. 2009). Since DTT is not present in our cells, Mikami et al were looking for physiologically relevant reducing substances and found that Thioredoxin (Trx) and dihydrolipoic acid (DHLA) in physiological concentrations are able to reduce 3MST. They have a similar active site to DTT with two redox-active cysteine residues and can release H_2S even better than DTT when added to 3MP mixed with the mitochondrial fraction of mouse brain. The H_2S production activity in the brain could be enhanced with the reduced form of Trx under physiological concentrations in a dose dependent way. Other physiological reducing substances like Glutathione (GSH), cysteine, NADH and CoA were tested, but they didn't show increased H_2S production, even the ones with a higher redox potential than Trx and DHLA and even at high concentrations, suggesting that a disulfide is needed to interact with 3MST and to release H_2S (Mikami et al. 2011).
However, Yadav et al showed that 3MST can also couple to monothiols like 2-mercaptoethanol, GSH, cysteine and homocysteine for the sulfur transfer. In contrast to dithiols, in monothiols two equivalents are needed to release H_2S from the persulfide. In this study they used purified 3MST enzyme, which could explain the discrepancy in the

previous results, where they used cell extracts where other enzymes could compete for the thiols (Yadav et al. 2013).

CAT produces 3MP from L-cysteine via a transamination reaction, which requires a NH_2 acceptor, for example α-ketoglutarate (α-KG). It's the suggested pathway for H_2S production in the brain, since H_2S was still produced in brain homogenates of CBS KO mice (Shibuya et al. 2009) and when no α-KG was present, the mitochondrial fraction produced only little H_2S (Mikami et al. 2011). 3MST is not PLP dependent like CGL and CBS, but CAT shows PLP dependence, which makes H_2S production through all these pathways requiring PLP (Shibuya et al. 2009).

In contrast, DAO doesn't require PLP to produce 3MP. The other major difference to the other H_2S producing pathways is that DAO utilizes D-cysteine instead of L-cysteine. L-cysteine is the form that is produced from methionine through reverse transsulfuration and there is no known pathway to transform L-cysteine into its racemate D-cysteine in our body. However, D-cysteine can be generated from L-cysteine by heat and alkaline conditions during food processing, which suggests that D-cysteine is present in our body upon food intake. DAO and 3MST are localized in kidney and cerebellum and this is also where H_2S production from D-cysteine could be confirmed (Shibuya et al. 2013).

The importance of both pathways involving 3MST still needs to be determined in vivo since in these in vitro experiments higher cysteine concentrations and optimal basic pH concentrations have been used.

Recently, Kimura et al showed that 3MST can produce H_2S_3, H_2S_2, and H_2S_5 in the brain, which can be characterized as polysulfides and are possibly involved in many signaling processes. With increased 3MP levels in the cells, the H_2S_3 levels dramatically increased in wild type mice, but not in 3MST-KO mice (Kimura et al. 2015).

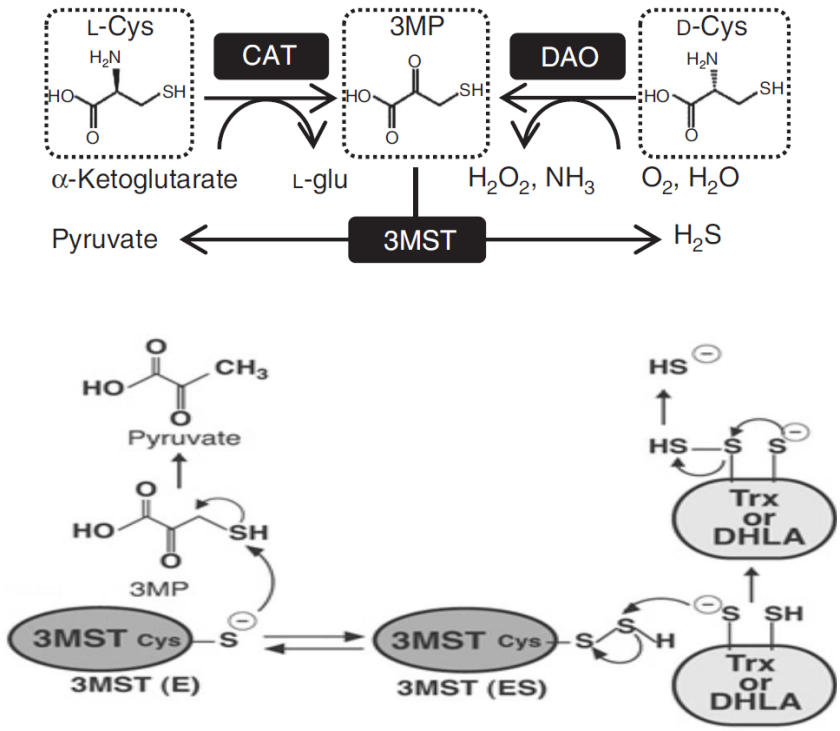

Figure 3 H_2S production by 3-MST (A) 3-mercaptopyruvate is produced enzymatically by CAT from L-Cysteine or by DAO from D-Cysteine. 3MST then leads to the production of H_2S from 3MP. **(B)** 3MST reacts with 3MP and forms an enzyme bound persulfide intermediate. Thioredoxin (Trx) or dihydrolipoic acid (DHLA) can reduce the persulfide releasing H_2S.
A: from (Shibuya et al. 2013), B: adapted from (Mikami et al. 2011)

1.2.4. Non-enzymatic H_2S production

Non-enzymatic H_2S production can occur through the reduction of sulfur containing compounds. In human erythrocytes, reduction of glutathione and elemental sulfur was observed when NADH or NADPH were present to serve as reducing equivalent (Searcy und Lee 1998).

Thiosulfide can be generated from sulfide through non-enzymatic oxidation and subsequently reduced to H_2S by different enzymes and possibly also non-enzymatically through a reductive reaction involving pyruvate (Kolluru et al. 2013). Most certainly also persulfides and polysulfides can release H_2S under suitable reducing conditions. Peng et al could recently confirm that H_2S can be released from polysulfides after oxidation through NADH models (Peng et al. 2017). H_2S release from iron-sulfur-cluster only happens under acidic and not under physiological conditions (Cuevasanta et al. 2017).

1.2.5. Transsulfuration pathway – the canonical reactions of CBS and CGL

Like mentioned previously, the main reaction catalyzed by the enzymes CGL and CBS are their non H_2S producing reactions in the reverse transsulfuration pathway.

Reverse transsulfuration and transsulfuration are the processes for interconverting the sulfur amino acids cysteine and methionine. In bacteria and plants transsulfuration takes place, transforming cysteine into homocysteine and then methionine. In vertebrates and funghi the opposite process of reverse transsulfuration occurs, generating cysteine as a final product. It is the only known route for biosynthesis of cysteine in vertebrates, turning cysteine into an essential amino acid upon lack of methionine or when there is a deficiency in the reverse transsulfuration pathway.

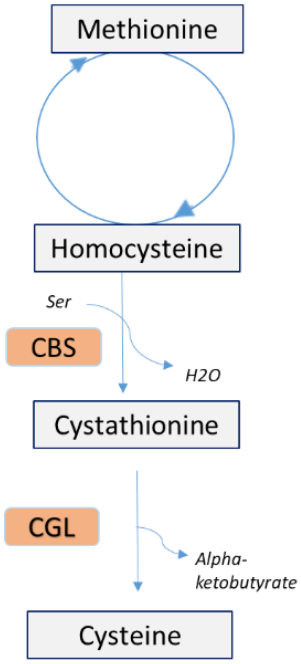

Figure 4 Reverse transsulfuration pathway. The reverse transsulfuration pathway in vertebrates converts methionine into cysteine. In the methionine-homocysteine-cycle methionine gets converted into homocysteine which can be reconverted back into methionine or enter the reverse transsurlfuration pathway, with its enzymes CBS and CGL to produce cysteine.

The conversion of methionine into homocysteine happens through the methionine homocysteine cycle. Besides getting reconverted back to methionine, most of the homocysteine enters the reverse transsulfuration pathway for the biosynthesis of cysteine.

CBS catalyzes the first step, the conversion of homocysteine with serine into cystathionine. This is an irreversible reaction, making the flow unidirectional from methionine to cysteine. CGL is able to hydrolyze the cystathionine to form cysteine, α-ketobutyrate and ammonia. While cysteine can be used for the synthesis of glutathione (GSH), taurine or the production of H_2S, α-ketobutyrate is further catabolized entering the tricarboxylic acid cycle (TCA) and producing ATP.

It is not clear how the enzymes are regulated to switch between the canonical transsulfuration reaction and the H_2S producing reactions. Cysteine is the product of the canonical CGL reaction but the substrate for the H_2S producing reaction. A similar question arises with CBS: Homocysteine is used for both reactions, together with serine for the canonical and with cysteine for the H_2S producing reaction. It is not known if and how CBS can switch its substrate preference from serine to cysteine (Kabil und Banerjee 2014).

This also leads to further questions and to a potential reason why the CBS contribution to H_2S production could be higher than expected. In a CGL knockout, cysteine synthesis via the transsulfuration pathway is inhibited, leading to a lack of cysteine, which is one of the substrates needed for H_2S production through CBS (Kabil und Banerjee 2014)

1.2.6. Pharmacological inhibitors of H_2S producing enzymes

Different inhibitors of the H_2S producing enzymes CGL and CBS, are known and have been widely used. While some of them are specific for CGL, others inhibit both. Until now no specific CBS inhibitor and no inhibitor for 3MST is available.

Propargylglycine (PAG)

PAG is an antibiotic produced by *Streptomyces sp.* and is a specific inhibitor for CGL. PAG irreversibly binds to CGL and doesn't allow the substrate to bind to the enzyme's active site. It also binds to a tyrosine residue that facilitates the substrate release (Sun et

al. 2009).

IC_{50} for PAG have been reported with 40 to 55 µM (Asimakopoulou et al. 2013; Mok et al. 2004) However, when used in functional assays, millimolar concentrations are needed to inhibit CGL activity, typically 1-10mM (Asimakopoulou et al. 2013). It can also be used for injection into animals at concentrations of 20-100mg/kg, where it was shown to suppress H_2S production in vascular tissues (Zhao et al. 2003).

PAG doesn't inhibit CBS and seems to be selective for CGL, but it is possible and has been reported that it can also inhibit other enzymes and reactions in the high concentrations which are needed to fully block CGL (Asimakopoulou et al. 2013).

Aminooxyacetic acid (AOAA)

AOAA is a general aminotransferase inhibitor, inhibiting both CGL and CBS. It has also been suggested to inhibit CAT, which also makes it an inhibitor for the third pathway of endogenous H_2S production (Teraoka et al. 1993). In other fields, AOAA has also been used as inhibitor of oxidative phosphorylation, protein synthesis and amino acid transport, which makes its use in functional assays problematic (Whiteman et al. 2011).

Hydroxylamine (HA)

HA is an intermediate of biological nitrification and is a product of the enzymatic oxidation of NH_3. It has been used as a general inhibitor of for haem-containing enzymes in higher like CBS eukaryotes. However, Asimakopoulou et al shows that HA inhibits CBS but is 60-fold more selective towards CGL (Asimakopoulou et al. 2013).

B-cyano-L-alanine (BCA)

BCA is also handled as CGL inhibitor, in contrast to PAG it binds CGL reversible. BCA is a neurotoxic agent in poisonous legumes and it can lead to neurolathyrism in humans (Whiteman et al. 2011).

BCA is thought to avert binding of the cofactor PLP to the enzyme (Whiteman et al. 2011). Similarly to PAG, millimolar concentrations are needed to completely inhibit CGL and this

means that probably other enzymes are also inhibited. Asimakopoulou et al. reported that concentrations above 1 mM also inhibit CBS.

1.3. Catabolism and H_2S signaling

Cellular H_2S levels are not only regulated by its biosynthesis but also by the rates of oxidation and detoxification, which mostly takes place in the mitochondria. In mitochondrial H_2S oxidation, four enzymes are known to be involved, forming persulfide, sulfite, thiosulfate and sulfate (*Figure* 5) (Tiranti et al. 2009).

H_2S was also reported to directly oxidize to thiosulfate when O_2 and transition metals are available. Another way leading to less H_2S production is, if the substrate cysteine is depleted, for example via cysteine dioxygenase (CDO) (Rose et al. 2017).

In recent years it was found that H_2S interacts with –SH groups of cysteine in proteins and that this is a common post-translational modification, mediating various reported physiological effects of H_2S (Mustafa et al. 2009). This binding to proteins or to other sulfides could also be a way to store H_2S.

1.3.1. Mitochondrial H_2S oxidation

In the first step of mitochondrial H_2S oxidation, the flavoprotein sulfide quinone oxidoreductase (SQR) in the internal mitochondrial membrane transfers electrons from H_2S to the coenzyme Q in the electron transport chain (ETC). SQRs contain flavin adenine dinucleotide (FAD) that acts as a cofactor and accepts two electrons from H_2S (forming $FADH_2$) and transferring them to ubiquinone, entering the ETC. H_2S was the first inorganic reducing substrate found for oxidative phosphorylation in mammals (Goubern et al. 2007). The oxidation of H_2S by SQR leads to a protein bound persulfide as an intermediate and the persulfide is then transferred to an acceptor. Sulfite and GSH have been proposed as physiological persulfide receptors. Transfer to GSH leads to the generation of GSH persulfide which is a substrate of the sulfur dioxygenase ETHE1, that transforms the persulfide into sulfite (Libiad et al. 2014). It has also been suggested that ETHE1 can act directly on the SQR-persulfide and turn it into sulfite without the transfer to an intermediate

molecule like GSH (Kabil und Banerjee 2014). Transfer of a persulfide to sulfite happens through a protein of the rhodanase family, leading to the formation thiosulfate (Jackson et al. 2012).

In mammals ETHE1 and the sulfurtransferase rhodanese are both located in the mitochondrial matrix and in some prokaryotes, they even form fusion proteins, suggesting that their activities are linked (Melideo et al. 2014). Sulfite can be further oxidized to sulfate by the enzyme sulfite oxidase.

High but sublethal doses of administered sulfide in mammals are oxidized right away and excreted as thiosulfate and sulfate (Curtis et al. 1972). Rates and distribution of thiosulfate and sulfate are very tissue specific. While in rat liver mostly sulfate was created, in the lung it was predominantly thiosulfate and in the kidney a mixture of both (Bartholomew et al. 1980).

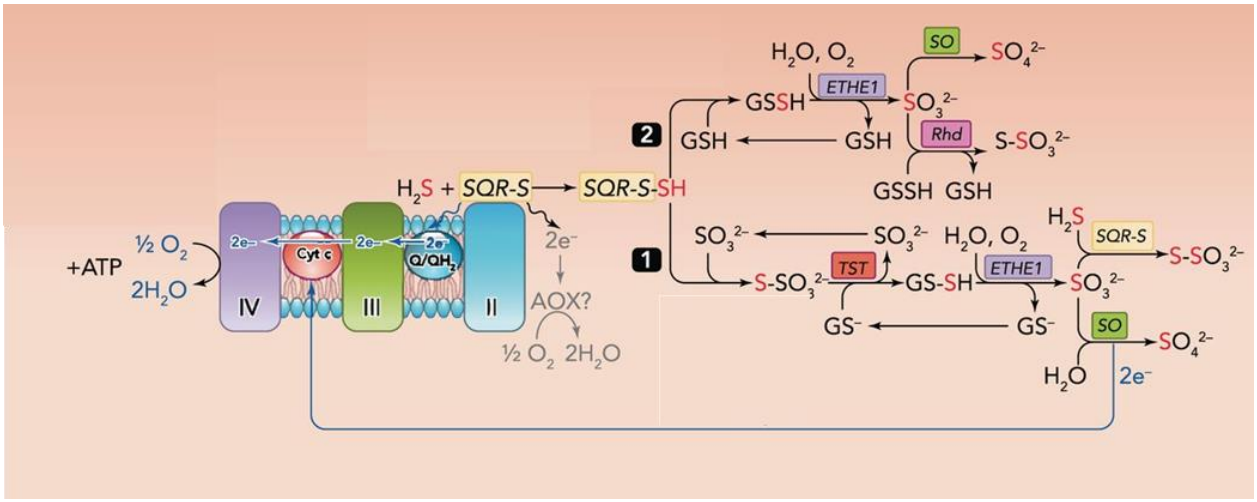

Figure 5 Mitochondrial H_2S oxidation. H_2S binds to the enzyme sulfur quinone oxidoreductase (SQR) forming a persulfide (SQRS-S). During this process electrons are transferred into the electron transport chain through ubiquinone (Q). The persulfide can either be transferred to glutathione (GSH) or sulfite ($S_2O_3^{2-}$), forming glutathione persulfide (GSSH) or thiosulfate ($S_2O_3^{2-}$). GSSH can then be oxidized by the sulfur dioxygenase ETHE1 to sulfite, which can be further oxidized by sulfite oxidase to sulfate (SO_4^{2-}). The enzyme rhodanase (Rhd) can also catalyze the formation of thiosulfate from sulfite and GSSH.
Adapted from (Olson und Straub 2016)

It is interesting that H_2S can on the one hand act as a substrate for the coenzyme Q of the ETC, but on the other hand is very toxic to the complex IV at high concentrations, leading to a block of the ETC. Additionally, in low concentrations, H_2S can stimulate electron transport in the mitochondria through inhibiting the phosphodiesterase 2A and

subsequently increasing the cAMP concentration (Módis et al. 2013). This suggests a high dose dependence of H_2S with low levels increasing mitochondrial oxygen consumption and high concentrations decreasing it or blocking it completely.

Mutations in the ETHE1 gene lead to the hereditary disease ethylmalonic encephalopathy. Mouse knockouts of ETHE1 lead to the same features and highly elevated amounts of thiosulfate is excreted. In KO tissues concentrations of thiosulfate and sulfide were very high (Tiranti et al. 2009).

1.3.2. Direct reactions of H_2S with oxidants

Its reduction potentials allows H_2S to react with different biologically relevant oxidants, producing sulfate (SO_4^{2-}), sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$) and elemental sulfur (S_8). These reactions with oxygen are pH-dependent and require a trace metal (O'Brien und Birkner 1977; Cuevasanta et al. 2017).

One-electron oxidants lead to the generation of sulfanyl radical ($HS^{\bullet}$) which can react with different electron donors like GSH, with another $HS^{\bullet}$ or with O_2. Lots of these reactions are parts of radical chain reactions. Also two-electron oxidants are able to react with H_2S. This shows that oxidation of H_2S can be biologically relevant, however, alternative reductants like thiols are usually available in tissues at higher concentrations than H_2S. Probably H_2S consumption through these reactions is only of minor importance. Nevertheless, the scavenging of reactive oxygen species through H_2S, making it an antioxidant, is probably of high importance (Cuevasanta et al. 2017).

1.3.3. Binding and reducing of metal centers

H_2S can bind to metal centers and reduce them. The iron atom in the center of hemeproteins like the cytochrome c oxidase, hemoglobin or myoglobin act as an electron acceptor and oxidized sulfur species are formed (Vitvitsky et al. 2015; Bostelaar et al. 2016). Ferric hemoglobin and myoglobin oxidizing H_2S can also lead to iron-bound polysulfides through a complex mechanism (Bostelaar et al. 2016).

Binding of H_2S to ferric heme can also lead to the formation of stable complexes and inhibition of the enzyme. This is the case in the block of the electron transport chain through binding of H_2S to cytochrome c oxidase (Guidotti 2015).

Also the binding of zinc or copper centers has been reported. Centers in Cu/Zn superoxide dismutase can be reduced by H_2S, and angiotensin converting enzyme can be inhibited through binding of H_2S to its zinc center (Talaei et al. 2012).

1.3.4. S-sulfhydration

H_2S can react with –SH groups to form persulfides in a reaction called “sulfhydration”, which has recently been categorized as a novel posttranslational modification for proteins. Through this process, similar to nitrosylation with NO, H_2S is suggested to have signaling properties. Sulfhydration can occur at the –SH group of reactive cysteines in proteins, forming protein persulfides (Paul und Snyder 2015). It largely takes place in vivo and has been described in enzymes, receptors, transcription factors and ion channels, promising broad physiological effects (Meng et al. 2017). 10-25% of extracted liver proteins were shown to be S-sulfhydrated under physiological conditions (Mustafa et al. 2009).

Many reported physiological actions of H_2S could go back to this mechanism, for example the persulfidation of K_{ATP} leading to vasodilation or of GAPDH and NF-κB inhibiting apoptosis (Filipovic 2015). In various diseases and pathological conditions, like cardiovascular diseases or neurodegeneration, it was shown that sulfhydration patterns were different (Paul und Snyder 2015). Persulfides are not very stable, making them hard to detect and to investigate the mechanism of S-sulfhydration.

S-sulfhydration by H_2S can occur on cysteine sulfidic acids or cysteine disulfides and under oxidation conditions also on cysteine thiols. Under physiological conditions, H_2S cannot react with thiols because they both have an oxidation state of -2.

Recently is has been suggested that sulfane sulfurs, as prevalent in persulfides or polysulfides, may play a more important role in S-sulfhydration than H_2S itself. The inner sulfur of polysulfides is 0, which favors binding to other sulfur atoms like thiols (Meng et

al. 2017; Takano et al. 2016). A lot of H_2S mediated reactions and health benefits may therefore be mediated by polysulfides.

1.3.5. Interplay of H_2S with NO

It has been reported that H_2S can crosstalk with NO, leading to synergistic effects and to angiogenesis (Coletta et al. 2015), vascular smooth muscle relaxation and also the control of the intestinal contractility (Teague et al. 2002). The interaction of H_2S and NO leads to the generation of HNO and SSNO. The former can activate transient receptor potential Ankyrin 1 (TRPA1) channels (Eberhardt et al. 2014), the second relaxing vascular smooth muscles (Cortese-Krott et al. 2015).

In a lot of these studies high levels of polysulfides were detected and Miyamoto et al could recently show that polysulfides (H_2S_2 and H_2S_3) can be generated by the interaction of H_2S and NO. They also showed that these polysulfides are mediating as signaling molecules and activate TRPA1 channels (Miyamoto et al. 2017). The polysulfide generation is very fast since H_2S immediately reacts with NO when present.

1.4. H_2S and dietary restriction

Dietary restriction (DR) is the oldest intervention known to increase lifespan, improve metabolic fitness and stress resistance in a variety of species. It slows aging and age-associated diseases, imparting an improvement in health and reducing risk factors for diabetes, cardiovascular disease and cancer (Fontana et al. 2010).

DR can be a reduction of total food intake, also called caloric restriction (CR), or restriction of dietary components like proteins (protein restriction - PR) or specific amino acids such as methionine (methionine restriction - MR) without enforced food restriction. Also temporal variations of food intake like intermittent fasting are dietary restriction regimens. In rodents, MR also needs a restriction in cysteine levels.

CR effects have been reported in yeast, flies, worms, rodents, monkeys and humans (reviewed by Fontana et al. 2010; Katewa und Kapahi 2010). Also MR could extend

lifespan from yeast to rodents (Ruckenstuhl et al. 2014; Grandison et al. 2009; Miller et al. 2005), but it is not clear to what degree amino acid restriction on ad libitum basis and total caloric restriction share underlying mechanisms of protection. In flies and mice, addition back of methionine or cysteine could abrogate the effects of CR, suggesting that the mechanisms are overlapping (Grandison et al. 2009; Hine et al. 2015).

Recently, the Mitchell lab could find a link between endogenous H_2S by the transsulfuration pathway and DR in multiple organisms. DR, which led to longevity extension in yeast, flies and worms and to improved stress resistance in rodents, resulted in increased H_2S production capacity (Hine et al. 2015). When the CGL inhibitor PAG was used to block H_2S production, benefits of DR on stress resistance, using a hepatic ischemia reperfusion injury (IRI) model, were abrogated and CGL knock out mice didn't gain protection upon DR. On the other hand, CGL overexpression via adenoviral-mediated gene delivery in mice led to increased H_2S production and in improved outcome upon hepatic IRI (Hine et al. 2015).
Prior to this, Kabil et al could already connect CBS expression and activity with dietary restriction benefits in fruit flies. Overexpression of CBS could increase the lifespan independent of diet, and knock down of CBS abrogated the DR mediated lifespan extension (Kabil et al. 2011).
This suggests that increased TSP and increased H_2S production is at least partly responsible for the benefits of DR.

However, the question arises if the beneficial effects of an increase in the enzymes of the TSP are due to increased H_2S production or through influencing cysteine and methionine levels. Methionine affects protein translation since it is always the first amino acid incorporated into the growing polypeptide chain and also methylation, which plays a role in gene silencing. Cysteine is a precursor for glutathione (GSH), the most important regulator of the redox state of the cell. People with genetic defects in the TSP show high homocysteine and low GSH levels and have an increased predisposition for age-related diseases (Beyer et al. 2004; Isobe et al. 2005).

Evidence for the relevance of H_2S in DR mediated protection was found by the Mitchell group when they showed that exogenous H_2S prior to ischemia in ad libitum fed mice was

sufficient for protection in this model of hepatic IRI and showed less liver damage (Hine et al. 2015). Also in worms it could be shown that exogenous H_2S can extend the lifespan of worms (Miller und Roth 2007). In mice and rats dietary restriction upregulated the TSP and H_2S levels, but not GSH, suggesting that H_2S production is the main reason for upregulation of the enzymes of the TSP (Hine et al. 2015; Wang et al. 2016).

1.5. Intracellular H_2S pools

H_2S that is not directly catabolized is either free sulfur or can be stored in cells and released or mobilized in response to different triggers. The two main forms of sulfur storage are acid-labile sulfur and bound sulfur, also called sulfane sulfur.

1.5.1. Free sulfur

Free sulfur is dissolved H_2S gas and in solution there is an equilibrium $H_2S \leftrightarrow HS^- \leftrightarrow S^{2-}$. With pK_as of 7 and 17 there is no S^{2-} present in biological tissues and at physiological conditions at 37°C and pH 7.4 intracellular there should be about equal amounts of H_2S and HS^- and extracellular around 20 % H_2S and 80 % HS^-. Free H_2S levels are suggested to be very low due to its fast reactions with other molecules (Wang 2012).

1.5.2. Sulfane sulfur

In sulfane sulfur, also called bound sulfur, a sulfur is covalently bound to another sulfur. This is the case in polysulfides (R-S-S_n-R) and persulfides (-SSH), thiosulfate (S_2O_3) and elemental sulfur (S_8).

Bound sulfur can be easily liberated as hydrogen sulfide ions (HS^-) under reducing conditions, for example after addition of DTT. Upon exogenous application of free H_2S to homogenates of brain, liver and heart, more H_2S could be released upon treatment with DTT (Ishigami et al. 2009). This shows that applied H_2S can be stored in sulfur structures, suggesting the same for endogenously produced H_2S that is not directly utilized in signaling processes. It could be shown that the amounts of bound sulfur increased upon overexpression of CAT/MST (Ogasawara et al. 1994) and CSE (Mustafa et al. 2009) when cysteine was present.

The mechanism of this absorption of H_2S and production of sulfane sulfur is probably mostly through post-translational sulfhydration of proteins or the consequence of the mitochondrial oxidation pathway. Time of absorption of exogenous H_2S varied between different tissue homogenates and also the release with DTT was slower in some tissues compared to others (Ishigami et al. 2009).

Recently an enzyme was found that is able to synthesize persulfides from the substrate cysteine. Cysteinyl-tRNA synthetases (CARSs) were shown to be the principal cysteine persulfide synthases in mammals and can mediate mitochondrial functions through this action (Akaike et al, in press).

Another possibility to produce polysulfides is directly through the H_2S producing enzyme 3MST, which was recently reported to be able to produce H_2S_3, H_2S_2, and H_2S_5 (Kimura et al. 2015). These polysulfides are small, probably diffusible, not bound to proteins and involved in many signalling reactions (Kimura 2016). When 3MST is producing H_2S, an enzyme bound persulfide is always an intermediate, since only in the presence of a reductant H_2S is released. Persulfide transmission between proteins is an evolutionary conserved reaction and could also be occurring in this case (Greiner et al. 2013; Mikami et al. 2011).

Also other groups could show that bound sulfur can have signaling properties and physiological roles in mammalian cells (Koike et al. 2015; Kimura et al. 2013; Greiner et al. 2013). Now it is presumed that some of the reported effects of H_2S may actually have been due to polysulfides.

Like previously mentioned, thioredoxin and dihydrolipoic acid can reduce persulfides and release H_2S under physiological conditions. Other physiological reducing compounds like cysteine and glutathione could lead to a release under alkaline conditions, where their reducing capacity is increased (Miyamoto et al. 2017). If H_2S is stored in bound sulfur structures, it can be presumed that the release is also controlled, regulating the amount of bioavailable free sulfide (Ishigami et al. 2009). Others however think, that bound forms are more important to store exogenously administered sulfide, removing toxic concentrations from circulation (Wintner et al. 2010).

1.5.3. Acid-Labile Sulfur

Acid-labile sulfide occurs mainly in the form of iron-sulfur complexes (Fe-S). The name already tells that acid-labile sulfur is released under acidic conditions. In vitro it could be shown that 5.4 was the critical pH to release H_2S from iron-sulfur clusters and the greatest release could be observed at a pH of 1.5 (Ishigami et al. 2009).

Since iron-sulfur clusters are mostly prevalent in mitochondrial enzymes and the pH of mitochondria is at all time above 7, there is no release of H_2S under physiological conditions.

1.6. Detection of endogenous H_2S

Its detection is one of the major difficulties of working with H_2S. H_2S is volatile and reacts very fast and it can be bound to other molecules for storage. Therefore, besides the measurement of free sulfide, the sulfur pools or H_2S production can be measured.

1.6.1. Measuring free sulfide

The main methods to detect free H_2S in tissue, cells or body fluids are the use of spectrophotometry, sulfide ion-specific or polarographic electrodes or chromatography with subsequent mass spectrometry. Recently more and more fluorescent probes are designed and used to detect H_2S.

In the past, the methylene-blue method has been the most commonly used technique to measure sulfide levels. Originally developed in 1883 for the detection of sulfide in aquatic samples (Fischer 1883), it has been modified for the use in biological samples. The dye is formed by the reaction of H_2S with a metal, subsequent acidification and reaction with N,N-dimethyl-p-phenylenediamine (NDPA) and can be detected by a spectrophotometer at 670 nm. It has been argued that the sensitivity for low H_2S concentration is not good and that through the acidification step H_2S can be liberated from other forms of sulfide like the acid labile pool (Kolluru et al. 2013).

For detecting H_2S in blood or cell culture media, sulfide-ion specific electrodes (ISEs) have been widely used. They are easy to use and show low detection limits. Since they are only sensitive to S^{2-}, alkaline conditions are needed to shift the equilibrium from H_2S to S^{2-}.

This could again lead to erroneous elevated measurements due to the release of H_2S from proteins due to desulfuration (Whitfield et al. 2008).

Similarly to other gas molecules like O_2, NO or CO, there are polarographic H_2S sensors for measuring H_2S levels in real time. Its use has been reported on cellular, tissue and organ levels and the sensitivity is high. They are highly specific for free H_2S and not for other sulfide pools since anode, cathode and electrolyte are behind a permeable membrane where only free H_2S can pass (Doeller et al. 2005).

Different fluorescent dyes have been developed to detect H_2S. Several are based on the reduction of different azide or nitro compounds by H_2S or another reactions of H_2S with a compound that leads to a fluorescent product (Singha et al. 2015; Yu et al. 2012; Montoya und Pluth 2012; Das et al. 2012). However, most of them show pretty high detection limits and high background signals. Most have excitation-emission wavelengths between 450 and 700 nm, near tissue autofluorescence wavelengths (Guo et al. 2015).

A widely used method to detect H_2S is chromatography, nowadays mostly HPLC (high-performance liquid chromatography) is used. HPLC analysis can be conducted after sulfide conversion to methylene blue or to the monobromobimane derivative or after labeling with o-phthalaldehyde (OPA) (Wang 2012).
Very low detection limits of 5 nm are achieved with a method combining MBB and HPLC. Under basic conditions monobromobimane reacts very rapidly with H_2S, producing sulfodibimane (SDB), a very stable and more hydrophobic compound. With reverse phase HPLC, SDB is separated and analyzed by fluorescent detection (Shen et al. 2011).

1.6.2. Measuring the activity of H_2S producing enzymes

Instead of measuring the absolute H_2S that is present, the H_2S production through its enzymes can be measured. Usually these assays are in vitro assays, adding the substrates and cofactors in excess. Therefore it's also not the actual H_2S production in the tissue but more the potential activity of H_2S generating enzymes, also called the H_2S production capacity. Tissue or cell samples are homogenized and incubated with cysteine and the cofactor PLP and H_2S generation at 37°C is measured. Measurement can be done by transformation of the H_2S to methylene blue or by capturing H_2S with a lead

acetate paper. Lead acetate forms upon reaction of H_2S with lead sulfide, which is a dark precipitate (Stipanuk und Beck 1982; Wang 2012; Hine und Mitchell 2015).

1.6.3. Measuring bound sulfur levels

H_2S or HS^- are very reactive within biological matrices, suggesting that most of the sulfide is present in sulfur pools.

To measure the bound sulfur, H_2S is released from its pools with a reducing agent like DTT and then quantified with a method like MBB (Togawa et al. 1992). Alternatively to DTT, the reducing agent tris (2-carboxyethyl) phosphine hydrochloride (TCEP) can be used to cleave the disulfide bonds and liberate the sulfur. TCEP has the advantage that it can be used in a wide range of pH and it doesn't contain a thiol moiety which could lead to sulfide contaminants (Han und Han 1994; Shen et al. 2012).

Recently, due to the evidence that persulfides and polysulfides play an important role in signaling processes, different methods based on biotin-switch were developed to detect protein S-sulfhydration. The first described method involves alkylation of the proteins to differentiate between thiols and persulfides by blocking the thiols, and subsequent conjugation to biotin (Mustafa et al. 2009). In two other methods they suggest that both thiol (-SH) and persulfide (-SSH) units are blocked by an alkylating agent, then persulfides are reduced by DTT to distinguish them and subsequently they are labeled with biotin (Krishnan et al. 2011; Dóka et al. 2016). An additional method blocks persulfides and thiols in a first step, the separation is then conducted with a tag-switch reagent that uses the enhanced reactivity of persulfides to certain nucleophiles to label only the persulfides (Zhang et al. 2014).

1.6.4. H_2S measurements in blood serum/plasma

Over the years the reported levels of free H_2S in blood have varied a lot between studies. Mostly the reported values have been in the range of 20-50 µmol/l (Suzuki et al. 2017), but also more and more studies show levels below 10 µM and down in the nanomolar range (Shen et al. 2012; Peter et al. 2013; Kovačić et al. 2012; Whitfield et al. 2008). Differences are mostly due to the different measurement techniques. Most methods, like the common methylene blue method are very harsh and could include release of stored

H_2S, leading to higher levels (Olson 2009). The previously described very sensitive method combining MBB, HPLC and fluorescent detection is now widely used and reports free H_2S levels in plasma of around 1 µM and below (Wintner et al. 2010; Shen et al. 2011).

Also other problems occur when working with plasma. Storage and freeze-thaw cycles could lead to a loss of free H_2S and hasn't been determined. Additionally, Peter et al claim that the used anticoagulant can change the measurement. Using EDTA collection tubes, they measured four fold higher H_2S in healthy blood donors than with lithium heparin collection tubes, which they attributed to increased hemolysis (Peter et al. 2013).
In vitro, the fast decay of H_2S could be shown after administration of exogenous sulfide in culture solution. The H_2S concentration rapidly decreased from micromolar concentration to undetectable levels (Hu et al. 2009). Another study used ^{35}S-sulfide and measured it's consumption by blood after injection into rats. After 5 minutes only 11% remained in the blood. They could also show in vitro that 5 minutes after the addition of ^{35}S-sulfide to blood, over 70% was bound to protein, half of it to plasma proteins, the other half to proteins in red blood cells (Curtis et al. 1972) .

The enzymes CBS and CGL have been reported to be present in human plasma in low concentration and their activities could be detected by HPLC-MS (Krijt et al. 2011; TAGUCHI et al. 1995).

1.7. Previous work leading to this work

The Mitchell group widely used the lead acetate method to compare H_2S production capacity between tissue samples. With this method it could be shown that dietary restriction increases the H_2S production capacity.

Like previously described, H_2S is strongly related to vascular health, but measurement of H_2S levels in plasma or serum is still difficult, time consuming and often erroneous. Since H_2S producing enzymes are present in plasma and enzymatic activities could be detected, it was attempted to use the lead acetate method to detect H_2S production capacity in plasma. With high cysteine levels a signal could be detected and subsequently this method was used to compare H_2S production capacity between patients with vascular

disease and their age-, sex- and hypertension matched controls. The 115 patients had peripheral artery disease and needed to undergo surgery (carotid endarterectomy, open lower extremity arterial revascularization or major leg amputation). In the patients with vascular diseases, H_2S production capacity was significantly reduced compared to the healthy controls. Additionally, a difference in survival after surgery could be detected: preoperative plasma H_2S production capacity was significantly lower in patients with mortality during a 2 year follow up than in those alive and the percentage of survival in patients with H_2S production levels lower than the median was lower compared to patients with H_2S levels higher than the median.

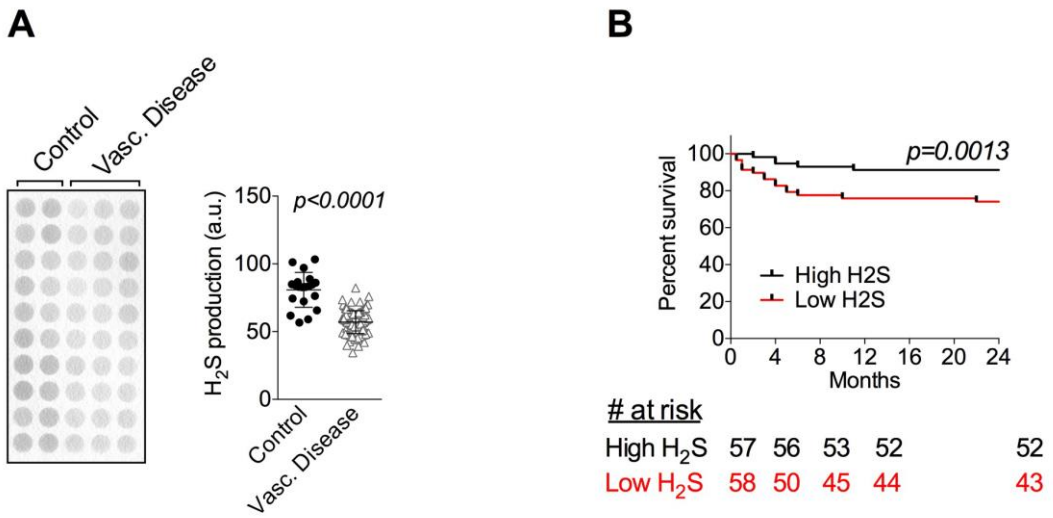

Figure 6 Circulating H_2S production is reduced in patients with vascular disease and correlates with survival. (**A**) Representative plasma H_2S production capacity (**left**) and quantification (**right**) from human patients suffering vascular disease (n=115) versus healthy age-matched individuals (n=20) as detected by the lead acetate method. (**B**) Kaplan-Meier survival curve for patients with low and high plasma H_2S before undergoing vascular surgery procedure. P value is derived from log-rank calculation.

1.8. Aim of this work

The aim of this work was to further characterize the lead acetate assay in serum/plasma, measuring H_2S production capacity and confirm the correlation between H_2S production capacity and vascular health.

During this process we discovered, that the added cysteine is not used as the substrate for the H_2S producing enzymes like in liver extracts but makes use of its reducing capacity to slowly release H_2S from sulfane sulfur in the serum. We could confirm a relation

between H_2S and vascular health, showing reduced sulfane sulfur and free sulfide levels in patients with peripheral artery disease.

In the second part of this work we wanted to examine the effects of different dietary restriction regimens, which lead to lifespan extension and various health benefits, on hepatic H_2S production capacity. While caloric restriction was previously shown to increase H_2S production capacity, we could show that there is no alteration upon protein restriction. In contrast, methionine restriction does increase H_2S production capacity but probably through a different mechanism than caloric restriction.

We could show once more the physiological relevance of H_2S and its potential importance on health and disease in humans. Furthermore we could confirm that endogenous H_2S production can be modulated by factors like diet.

2. Material and methods

2.1. Materials

2.1.1. Biological material

All measurements in liver were conducted with mice livers and all mouse work was performed with the approval of the Harvard Medical Area Institutional Animal Care and Use Committee (IACUC). If not mentioned otherwise, wildtype mice with a B6D2/F1 hybrid background (purchased from Jackson laboratories) were used. CGL knockout livers or serum/plasma are from mice bearing a targeted gene disruption of the CGL gene and are from Dr. Rui Wang at the University of Saskatchewan, Canada (Yang et al., 2008).

For serum and plasma measurements either mouse serum or plasma (with EDTA as anticoagulant) or cosmic calf serum (purchased from GE Healthcare Life Sciences) was used. Human plasma of the PAD patients were obtained from Keith Ozaki from the Brigham and Women’s hospital in Boston.

2.1.2. Chemicals and kits

Table 1. Chemicals and substances used in this work

Chemical	Source
Aminooxyacetic acid (AOAA)	Sigma
Beta-mercaptoethanol	Thermo Fisher (21985023)
Chloroform	Sigma (288306)
D-Cysteine	Sigma (30095)
DL-Dithiothreitol	Sigma (43815)
DNase I	Thermo Fisher
EDTA (0.5M, pH 8)	Corning (46-034)
Ethanol	Koptec (V1001)
L-Cysteine	Sigma (C7352)
L-Homocysteine	Sigma (69453)
Lead (II) acetate trihydrate	Sigma (215902)
Methanol	VWR (BDH-1135-1LP)
PageRuler™ Prestained Protein Ladder	Thermo Scientific (26616)
Proteinase K Solution (20 mg/mL)	Thermo Scientific
Pyridoxal 5'-phosphate hydrate	Sigma (P9255)
Reduced Glutathione	Sigma (G4251)
Sodium hydrosulfide hydrate (NaHS)	Sigma (161527)

Table 2. Purchased kits used in this work

Kits	Source
BCA Protein Assay Kit	Pierce/Thermo Fisher (23227)
SilverQuest Silver Staining Kit	Invitrogen/Thermo Fisher (LC6070)
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific/ Thermo Fisher (34095)

Table 3. Antibodies used in this work

Antibody	Source
CGL	Abcam (ab151769)
CBS	Abcam (ab135626)
GAPDH	Santa Cruz (365062)
Vinculin	
HRP conjugated goat anti-rabbit	Dako (P0448)
HRP conjugated goat anti-mouse	Dako (P0447)

2.1.3. Buffers

Table 4. Buffers used in this work

Buffer	Source/Composition
Phosphate buffered saline	Corning/VWR (45000)
Passive Lysis Buffer	Promega (E1941)
Laemmli sample buffer (2x)	Biorad (1610737)
Electrophoresis Buffer	250 mM Tris, 1.92 M glycin, 1 % SDS
Transfer buffer	25 mM Tris, 192 mM glycin, 10% methanol
10x TBS(-T), pH 7.4	200 mM Tris, 150 mM NaCl, 0.2% Tween 20
Antibody binding Buffer	5 % BSA in TBS-T
Stacking Gel	5 % acrylamide mix, 125 mM Tris (pH 6.8), 0.1 % SDS, 0.1 % APS, 0.01 % TEMED
Running Gel	12.5 % acrylamide mix, 375 mM Tris (pH 8.8), 0.1 % SDS, 0.1 % APS, 0.01 % TEMED

2.1.4. Laboratory equipment

Table 5. Expendable material used in this work

Expendables	Source
96-well plate	Corning
384-well plate	Corning
8-strip well format tubes	Denville
Whatman Filter Paper	Sigma Aldrich
20K MWCO Protein Concentrators	Pierce/Thermo Fisher
Zeba Spin Desalting Columns 7K	Thermo Fisher
PDVF membrane	Merck Millipore
2mL vials with PTFE screw cap	Fisherbrand/ Thermo Fisher
4–15% Mini-PROTEAN® TGX™ Precast Gel	Biorad

Table 6. Instruments used in this work

Instrument	Source
Scale	OHAUS
Tissue grinder	IKA T10 Ultra-Turrax
Incubator	VWR
Vacuum Oven	VWR
UV-Vis Plate Reader	Biotek Synergy 2
Micro-Centrifuge	VWR
Centrifuge	Beckman (GS-6R)
Heat block	VWR
pH meter	Mettler Toledo, Seven Easy
Vortex	VWR Vortex Genie 2
Waterbath	Precision 180 series
Electroblotting power supply	Biorad Power Pac300
Electrophoresis chamber	Thermo EC
SIFT-MS	Syft Technologies
qPCR machine	Biorad CFX 384 Real Time System
Thermocycler	Biorad S1000

2.2. Methods

2.2.1. Preparation of liver protein extracts

After harvesting, livers are immediately flash frozen in liquid nitrogen and stored at -80 °C until homogenization. During processing of the livers they are always placed on dry ice. With a disposable razor a segment of approximately 70 - 100 mg is cut off and placed in 300 µl of pre-cooled fresh 1x solution of passive lysis buffer in deionized H_2O. Immediately homogenization with the tissue grinder is started until the sample is thoroughly homogenized. Homogenized tissue is transferred to a 1.5 ml tube and flash frozen in liquid nitrogen, after thawing at room temperature this cycle is repeated. Samples are spun down in a microcentrifuge for 10 minutes at 14,000 x g and the supernatant is transferred into 8-strip well format tubes and flash frozen again.

The protein concentration is determined making a 1:30 dilution of the liver homogenate in deionized water and the working and standard BSA solutions are prepared according to the Pierce BCA Protein Assay Kit. 5 µl of the diluted homogenized sample and 5 µl of the standard dilutions are used and 200 µl of the assay solution is added. After incubation of the plate, covered in aluminum wrap for approximately 20 minutes at 37°C, the absorbance is measured at 562 nm in a UV-Vis spectrophotometer.
According to the standard curve the protein concentration is determined and normalized to 10 or 20 µg/µl in each sample.

2.2.2. Lead acetate assay

2.2.2.1. Preparation of lead acetate filter papers

Whatman filter paper are cut into the size dimensions of a 96-well plate. They are then placed in a large glass pyrex dish with 20 mM lead (II) acetate trihydrate in deionized H_2O and soaked for at least 20 minutes. The soaked papers are then dried in a vacuum oven at 110 °C for approximately 30 minutes. If they are not completely dry they are further dried at room temperature and then stored at room temperature in a dark and dry area.

2.2.2.2. Measurement of H_2S production capacity

For every measurement of H_2S production capacity a reaction solution is freshly prepared, dissolving the substrate L-cysteine and the cofactor pyridoxal 5'phosphate (PLP) in PBS. For liver measurements final concentrations of 10 mM of cysteine and 0.5 mM of PLP are used, adaptations of these concentrations are described for each experiment in this work.

For PLP, a stock solution of 10 mM can be prepared in PBS, aliquoted and stored at -20 °C. 10 mM is the highest concentration to completely dissolve the PLP in PBS and for its preparation vortexing and heating to 37°C might be necessary.

The assay can be done in 96-well or in 384-well plates, requiring reaction mix volumes of respectively 150 µl or 60 µl for each well.

In a 96-well plate 10 µl of a 10 µg/µl dilution of liver extract (100 µg protein) is used for a detectable non-saturating signal within 1-2 hours. In this work a dilution of 1µg/µl was used in some experiments for a slower reaction and to detect a signal after approximately 7 hours. In 384-well plates 5 µl liver extract (50 µg protein) is used.

When this assay was tried to be used for measuring H_2S production capacity in serum or plasma, undiluted serum/plasma was used. In 96-well plate 10-20 µl of serum or plasma was placed into each well, in a 384-well plate 5-10 µl. For serum and plasma measurements the cysteine concentration was 100 mM.

After all samples have been placed into the plate, the reaction mixture is added with a multi-channel pipette. The plate is gently vortexed with a plate vortexer and if air bubbles occur, which is especially the case in the 384 well plate, the plate is spun down for a short time. After ensuring that no residual or spilled liquid is on the surface of the plate, the lead acetate embedded filter paper is placed directly above the plate. The plate is then incubated at 37 °C with a heavy object like a heating block placed over the paper to make sure that the paper is in direct contact with the wells of the plate.

2.2.2.3. H_2S release from bound sulfur

To release H_2S from sulfane sulfur, the reducing agent DTT has to be added. A reaction mixture of 10 mM DTT and 1 % SDS is freshly prepared each time before undergoing the

assay. 10 µl of undiluted serum or plasma is placed into a 384-well plate and 50 µl reaction mixture is added with a multi-channel pipette. After ensuring that no residual or spilled liquid is on the surface of the plate, the lead acetate embedded filter paper is placed directly above the plate, a heavy object is put on top of the paper and incubation is at 55 °C.

2.2.2.4. Detection and quantification of the lead acetate signal

Due to the interaction of lead acetate with H_2S, lead sulfide is produced, which forms brown circles on the lead acetate paper. When the circles are detectable but not saturated, the paper is removed.

The lead acetate paper is scanned and the image file is analyzed for densitometry of each circle with the software ImageJ. The image is first inverted and same size areas of each circle are analyzed. A portion of the paper with no sample is used for background subtraction. The readout is Integrated Density and can be used to compare different samples between the same run.

2.2.3. Sample treatments

Protein digestion

To denature and digest proteins, serum and tissue samples were placed into 1.5 ml tubes, a 10% SDS solution was added to a final concentration of 1 % and Proteinase K was added to a final concentration of 100 µg/ml. The tube was then placed into a heat block at 55 °C for 1 hour, every 10-15 minutes the tube was gently inverted.

RNA and DNA removal

Serum was treated with RNase A in a final concentration of 80 µg/ml to remove RNA and with DNase I at a final concentration of 200 µg/ml to remove DNA. The serum was incubated for 30 minutes at 37 °C before using it the assay.

Lipid removal

To remove lipids from serum, chloroform has traditionally been used and we followed a protocol described by Castro et al., where it was shown that it removes 60 - 65 % of the lipids present in serum samples (Castro et al. 2000).

Cosmic calf serum (CCS) was mixed 1:1 with chloroform in a 1.5 ml tube and vortexed until a thick emulsion was obtained. After centrifugation at 1,000 x g for 30 minutes, the supernatant was removed from the lipid-chloroform layer by decanting it into another tube. This tube was centrifuged again at 10,000 x g for 45 minutes and the supernatant was used for the assay lead acetate.

AOAA inhibitor

The inhibitor of the H_2S producing enzymes aminooxyacetic acid (AOAA) was incubated with serum or liver extract in final concentrations of 0.05 to 1000 µM for 15 minutes prior to using it in the lead acetate assay.

2.2.4. Mouse diets

All diets were made using research diets protein-free base (D12450BSpx). Purified amino acids were added to the specified total protein content and amino acid composition using semi-purified amino acids (Ajinomoto). All diets were made in a gel form by adding agar to a 1% final concentration.

Complete and protein free diet:

The ad libitum fed mice consumed ~0.22 g food/kg bodyweight/day. For 50 % caloric restriction they got ~0.11 g food/kg bodyweight/day. They were either fed the complete diet or the protein free diet with the following compositions:

Table 7 Diet compositions of complete and protein free diet

	Complete diet	*Protein Free Diet*
Carbohydrate	72%	90%
Fat	10%	10%
Protein	18%	0%

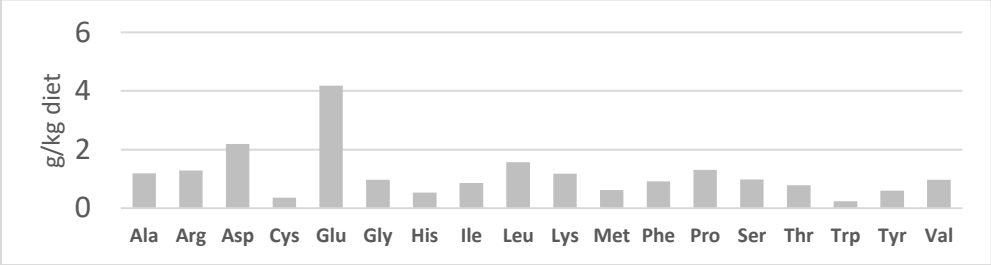

Figure 7 Amino acid composition of complete diet

Methionine restriction and MR control diet:

The following is the composition of the diet used in the MR experiment. The restricted group had methionine reduced from 0.46% of energy (~4.5g/kg diet) to 0.12% of energy (~1.0 g/kg diet).

Table 8 Diet composition in methionine restriction experiments

	Composition
Carbohydrate	76%
Fat	10%
Protein	14%

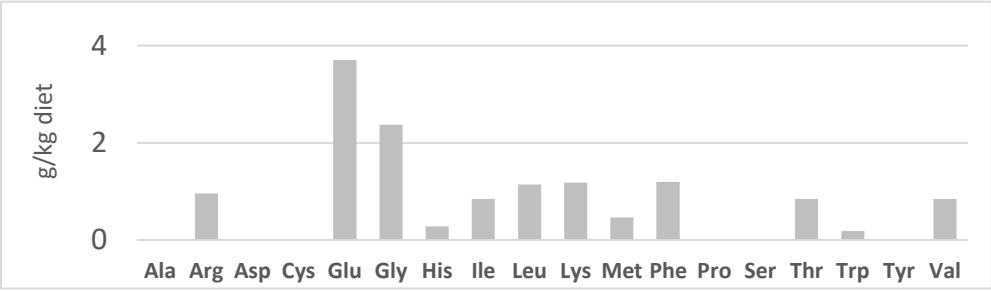

Figure 8 Amino acid composition of control diet in methionine restriction experiemts

2.2.5. H_2S measurements using SIFT-MS

SIFT-MS uses soft chemical ionization coupled with mass spectrometric detection for quantification of gases in real time with very low detection limits. We used it for headspace analysis in a closed vial that was directly coupled with the Syft^{TM} instrument. Measurement was monitored and analyzed in real time with the LabSyft software (Syft Technologies).

To measure H_2S, the reagent ion H_3O^+ was used, detecting products with the mass 19, 35, 37, 55 and 73. Time limit was set to 200 ms and the count limit to 10,000.

For measurement of H_2S using the H_2S donor NaHS, NaHS was diluted with distilled water into the closed reaction vial directly before the start of the measurement. The area under the curve (AUC) was determined to calculate the total amount of H_2S released.

To measure H_2S production capacity in liver, protein extracts were mixed with reaction mixture, containing 10 mM cysteine and 0.5 mM PLP diluted in PBS into the reaction vial. It was immediately coupled with the instrument and H_2S production could be monitored in real time.

2.2.6. Immunoblotting and staining

2.2.6.1. SDS-PAGE

A polyacrylamide gel was used for separation of the proteins according to their size. Either 12 % gels were casted or gradient pre-casted gels (Biorad) were used.

Plasma or serum were diluted 1:50 in PBS and then 1:1 in 2 x Lämmli Loading Buffer containing ß-mercaptoethanol and was heated for 7 minutes at 95 °C. 20 µl was loaded on the acrylamide gel.

Liver protein extracts were generated like previously described and further diluted with PBS and loading buffer to load 25 µg protein. Before loading, the sample was also heated for 7 minutes at 95 °C.

The electrophoresis took place in a Tris-Glycin-SDS running buffer. The PageRuler Prestained protein ladder was used as standard.

2.2.6.2. Silver staining

Silver staining of the proteins in the polyacrylamide gel was performed with the SilverQuest Silver Staining Kit. Silver staining is a very sensitive technique for protein staining, which is based on the chemical reduction of silver ions to metallic silver on a protein band, and it is reported to be 30-fold more sensitive than staining with coomassie. The detailed protocol can be found in the manual of the kit, steps contain fixation of the proteins in the gel and removing interfering ions and detergent from the gel, a sensitizing step to increase the sensitivity and subsequent staining with the staining solution. A developing step reduces the silver ions to metallic silver and makes the protein bands visible.

2.2.6.3. Western blot

In a western blot, the in the SDS-PAGE separated proteins in the polyacrylamide gel were transferred to a polyvinylidene fluoride (PDVF) membrane. After activation of the membrane in methanol, the transfer was conducted for approximately 90 minutes at 100 V in a tank-blot-system from BioRad with a methanol containing transfer buffer.

After the transfer, the membranes were cut and blocked with 5% milk powder in 1xTBS for 1 hour. The membranes then were incubated with the primary antibody overnight at 4 °C and afterwards with the secondary, horseradish peroxidase conjugated antibody for 1 hour under shaking conditions. Between each step three 5-minute washing steps with TBS-T were performed. The SuperSignal West Femto Chemiluminescent Substrate Kit was used for the development of the blots.

3. Results

3.1. Measuring sulfide derived from serum/plasma

The lead acetate assay, which was established for measuring H_2S production capacity in organ extracts also leads to a signal when used with blood serum or plasma. To evaluate the capacity of the H_2S producing enzymes, the substrate cysteine and cofactor PLP (vitamin B_6) are provided in excess and generated H_2S is detected with a lead acetate paper placed on top.

The significant differences that have been found with this assay between plasma from healthy controls and patients with peripheral artery disease (*Figure* 6) indicate that the biologically relevant H_2S production capacity was measured but nevertheless controls were needed for confirmation.

3.1.1. Lead acetate signal in liver and serum depends on different substrates and show unlike characteristics

While the major H_2S producing enzyme in liver is CGL, we wanted to evaluate which enzymes are mainly involved in H_2S production in serum and plasma. All the three H_2S producing enzymes can use the substrate L-Cysteine. However, the most relevant reaction from CBS uses L-Homocysteine and L-Cysteine, producing Cystathionine and H_2S. 3MST can also use D-Cysteine to produce H_2S in a different reaction. Therefore we tried different substrates in the lead acetate assay, all with and without the cofactor PLP to detect the amounts of H_2S that was produced. Besides wildtype mouse liver and serum we also used a CGL knockout mouse (*Figure* 9).

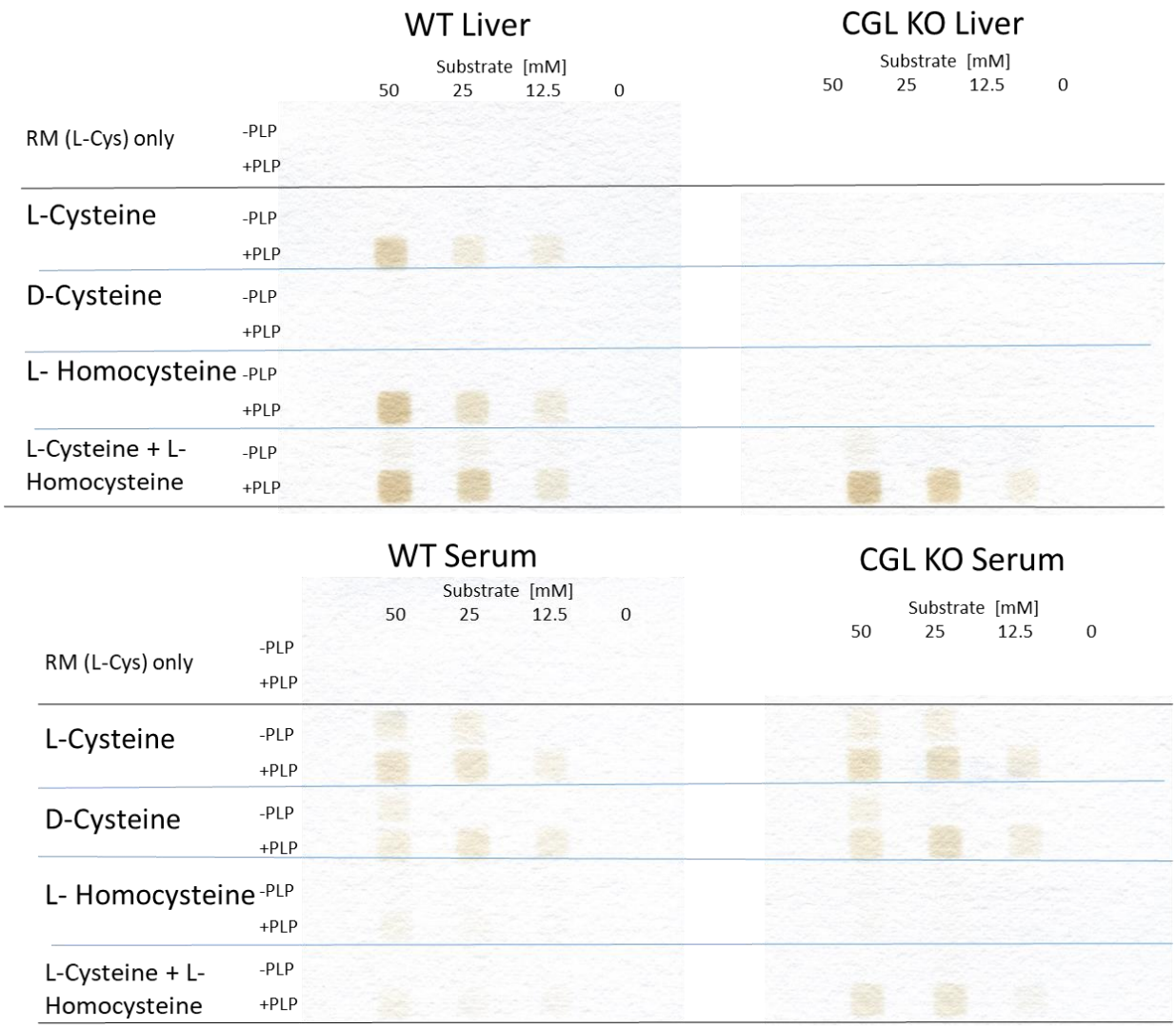

Figure 9 Signal in the lead acetate assay depends on different substrates in liver and serum. Substrates L-cysteine, D-cysteine, L-homocysteine or L-cysteine + L-homocysteine were added to liver extracts or serum of wildtype (WT) or CGL kockout (KO) mice with or without PLP. Lead acetate paper with liver extracts (10µg/µl protein) was incubated for 2 hours, with undiluted serum for 8 hours at 37°C.

The results show major differences between serum and liver. In liver, where CGL is the dominant enzyme, H_2S production with CGL's substrates L-cysteine and L-homocysteine was seen in the wildtype and was not detectable in the CGL KO. Use of L-cysteine and L-homocysteine together favors the CBS reaction, this explains why an H_2S production in the CGL knockout was still detactable when adding the two substrates together. CBS and CGL both need PLP as a cofactor and can't use L-cysteine's enantiomer D-cysteine as a

substrate for H_2S production, which is why we see no signal with D-cysteine in the liver extracts.

In serum we can see three major differences. First of all the reaction doesn't seem to be PLP dependent, since H_2S production can also be observed without addition of PLP, although to a lower degree. The other main differences are, that L-cysteine and D-cysteine both seem to lead to H_2S generation and the CGL knockout serum is not different from the wildtype.

This experiment suggests that CGL is not the main H_2S producing enzyme in serum. The H_2S generation without PLP could be explained with enough PLP present in the serum itself. H_2S production from D-cysteine has been observed in the past from a non PLP depended pathway using the third H_2S generating enzyme 3MST, which has to work in line with the enzyme DAO that uses D-cysteine to generate 3-mercaptopyruvate (3MP), the substrate for 3-MST (Shibuya et al. 2013).

To further compare the reactions between liver and serum we had a look at the concentration dependence of substrate and cofactor, adusting cysteine and PLP levels (*Figure* 10).

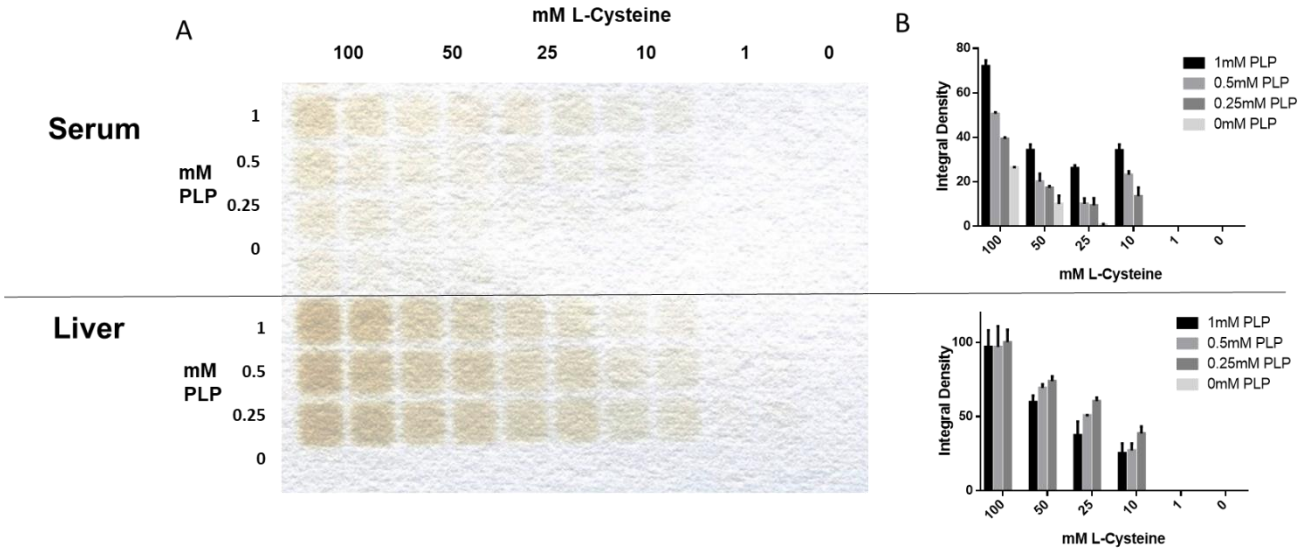

Figure 10 L-Cysteine and PLP concentration dependence. Lead acetate paper (A) and quantification of the signal (B) from liver extracts and serum using different concentrations of substrate cysteine and cofactor PLP. Liver extracts were diluted to a protein concentration of 1 µg/µl. Lead acetate paper was incubated for 7 hours at 37 °C.

Major differences between the H_2S signal in liver and serum were found. While both are cysteine dependent, the dependence of PLP looks different. In liver, PLP is essential for the reaction, but it is saturated with low concentrations. When no PLP is added, the enzymes cannot produce any H_2S which shows no signal on the lead acetate paper, a stable signal and no further increase can be observed upon adding 0.25, 0.5 or 1 mM PLP. Contrarily in serum, there is a signal without the addition of PLP, but increasing concentrations of PLP additionally increase the signal on the lead acetate paper.

3.1.2. Lead acetate assay in serum is not measuring enzymatic activity

Aminooxyacetic acid (AOAA) is a general inhibitor for aminotransferases and inhibits both CGL and CBS (Asimakopoulou et al. 2013). We used it to determine if H_2S generation can be inhibited in liver extracts and serum samples, which we incubated for 15 minutes with AOAA before using it in the lead acetate assay (*Figure* 11).

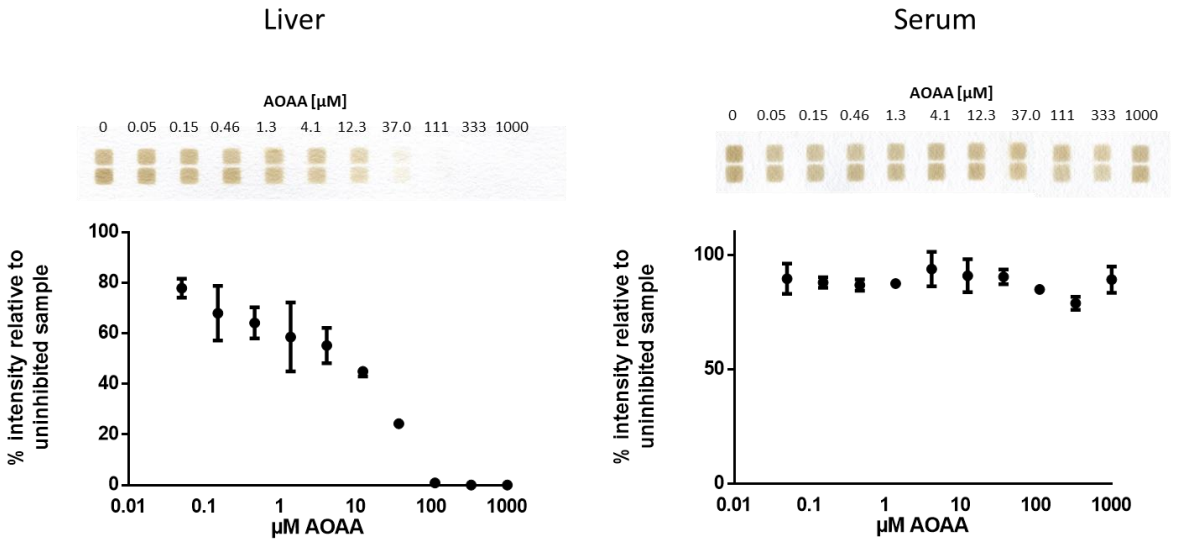

Figure 11 Enzyme-inhibitor AOAA successfully inhibits the signal in liver extract but not in serum. Lead acetate paper and quantification of the signal upon treatment of the liver extract and serum for 15min prior to its use in the assay. For the reaction, 100 mM cysteine and 0.5 mM PLP were added and the lead acetate paper was incubated at 37 °C for 2 hours with the liver extract (10 µg/µl) and 7 hours with serum.

While AOAA led to a dose dependent decrease in H_2S production in liver and to a total inhibition at 100 µM, we did not observe a reduction in H_2S generation in serum, even at inhibitor concentrations as high as 1 mM.

There is no specific inhibitor known for 3-MST, but AOAA inhibits not just CBS and CGL but also numerous other enzymes and presumably also CAT, which is part of the H_2S generation in the CAT/3MST pathway. If the H_2S generation in serum is through one of the three known enzymes, we would expect a decrease in the signal, which we could not detect.

Since the previous experiments indicate that we are either measuring H_2S production from a different enzyme or non-enzymatic H_2S generation, we denatured the protein prior to using it the lead acetate assay (*Figure* 12).

To denature the proteins we placed the sample for 1 hour on 55 °C and additionally added proteinase K and 1 % SDS to make sure all proteins are digested. Each of these conditions separately should remove all the enzymatic activity. The liver extract showed the expected result upon treating it under these conditions and didn't give any H_2S signal on the lead acetate paper. In contrast, denatured serum didn't only not remove the signal, it even increased it.

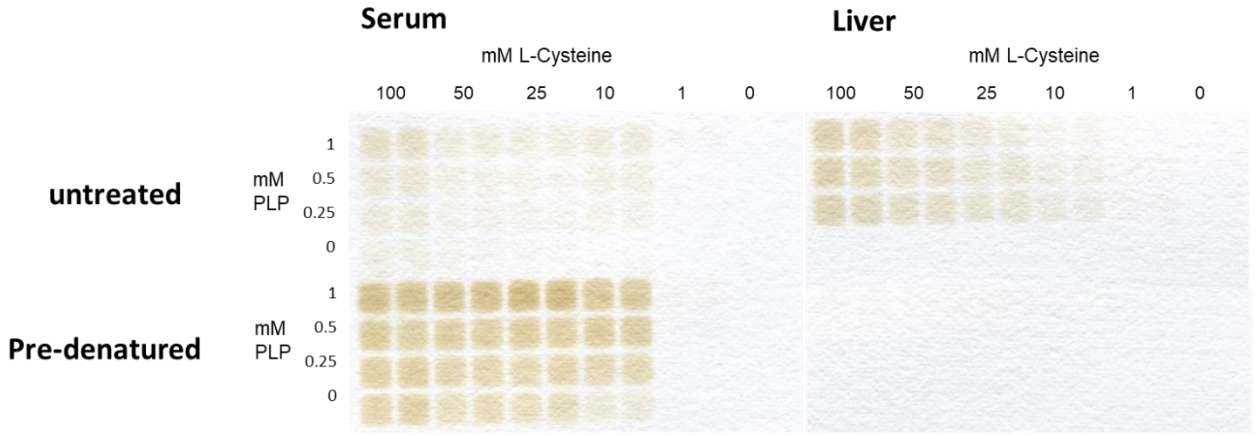

Figure 12 Denaturing of the sample increases the signal in serum while it removes it in liver. Serum and liver protein extract were incubated with different concentrations of L-cysteine and PLP. Denaturing of the sample was for 1 hour at 55 °C with 1 % SDS and proteinase K. Liver extracts were diluted to a protein concentration of 1 µg/µl and the lead acetate paper was incubated for 7 hours at 37 °C.

All these experiments together suggest that the signal we are measuring in serum when adding cysteine and PLP is not enzymatic H_2S production.

3.1.3. Small ions, nucleic acids and lipids don't cause the signal measured in serum by the lead acetate assay

If the signal we are observing is not enzymatic H_2S production, we need to confirm that the darkening of the lead acetate paper is not due to other macromolecules or small ions present in the serum. Small ions like iron or calcium are present in relatively high concentrations in serum and they are not only known to regulate enzymes, but could also have an effect on sulfur release, for example through directly interacting with PLP and cysteine. With a desalting column we removed all small ions and molecules below 7 kDa. Nucleic acids were removed with DNase and RNase and lipids were extracted with chloroform (*Figure* 13).

Removing salts, nucleic acids or lipids did not have any effect on the darkening of the lead acetate paper upon addition of cysteine and PLP, confirming that they are not the reason for the observed signal.

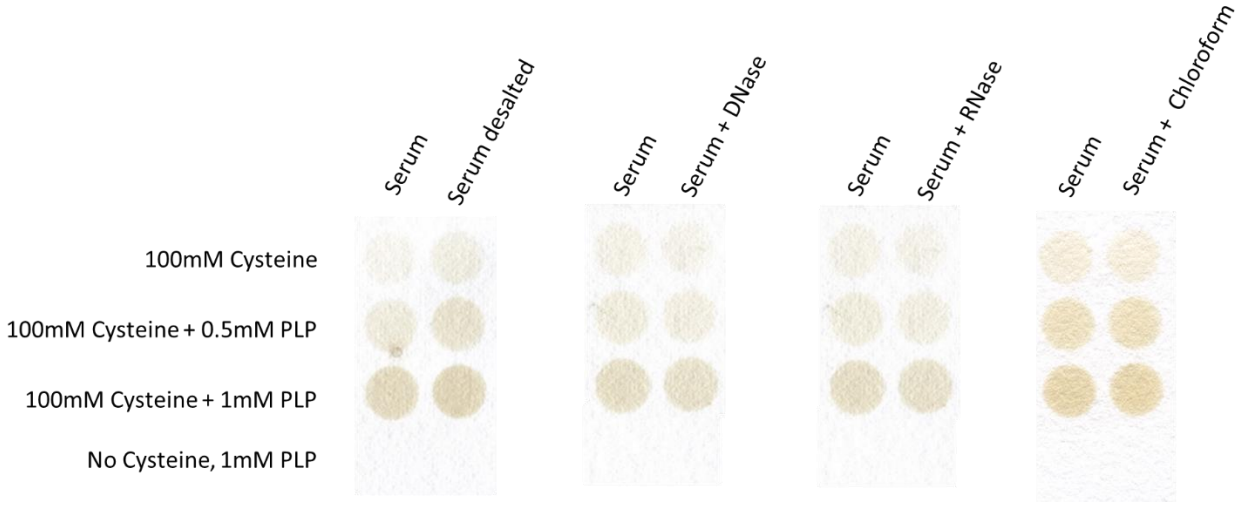

Figure 13 Small ions, nucleic acids and lipids don't cause the signal measured in serum by the lead acetate assay. Image of lead acetate paper showing the reaction of serum and serum where respectively salts, DNA, RNA or lipids were removed. Samples were incubated with the stated amounts of cysteine and PLP for approximately 7 hours at 37 °C with a lead acetate paper overlay.

3.1.4. H_2S is released upon addition of the reducing agent DTT

H_2S could also be generated through release from its sulfur pools.

Acid labile sulfur is released at a pH below 5.4. The high cysteine concentrations of 100 mM used in this assay lead to an acidification of the phosphate buffered saline (PBS), but the buffering capacity was high enough to always keep a pH of 6 or higher. Together with serum, which has a buffer capacity on its own, pH was kept at > 7, even with 100 mM cysteine. Lower Cysteine concentrations did not result in a change of the pH of PBS at 7.4. When we tried a higher pH (7-9), it led to an increased signal, indicating that if H_2S is released from its bound forms, it is not acid labile sulfur but sulfane sulfur which is present as persulfides, polysulfides or thiosulfate. H_2S can be released from sulfane sulfur upon addition of a reducing agent which usually perform better in a more alkaline environment.

Cysteine is not only a substrate for the enzymes, it also has a reducing capacity. If the signal we are seeing is due to the reducing capacity of cysteine, we should also see H_2S generation when using the reducing agents Dithiothreitol (DTT) or Glutathione (GSH). Therefore we performed the same assay, replacing cysteine with adding DTT and GSH (*Figure* 14).

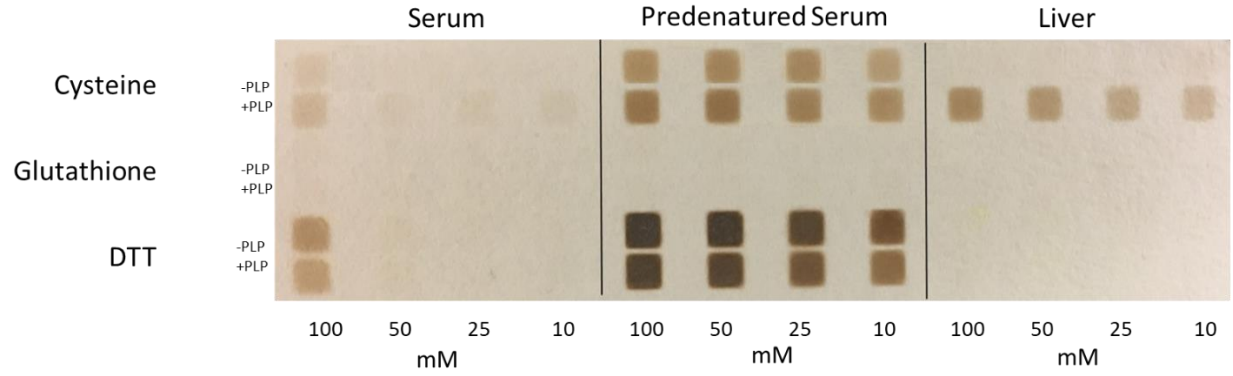

Figure 14 Effect of reducing agents on H_2S release. Serum, denatured serum and liver protein extract were incubated with different concentrations of L-cysteine, glutathione and DTT with and without PLP. Denaturing of the sample was for 1 h at 55 °C with 1 % SDS and proteinase K prior to using it in the assay. Liver extracts were diluted to a protein concentration of 1 µg/µl and the lead acetate paper was incubated for 7 hours at 37 °C.

Addition of DTT to serum led to a signal that was stronger than the one observed with cysteine. In liver, where the reaction is clearly enzymatic, no darkening of the paper could be seen after addition of DTT and only with its substrate cysteine and the cofactor PLP

H_2S was enzymatically generated and a cysteine dependent signal could be observed. DTT addition resulted in a stronger signal compared to cysteine in untreated serum but also in the denatured serum. As observed before, the signal in serum could be increased with sample denaturing prior to the assay and this was true for adding cysteine and also DTT. Both show a dependence on the concentration of cysteine or DTT respectively.
This same pattern when using cysteine and DTT in serum indicates that the underlying mechanisms are identical and the signal is most likely depending on their reducing capacity.

Glutathione in contrast, could not release any H_2S, which is a surprise if we look at the reducing capacity of GSH, which is higher than the one of cysteine. Also with the reducing agent TCEP, which has widely been used to reduce bound sulfur, we could not detect any H_2S, even at concentrations as high as 50 mM (not shown). This could have different reasons, for example steric hindrances.

According to this we hypothesize that we are measuring H_2S that is released slowly from sulfhydrated proteins and other sulfane sulfur species. Depending on the protein size and conformation or the polysulfide length, the suflur could be protected and this could explain the slow and time dependent release.

3.1.5. H_2S signal observed in the lead acetate assay might come from a high molecular weight molecule

We further conducted centrifuge filtration with a spin column keeping molecules bigger than 30 kDa in the upper fraction and letting molecules smaller than 30 kDa pass. We used this spin column for serum and denatured serum and used the fractions in the lead acetate assay adding cysteine and PLP (*Figure* 15).

We expected that upon protein digestion with SDS and proteinase K at high temperature, where all proteins are cut into very small pieces, we see the signal in the < 30 kDa fraction.

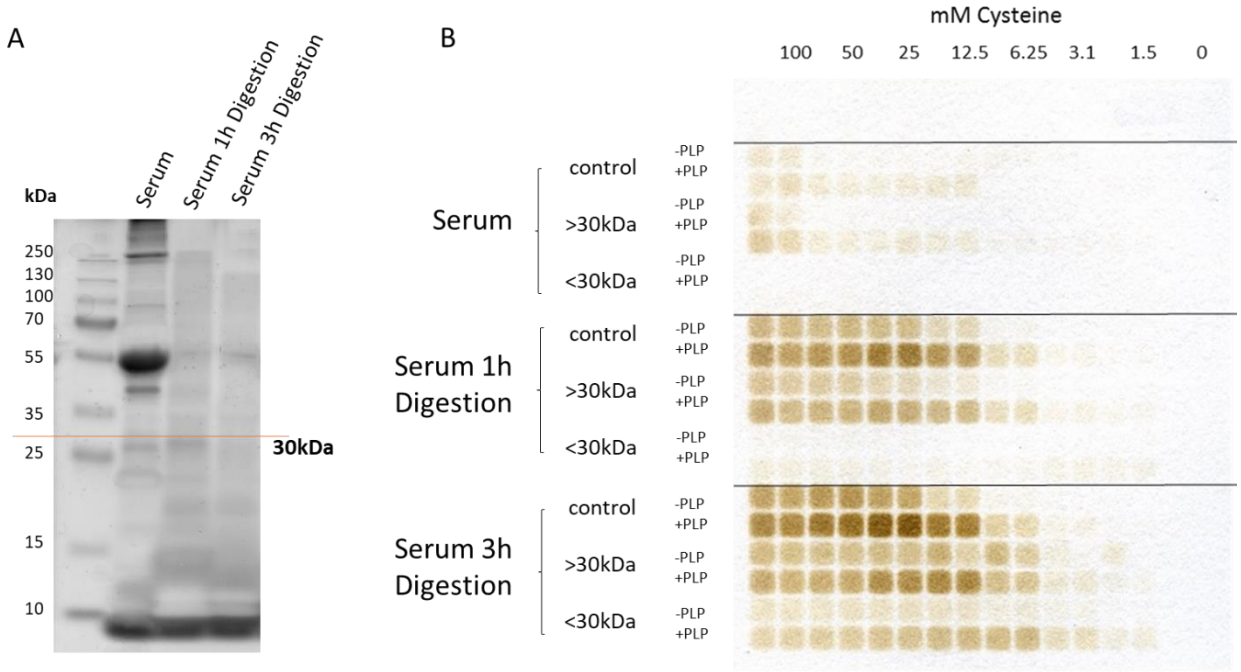

Figure 15 Lead acetate signal stays above a 30 kDa cut off after protein digestion. (A) Protein separation of serum and digested serum with gel electrophoresis, visualized by silver staining. **(B)** Lead acetate paper showing the reaction of serum and its fractions with different concentrations of cysteine with or without 0.5 mM PLP. Denaturing of the sample was for 1 h or 3 h at 55 °C with 1 % SDS and proteinase K. Centrifuge filtration was used to separate the high molecular weight fraction (>30 kDa) from the lower fraction (<30 kDa). Lead acetate paper was incubated for approximately 7 hours at 37 °C.

Despite our expectations, almost the entire signal stayed in the upper fraction even after protein digestion, indicating that the H_2S is released from a molecule that is bigger than 30 kDa (*Figure* 15B). The silver staining showed that using SDS and proteinase K at 55 °C successfully digested the proteins, since there was only a smear and no more bands visible (*Figure* 15A). Digestion at 55 °C for three hours instead of one hour did further increase the measured signal and also signal in the < 30 kDa fraction.

If this shows us that the signal is not derived from a protein-bound persulfide, it is suggested that the sulfur has to be released from high molecular weight polysulfides. Polysulfide chains of more than 10^5 sulfides have been described in plants and therefore high molecular weight polysulfides could also be present in mammals.

3.1.6. Assay adaptation for sulfane sulfur measurements to assess correlation of bound sulfur levels with vascular health

Because the lead acetate data presented in *Figure* 6 using plasma from PAD patient and control samples were generated using cysteine, which is both a potential substrate for H_2S production as well as a reductant, capable of releasing stored bound sulfur, we next adapted our assay using DTT to specifically measure sulfane sulfur release. 10 mM DTT were added to the serum under denaturing conditions, incubating at 55 °C and with SDS present. Dilutions of serum in PBS were made to validate this method (*Figure* 16).

With serum dilutions, the assay showed linearity down to 25% serum. After longer incubation, linearity between lower dilutions could be observed, but the signal of the higher dilutions and the undiluted serum (100% serum) reached saturation, which is why no differences could be detected between the dilutions (not shown). Therefore this assay is not applicable for total quantification of bound sulfur that is present but to compare different samples with similar levels.

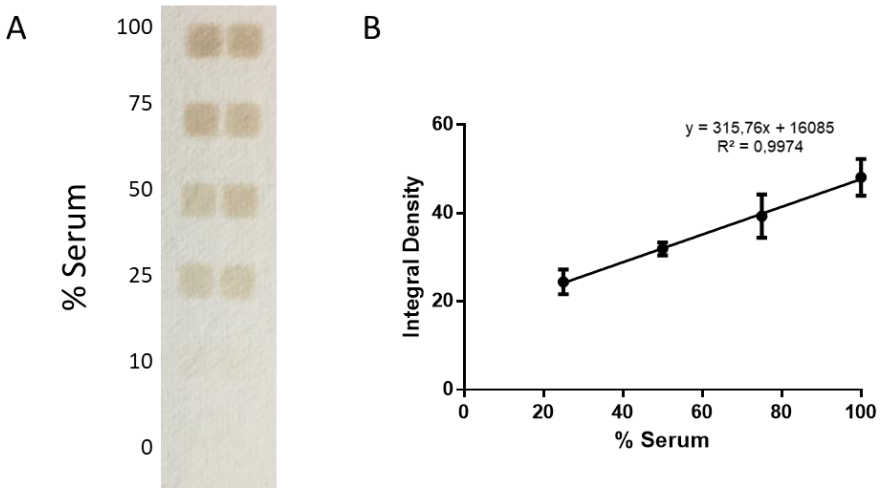

Figure 16 Lead acetate assay to detect H_2S release from bound sulfur. Lead acetate paper (A) and quantification of the signal (B) from serum dilutions treated with DTT and SDS, incubated for 4 hours at 55°C. Released H_2S shows linearity from 25% to 100% serum.

With the adapted assay we went back to the plasma samples from patients with vascular disease, which were analyzed with the previous assay with the objective to measure H_2S

production capacity. Now knowing that upon addition of high concentrations of cysteine and PLP we did not measure enzyme activity but more likely a release of sulfane sulfur. With the newly designed method we wanted to confirm this and determine, if the results correlate with the previous assay.

The 115 plasma samples from patients with peripheral artery disease and the 20 age-, sex- and hypertension-matched controls were treated with DTT under denaturing conditions and H_2S release was measured by lead acetate paper overlay (*Figure* 17).

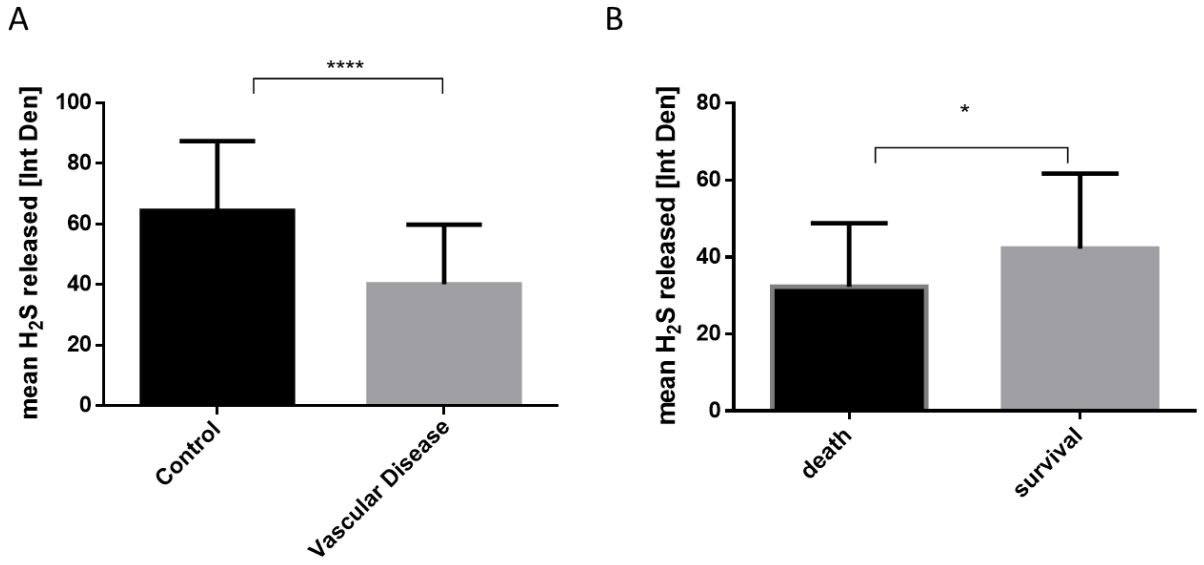

Figure 17 Sulfane sulfur levels are reduced in patients with vascular disease compared to controls and correlate with surgery outcome. Quantitation of lead acetate paper measuring the release of H_2S from suflane sulfur upon addition of DTT and SDS after incubation at 55°C for 4 hours. Controls (n=20) show higher mean H_2S release than patients with vascular disease (n=112) and among the patients, mean H_2S release was lower in patients with mortality (n=19) during the 2 year follow up than in patients that survived. Error bars indicate standard deviation (SD). **** $p < 0.0001$; * $p < 0.05$, using student's t-test.

The results correlated with the findings from the previous assay, indicating that the denaturing assay adding DTT and the assay adding cysteine and PLP did measure the same thing, namely H_2S release from bound sulfur (*Figure* 6, *Figure* 17).

We showed that bound sulfur levels were significantly reduced in plasma of patients with peripheral artery disease prior to undergoing vascular surgery compared to controls in no need for surgery (mean 40 ± 2 vs 64 ± 5) (*Figure* 17A). Furthermore, among patients that underwent surgery, polysulfide levels were significantly lower in patients with mortality

during a two year follow up (mean 32 ± 4; n=19) than of those alive (mean 42 ± 2, n=93) (*Figure* 17B).

This relation between bound sulfur levels in plasma and vascular health got further confirmed by our collaborator Peter Nagy from the National Institute of Oncology in Budapest, who determined the free sulfide levels in these plasma samples (*Figure* 18A). Using a very sensitive technique based on monobromobimane derivatisation and HPLC separation they could show that free sulfide levels were significantly reduced in patients with vascular disease compared to healthy controls (0.75 ± 0.03 µM vs 0.95 ± 0.06 µM).

Also CBS activity was measured in these samples by Peter Nagy's group (*Figure* 18B). The canonical non-H_2S-producing reaction of CBS was analyzed, using labeled serine which is converted into labeled cystathionine and can be detected by HPLC-MS. No differences in enzyme activity was observed between healthy people and patients with vascular disease.

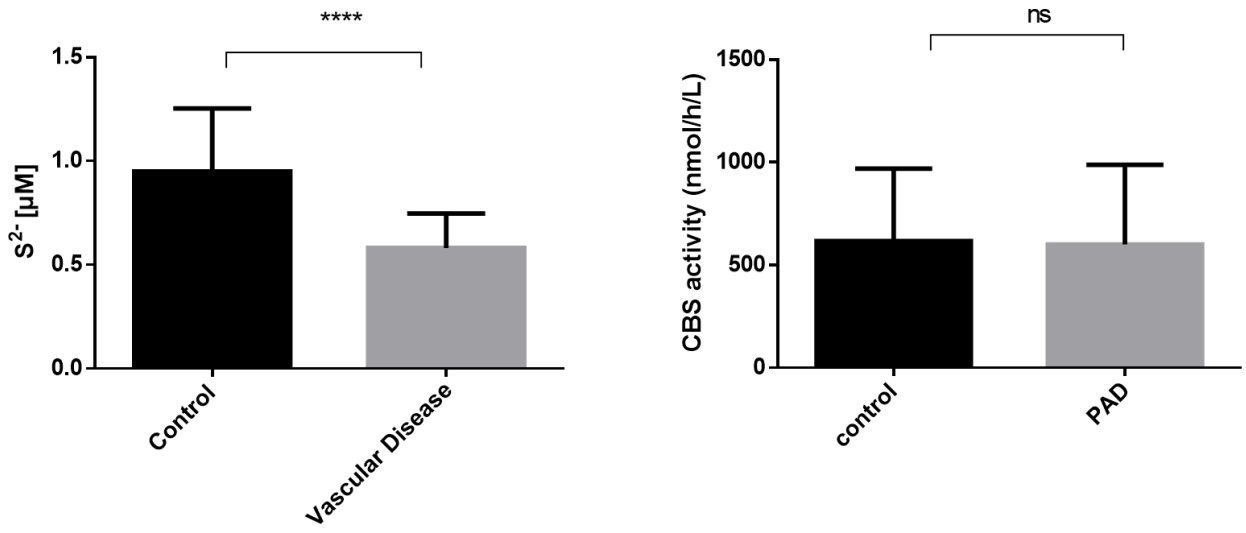

Figure 18 Free sulfur levels are decreased in patients with vascular disease while CBS activity is not altered. Free sulfur levels were measured by a method based on MBB and HPLC/MS, controls (n=20) show higher levels than patients with peripheral artery disease (n=112). CBS activity is not altered between controls (n=11) and patients with peripheral artery disease (n=80). Error bars indicate standard deviation (SD). **** $p < 0.0001$; ns not significant, using student's t-test

3.2. Hepatic H_2S production capacity is differently modulated by different dietary restriction regimens

It has been previously shown by the Mitchell group and also other groups that caloric restriction increases H_2S production capacity via increased mRNA and protein expression of CGL. However, whether other dietary regimens with similar longevity benefits as caloric restriction, for example methionine restriction and protein restriction, also increase H_2S production capacity remains unclear and has been investigated in this work.

3.2.1. Restriction of total calories and not only protein is needed to increase hepatic H_2S production capacity

Mice were set on a one week dietary intervention with either complete diet containing 18% protein or a protein free diet, both ad libitum fed and on 50% caloric restriction. Afterwards, mice were sacrificed and H_2S production capacity was measured with the lead acetate assay using liver extracts (*Figure* 18).

We could confirm previous findings, showing that 50% caloric restriction increases hepatic H_2S production capacity. In contrast, we couldn't observe any significant differences between ad libitum fed mice with 18% protein (complete diet) and with a protein free diet. Caloric restricted mice on a protein free diet also showed significantly increased H_2S production capacity compared to the ad libitum complete diet (control).

Health benefits by caloric restriction and protein restriction are mostly thought to underlie the same mechanism, but looking at H_2S production capacity, restriction in total calories and restriction of only proteins don't lead to the same outcome, indicating differences in the mechanisms between the two regimens.

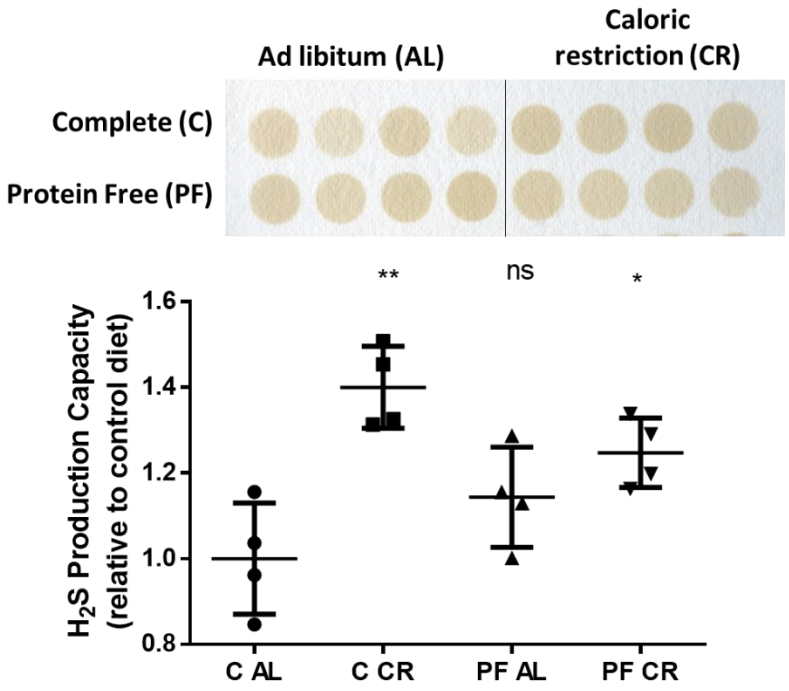

Figure 18 Restriction of total calories and not just protein is needed to increase hepatic H_2S production capacity. Lead acetate paper (A) and quantitation of the signal (B) showing H_2S production capacity measured in liver protein extracts. Mice were for one week either on a protein containing complete diet or on a protein free diet, with or without caloric restriction of 50%. Error bars indicate standard deviation (SD). ** $p < 0.01$; * $p < 0.05$, compared to ad libitum fed complete diet. n=4 for each group

3.2.2. Methionine restriction but not protein restriction leads to increased H_2S production capacity

Both protein restriction but also methionine restriction are known for their beneficial health effects and caloric restriction benefits were often attributed to the restriction in protein or even only in methionine and cysteine. To see if the H_2S production capacity is really not altered upon restriction in protein content, we measured it in liver extracts from two different experiments. In one experiment, mice were on a protein free diet for one week, in another on methionine restriction for one week (*Figure* 19).

To our surprise we found that methionine restriction led to a stable, more than 2.5 fold increase in H_2S production capacity compared to a control diet while protein free diet didn't alter enzymatic H_2S generation. Despite the lack of increase in H_2S production capacity,

the protein restricted mice did show numerous beneficial health effects. Compared to the protein containing complete diet they showed significant weight loss - especially fat mass – and a decline in triglycerides, total serum cholesterol and fasting plasma glucose.

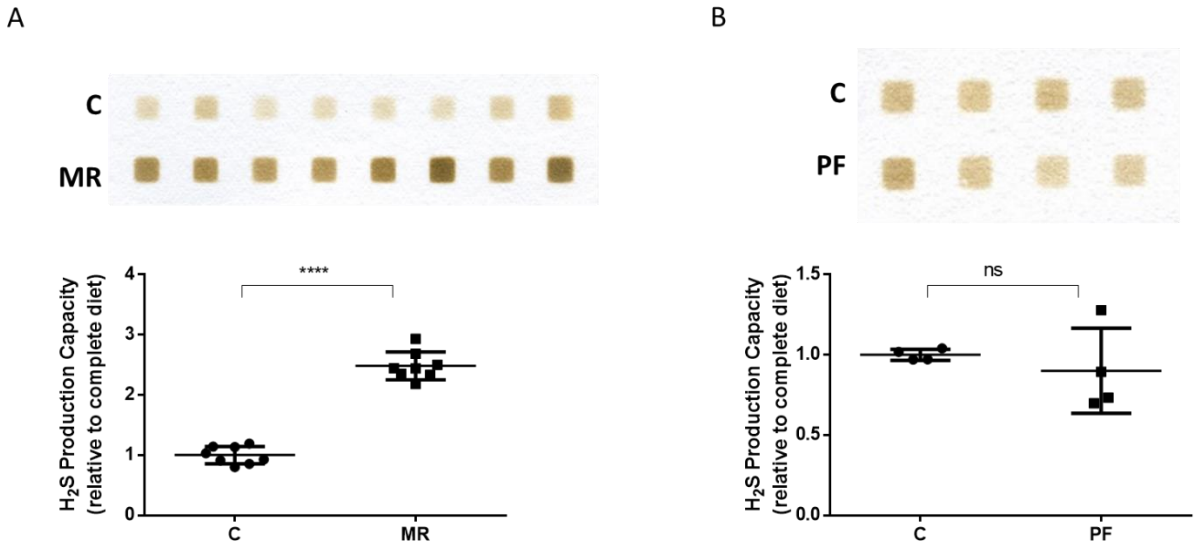

Figure 19 Methionine restriction but not protein restriction leads to increased H_2S production capacity in liver extracts. Lead acetate paper and quantification of H_2S production capacity from liver extracts after one week of a control diet (C) compared to methionine restriction (MR) **[A]** and a protein free diet (PF) **[B]**. Error bars indicate standard deviation (SD). **** $p < 0.0001$; ns not significant, compared.to ad libitum fed control diet.

We confirmed the increase in H_2S production capacity upon methionine restriction using the liver samples from this experiment at the Syft instrument. With selected-ion flow-tube mass spectrometry (SIFT-MS) we were able to measure H_2S production in real time when adding cysteine and PLP. SIFT-MS allows very low H_2S detection limits and we could show linear H_2S detection in a range of 5-log and down to 30 femtomol NaHS, which makes it a lot more sensitive than the lead acetate method (*Figure* 20 A).

Using SIFT-MS, we showed that methionine restriction led with our conditions to a stable average H_2S production in liver extracts of 0.275 ppm compared to 0.125 ppm in the control diet (*Figure* 20 B).

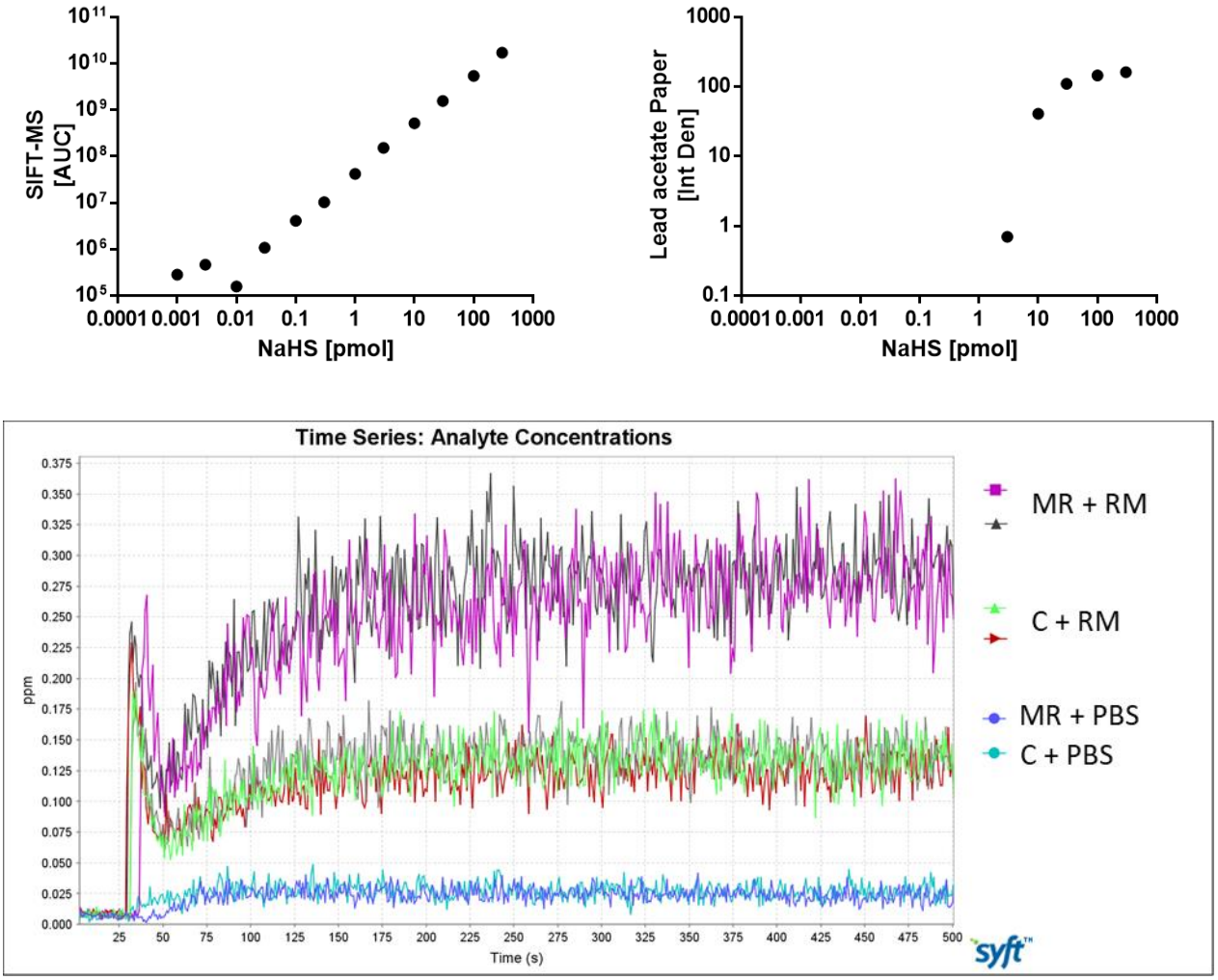

Figure 20 H_2S measurements with SIFT-MS [A] Comparison of H_2S detection range of SIFT-MS and lead acetate paper **[B]** Real time measurement of H_2S production with SIFT-MS: Hepatic H_2S production upon addition of reaction mix (RM) containing 100 mM cysteine and 0.5 mM PLP or PBS to liver extracts of mice upon a methionine restricted (MR) or a control diet (C). After measurement of the background for 30 sec, the vial was placed onto the machine, directly after addition of the reaction mixture to the vial.

3.2.3. Protein levels of CGL and CBS don't correlate with H_2S production capacity in methionine restricted mouse livers

H_2S production capacity is measuring the activity of the H_2S producing enzymes. Upon caloric restriction, it has been reported that an increase in protein and mRNA levels of CGL goes along with increasing H_2S production capacity.

We confirmed in western blots that in our caloric restricted liver samples, where we showed increased H_2S production, that there was also more CGL protein compared to the

ad libitum fed animals. Protein free diet compared to the 18% diet did not alter CGL expression, which also correlates with the lead acetate assay (*Figure* 21).

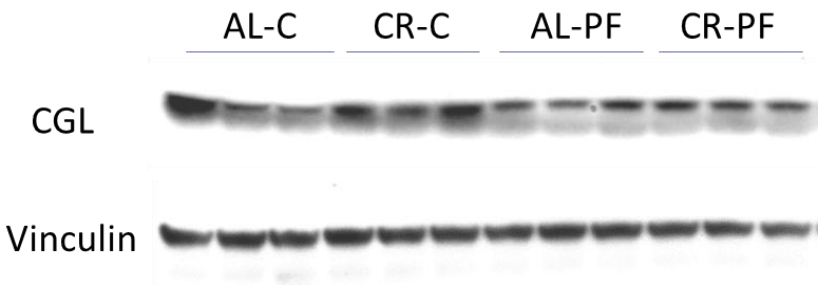

Figure 21 Caloric restriction increases CGL protein levels, protein restriction not. Western Blot of CGL from mice livers following one week ad libitum (AL) fed or 50% caloric restriction (CR) with a complete (C) or protein free (PF) diet. Vinculin was used as loading control.

We also measured the hepatic CGL levels in the methionine restricted animals, expecting increased CGL levels since we observed increased H_2S production capacity. Interestingly neither CGL nor CBS were upregulated upon methionine restriction compared to complete diet (*Figure* 22).

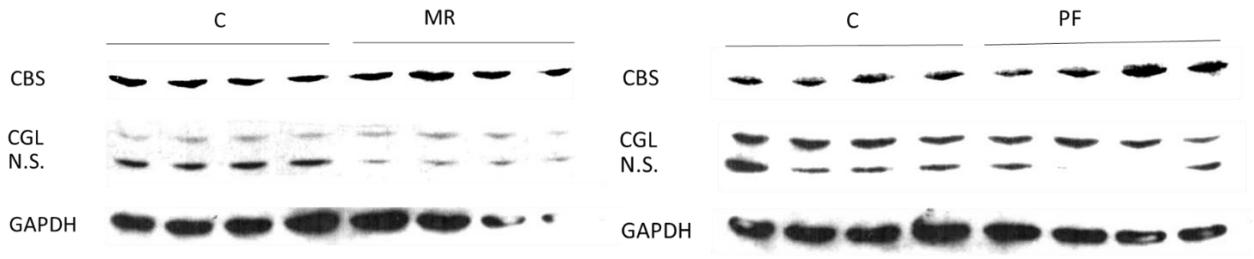

Figure 22 The H_2S producing enzymes CGL and CBS are not upregulated upon methionine restriction or protein free diet. Western Blot of CGL and CBS from mice livers following one week of ad libitum fed methionine restricted (MR) or protein free (PF) diet compared to their control diet. GAPDH was used as loading control, CGL antibody gives an unspecific band below the CGL band.

This unexpected result showing no upregulation in protein levels of CGL despite increased H_2S production capacity upon methionine restriction was confirmed by real time PCR, where we could show that CGL mRNA levels were even slightly decreased in MR compared to the control (*Figure* 23). No upregulation in transcription and protein levels of

H_2S producing enzymes indicates that H_2S production capacity can be also regulated post translationally.

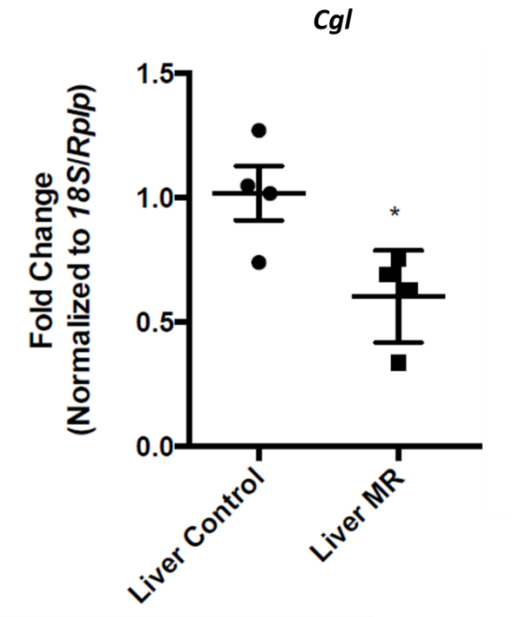

Figure 23 Expression of CGL is downregulated upon methionine restriction. Hepatic CGL mRNA levels following one week of ad libitum fed methionine restricted diet (MR) compared to the control diet containing methionine, normalized to 18S/Rplp. Error bars indicate standard deviation (SD). * $p < 0.05$, using student's t-test.

4. Discussion

The interest in H_2S in biology and medicine has been increasing during the last years and evidence for its physiological importance and beneficial effects on multiple organs could be found. Not only exogenously applied H_2S leads to benefits like vasodilation and neuroprotection, also the loss of endogenous H_2S production by its H_2S producing enzymes can result in cardiovascular diseases, oxidative stress and inflammation or neurodegeneration (Wang 2012).

One of the major problems in the H_2S research is its detection. As a gas it is volatile, reacts rapidly with other molecules and it has to be present in very low concentration, due to its toxicity at high concentrations. Additionally, it can be stored in different sulfur pools, being present as bound sulfur or as acid labile sulfur. The H_2S production capacity, which measures the activity of the H_2S producing enzymes, is another way to compare amongst

different tissue samples and requires the addition of excess amounts of substrate cysteine and cofactor PLP (Hine et al. 2015). Measurement of H_2S levels in blood serum or plasma samples have turned out to be quite complicated, potentially erroneous and time consuming, but body fluids are the only practical option for measurements in humans (Olson 2009).

In this work we tried using the lead acetate method to measure H_2S production capacity in blood serum or plasma. We found that even though serum results in a substrate-dependent signal on the lead acetate paper, it was not enzymatic H_2S production but most likely a H_2S release from bound sulfur, catalyzed by the reducing capacity of cysteine. Our data suggests that H_2S in serum and plasma can be released from persulfides and polysulfides under denaturing conditions, dependent on a reducing agent. In our human plasma samples, the levels of released H_2S correlated with free sulfide levels measured by mass spectrometry. Additionally we found a correlation between bound sulfur and free sulfide levels with vascular health, being reduced in patients with peripheral artery disease compared to healthy control samples.

Finally we investigated the effects of different dietary restriction regimens which lead to lifespan extension and increased stress resistance on hepatic H_2S production capacity in mice. Interestingly, we found that a restriction in proteins alone doesn't increase H_2S production capacity or the expression of H_2S-producing enzymes like a restriction in total calories. Methionine restriction, on the other hand, increased H_2S production capacity but without affecting enzyme levels, likely through changes in activity due to post translational modifications.

4.1. Sulfide measurements in blood serum/plasma

When the efficacy of the lead acetate method measuring H_2S producing capacity was first tested in blood serum, we found a substrate dependent H_2S production upon addition of cysteine and cofactor PLP similar to the one in liver samples, even though at much lower levels and with higher substrate concentrations. However, in a line of additional experiments evidence accumulated that this signal in serum was not caused by enzymatic activity. The reaction in serum showed major differences to the one in liver: PLP was not

essential but led to a concentration dependent increase in signal. D-cysteine, L-cysteine's enantiomer, which can't be used by the major H_2S producing enzymes CBS and CGL as a substrate, leads to H_2S production in serum. Additionally, AOAA, an inhibitor of CBS and CGL, was not able to inhibit H_2S production in serum while the inhibition in liver is dose dependent and removes all signal at concentrations of ≥100 µM. The final evidence that we didn't observe enzymatic H_2S production, also not from another enzyme, we got upon protein digestion with SDS/proteinase K at 55°C, when the signal was not only still present, but also increased. Each of these conditions alone should lead to the disruption of the entire enzymatic activity, but we can still observe an even stronger H_2S signal on the lead acetate paper.

If DTT is used instead of cysteine, it also increases the H_2S production, which suggests that the signal is depending on the reducing capacity of cysteine and DTT. Reducing agents can lead to the release of H_2S from the bound sulfur pool, reducing persulfides and polysulfides. What has to be noted is, that the use of DTT or other reducing agents to release H_2S from bound sulfur was previously described as a very fast reaction (Shen et al. 2012). This part we would miss with this lead acetate paper overlay since at least one minute passes between adding the DTT or cysteine mixture to all samples and the paper overlay. Additionally, the signal we observe here is generated over a long period of time and needs an incubation of several hours to get a detectable darkening of the paper. It seems that H_2S is released very slowly, which can be explained through the protein configuration, the position of the persulfide or the length of the polysulfide.

We also have evidence that the signal we observe is from high molecular weight polysulfides, since it remains above a 30 kDa molecular weight cut-off by centrifuge filtration even after protease digestion of the serum. However, this needs to be confirmed with different methods because the spin columns with a molecular weight cut-off which were used here, are usually designed as protein concentrators to keep the bigger proteins in the upper fraction. Therefore it could be that also digested, smaller proteins are kept above. However, two different columns from different manufacturer with a MWCO of 20 kDa and 30 kDa have been used during this study and both show the same result. Polysulfides can be very long chains and since they can reach weights of >30 kDa in plants, it is possible, that they also exist in human serum and plasma.

Contrary to expectations, other reducing agents like glutathione and TCEP did not result in release of H_2S from serum. TCEP is used in an increasing number of publications instead of DTT to reduce bound sulfur and release H_2S, showing strong reducing capacity over a wide pH range (Han und Han 1994). However, TCEP has been reported to consume sulfur, especially when the release is slow like in this case, TCEP could prevent the darkening of the lead acetate paper. Reduced glutathione could have steric hindrance issues averting interaction with the sulfur. Nevertheless, both reducing agents that can be used, cysteine and DTT, contain a thiol group on its own. This is one of the reasons why many groups switched from DTT to TCEP, because there is the possibility that a sulfur signal is derived from the own thiol group (Togawa et al. 1992). In all our controls, the reaction mixture of cysteine and DTT alone or with PLP or SDS did only result in a very low signal after incubation of more than 24 hours at 37 °C or 55 °C and in no signal during the incubation time used in these experiments. Compared to the signal with serum, which is a lot stronger, this suggests that sulfur contamination from the thiol group of the reducing agent itself is not the reason for the signal. Regardless, additional controls to exclude this possibility need to be undertaken because there is also a chance that there is a component in serum that helps releasing H_2S from DTT or cysteine.

In all the experiments adding cysteine it could be clearly shown, that the addition of PLP increases the signal. This first let us believe that an enzymatic reaction is the cause because its function as cofactor. After proofing, that we don't measure a biological reaction involving enzymes, but a chemical reducing reaction, we still cannot explain why PLP can increase the signal in both untreated and denatured serum. In contrast, with DTT as a reducing agent, PLP was not able to further increase the signal.

After adapting the previous lead acetate method and designing a new assay which is more specific for the release of H_2S from bound sulfur, where DTT and SDS were added and incubated at 55°C, we used both assays in more than 100 human plasma samples with vascular diseases. All patients were diagnosed with peripheral artery disease and had to undergo surgery (carotid endarterectomy, lower extremity arterial revascularization or major leg amputation). Compared to healthy, age-, sex- and hypertension-matched controls, the patients with vascular disease led in both lead acetate assays to a significantly reduced signal, suggesting reduced levels of bound sulfur. This confirms

again the hypothesis, that with the assay adding cysteine and PLP, it was also the release from bound sulfur that has been measured. A further confirmation that biological differences in sulfide levels are measured with this assay settings was achieved, when our collaborators confirmed, that also the free sulfide levels were significantly reduced in these PAD samples compared to the controls. This correlation of bound sulfur levels and free sulfide levels in plasma, at least in our samples, could suggest that there is an equilibrium of free H_2S and bound sulfur, indicating that excessive H_2S might be stored in bound sulfur structures.

Furthermore, the lead acetate paper based bound sulfur assay also showed a correlation within the patients between surgery outcome and bound sulfur levels. Patients with mortality during a two year follow up after surgery showed significantly lower bound sulfur levels than surviving patients. This indicates that bound sulfur levels could be a biomarker for vascular health and predict the severity of the disease and the risk of an intervention.

H_2S shows a lot of effects on the vascular system and is known for leading to vasodilation, preventing hypertension and promoting angiogenesis, therefore altered H_2S levels in patients with vascular disease are not unexpected. Not many data on bound sulfur levels in plasma is published, one reason is the current lack of sensitive polysulfide detection assays. Earlier this year Ikeda et al published a combined assay liberating sulfide and measuring it with a MBB based method and detected concentrations of approximately 8,000 µM of polysulfides in plasma, as a major pool they suggested albumin (Ikeda et al. 2017). However, this method is for low molecular weight sulfur species and taking in account that we might have very high molecular weight polysulfides, this number could be a lot higher.

The lead acetate assay cannot be used for total quantification, because the lead acetate paper reaches saturation at one point. We tried to adjust the serum volume and the DTT concentration to be able to capture the total H_2S released, but with the very slow release, the signal starts fading before everything is released. The final setting of the assay released H_2S from serum dilutions in a linear range, but it is important to remove the paper before saturation is reached.

Since reliable, quantitative techniques for measuring H_2S levels in human samples are

still under development, this assay could be an option to compare samples like plasma with similar sulfane sulfur levels, even though it can't be used for total quantification. The assay is very easy to perform and high throughput, with the possibility of measuring 384 samples in one plate at the same time.

4.2. H_2S production in dietary restriction regimens

Dietary restriction is the oldest intervention to extend lifespan and goes along with an increase in healthspan, protection against age related diseases and better stress resistance. This can be observed in different organisms from yeast to rodents (Fontana et al. 2010). The Mitchell lab has previously shown that endogenous H_2S production through the transsulfuration pathway is essential for caloric restriction benefits. Upon CGL deletion these benefits were abrogated and exogenous H_2S administered to ad libitum fed mice could restore the increased stress resistance. The lead acetate paper method has been used to measure H_2S production capacity in liver extracts by adding substrate cysteine and cofactor PLP, where they could show that more H_2S was produced from CR than AL fed mice (Hine et al. 2015).

Dietary restriction (DR) can be a reduction of total calories (CR), but also of only proteins (PR) or even just some amino acids like in methionine restriction (MR). In mice all three lead to lifespan extension and benefits like decreased body weight, fat mass, serum triglycerides and blood glucose. It has mostly been suggested, that the molecular mechanisms between these conditions are overlapping, especially since cysteine and methionine add back can abrogate the CR effects in mice. As mentioned before, also the increase in H_2S production capacity is abrogated upon this back addition, suggesting that also protein restriction and methionine restriction lead to an increase in TSP (CGL and CBS expression) and H_2S production.

Surprisingly, we discovered that protein restriction without restriction of total calories doesn't result in increased H_2S production capacity. After one week of ad libitum fed protein free diet no alteration in H_2S production could be detected compared to an ad libitum fed control diet containing 18% protein, while CR did lead to a significant increase. In another cohort, where this could be confirmed, we could show that even though no

difference in H_2S production was detected, the expected PR benefits like decreased body and fat mass and better serum lipid levels were present. It could be that an increase in H_2S production capacity demands a longer intervention, but since the other benefits are already present after one week, H_2S is not essential for the beneficial effects of a protein free diet. The necessity of a functional TSP and H_2S production for protection upon IRI with caloric restriction suggests, that the underlying mechanisms might differ. Speakman et al analyzed more than a hundred experiments in rodents over the past 80 years and came to the conclusion, that besides the clear lifespan extending effect of protein restriction, lifespan extension upon food restriction is due to caloric restriction and not reduced protein intake. They assume an independent impact, since protein levels need to be reduced by 80% (from 20% to 4%) to get a median lifespan increase by about 15% while caloric restriction by only half the amount (40%) increases lifespan by an average of 30% (Speakman et al. 2016). It will be very interesting to elucidate if a protection from IRI after a protein free diet is present and if yes, if it is also abrogated in a mouse with CGL deficiency to evaluate the impact of H_2S on this dietary restriction regimen.

Upon this interesting finding showing differences between restriction in total calories and in proteins on H_2S production capacity, it is even more surprising that a restriction in a specific amino acid resembles CR and not PR. In multiple experiments we could show that MR increases H_2S production capacity two to three fold compared to the control diet. The only difference between the MR and the MR control diet was the reduction in methionine from 0.46% of energy to 0.12% of energy, which led to this significant increase in H_2S production capacity, which is even more prominent than in CR.

We could have found an explanation for this paradoxical fact that CR and MR increase H_2S production capacity but not PR when looking at the protein levels of the H_2S producing enzymes. Like it was previously described, CGL protein and mRNA levels are increased upon caloric restriction and we could confirm this once more. Like expected, there was no difference in CGL and CBS levels upon protein restriction, but surprisingly also upon methionine restriction. The lack of increase in CGL protein levels was underlined by its mRNA levels, which were even slightly decreased upon MR. This discrepancy in expression levels of H_2S producing enzymes and H_2S production capacity shows a major mechanistic difference behind the increase in H_2S production capacity between caloric

restriction and methionine restriction. However, even though the increase in H_2S production capacity could be shown in multiple independent experiments, CGL/CBS expression levels upon MR have only been elucidated in one experiment and this lack of increase in expression levels needs to be confirmed in different cohorts.

If H_2S production capacity is increased independently of expression levels of the enzyme, the differences must be post-translational. Post-translational modifications allow a change in enzyme activities or in interaction with other molecules through addition of functional groups or cleavage of subunits. Modifications like these could have a major impact on enzymes like CGL, which undergo major structural changes upon binding of the cofactor, in this case PLP. Hydrogen bonds maintain the close contact between the subunits of the tetramer CGL and also between the enzyme and PLP (Sun et al. 2009). Post-translational modifications could favor or hinder these conformational changes, modulating the enzymatic activity. In the fruit fly, an involvement of posttranslational regulation of CBS was already suggested, since DR increases the CBS protein levels to a higher extend than the enzymatic activity (Kabil et al. 2011). In that case, where it must be a downregulation, the authors suggested modification by sumoylation or allosteric regulation by AdoMet of the components of the TSP (Koutmos et al. 2010; Kabil et al. 2006).

5. Conclusion and Outlook

Evidence for the importance of endogenous H_2S is constantly increasing and in this work we could show that plasma H_2S levels in bound sulfur and free sulfur are reduced in patients with vascular health. The lack of sensitive, technically easy and cost effective high throughput methods to detect H_2S in body fluids like plasma makes measurements in human difficult. The lead acetate paper method to capture released H_2S from bound sulfur structures upon adding DTT and SDS could be a good method to compare sulfane sulfur levels in plasma samples. Our next steps will be to confirm the bound sulfur measurement of this assay with an elaborated, more sensitive and quantitative assay. It will also be further investigated if high molecular weight polysulfides are present in plasma/serum and if they are causing the main signal.

Further studies need to be performed to evaluate the contribution of H_2S to vascular health and to elucidate if H_2S levels could even have the potential to be used as a biomarker in the future.

While endogenous H_2S production is essential for the benefits of CR, it remains to be explored if it plays a role in other dietary restriction regimens like PR and MR. Even though our results suggest that H_2S production capacity and also protein levels of H_2S producing enzymes are not altered upon a protein free diet, it remains to be elucidated if H_2S plays a role in the lifespan extension and the increased stress resistance caused by protein restriction. A main focus of future experiments is also to elucidate why H_2S production capacity in MR is upregulated and if there is really not accompanied by an increase in CGL and CBS protein levels. It is also essential to better understand how enzyme activity and H_2S levels are connected and if an increase in H_2S production capacity upon addition of excess substrate goes along with actual H_2S production and H_2S levels in the living cells.

The relevance of endogenously produced H_2S has only been a focus of research for the last 20 years and due to the complexity of the topic a lot remains to be elucidated. Many more experiments need to be conducted to understand, what impact this gasotransmitter has on maintaining physiological functions and on health and disease.

6. Abbreviations

3MP	3-Mercaptopyruvate
3MST	3-Mercaptopyruvate sulfurtransferase
AOAA	Aminooxyacetic acid
ATP	Adenosintriphosphat
BCA	B-cyano-L-alanine
BSA	Bovine serum albumin
CAT	Cysteine aminotransferase
CBS	Cysteine beta synthase
CGL	Cystathionine gamma lyase
CO	Carbon monoxide
CoA	Coenzyme A
CR	Caloric restriction
DAO	Diaminooxidase
DHLA	Dihydrolipoic acid
DR	Dietary restriction
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
ETHE1	Persulfide Dioxygenase
FAD	Flavin-Adenin-Dinukleotid
GAPDH	Glyceraldehyde 3-P dehydrogenase
GSH	Glutathione
H_2O	Water
H_2S	Hydrogen sulfide
HA	Hydroxylamine
HPLC	High performance liquid chromatography
IRI	Ischemia reperfusion injury
KO	Knockout
mM	millimolar

ml	Milliliter
µM	Micromolar
µl	microliter
MR	Methionine restriction
MWCO	Molecular weight cut off
NADH	Nicotinamidadenindinukleotidphosphat
NO	Nitric oxide
PAG	Propargylglycine
PBS	Phosphate buffered saline
PLP	pyridoxal 5' phosphate
ppm	parts per million
pmol	picomol
PF	Protein free
SIFT-MS	Selected-ion flow-tube mass spectrometry
SQR	sulfur quinone oxidoreductase
TCA	tricarboxylic acid
TSP	Transsulfuration pathway
TCEP	Tris(2-chlorethyl)phosphat
Trx	Thioredoxin
WT	Wildtype

7. References

Abe, K.; Kimura, H. (1996): The possible role of hydrogen sulfide as an endogenous neuromodulator. In: *The Journal of neuroscience : the official journal of the Society for Neuroscience* 16 (3), S. 1066–1071.

Asimakopoulou, Antonia; Panopoulos, Panagiotis; Chasapis, Christos T.; Coletta, Ciro; Zhou, Zongmin; Cirino, Giuseppe et al. (2013): Selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). In: *British journal of pharmacology* 169 (4), S. 922–932. DOI: 10.1111/bph.12171.

Bartholomew, T. C.; Powell, G. M.; Dodgson, K. S.; Curtis, C. G. (1980): Oxidation of sodium sulphide by rat liver, lungs and kidney. In: *Biochemical pharmacology* 29 (18), S. 2431–2437.

Beauchamp, R. O.; Bus, J. S.; Popp, J. A.; Boreiko, C. J.; Andjelkovich, D. A. (1984): A critical review of the literature on hydrogen sulfide toxicity. In: *Critical reviews in toxicology* 13 (1), S. 25–97. DOI: 10.3109/10408448409029321.

Beyer, Katrin; Lao, José I.; Carrato, Cristina; Rodriguez-Vila, Ana; Latorre, Pilar; Mataró, Maria et al. (2004): Cystathionine beta synthase as a risk factor for Alzheimer disease. In: *Current Alzheimer research* 1 (2), S. 127–133.

Bostelaar, Trever; Vitvitsky, Victor; Kumutima, Jacques; Lewis, Brianne E.; Yadav, Pramod K.; Brunold, Thomas C. et al. (2016): Hydrogen Sulfide Oxidation by Myoglobin. In: *Journal of the American Chemical Society* 138 (27), S. 8476–8488. DOI: 10.1021/jacs.6b03456.

Braunstein, A. E.; Goryachenkova, E. V.; Lac, N. D. (1969): Reactions catalysed by serine sulfhydrase from chicken liver. In: *Biochimica et biophysica acta* 171 (2), S. 366–368.

Bredt, D. S.; Snyder, S. H. (1989): Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. In: *Proceedings of the National Academy of Sciences of the United States of America* 86 (22), S. 9030–9033.

Bucci, Mariarosaria; Cirino, Giuseppe (2011): Hydrogen sulphide in heart and systemic circulation. In: *Inflammation & allergy drug targets* 10 (2), S. 103–108.

Castro, A. R.; Morrill, W. E.; Pope, V. (2000): Lipid removal from human serum samples. In: *Clinical and diagnostic laboratory immunology* 7 (2), S. 197–199.

CAVALLINI, D.; MONDOVI, B.; MARCO, C. de; SCIOSCIA-SANTORO, A. (1962): The mechanism of desulphhydration of cysteine. In: *Enzymologia* 24, S. 253–266.

Chen, Chang-qing; Xin, Hong; Zhu, Yi-Zhun (2007): Hydrogen sulfide: third gaseous transmitter, but with great pharmacological potential. In: *Acta pharmacologica Sinica* 28 (11), S. 1709–1716. DOI: 10.1111/j.1745-7254.2007.00629.x.

Chiku, Taurai; Padovani, Dominique; Zhu, Weidong; Singh, Sangita; Vitvitsky, Victor; Banerjee, Ruma (2009): H2S biogenesis by human cystathionine gamma-lyase leads to the novel sulfur metabolites lanthionine and homolanthionine and is responsive to the grade of hyperhomocysteinemia. In: *The Journal of biological chemistry* 284 (17), S. 11601–11612. DOI: 10.1074/jbc.M808026200.

Coletta, Ciro; Módis, Katalin; Szczesny, Bartosz; Brunyánszki, Attila; Oláh, Gábor; Rios, Ester C. S. et al. (2015): Regulation of Vascular Tone, Angiogenesis and Cellular Bioenergetics by the 3-Mercaptopyruvate Sulfurtransferase/H2S Pathway: Functional Impairment by Hyperglycemia and Restoration by DL-α-Lipoic Acid. In: *Molecular medicine (Cambridge, Mass.)* 21, S. 1–14. DOI: 10.2119/molmed.2015.00035.

Coletta, Ciro; Szabo, Csaba (2013): Potential role of hydrogen sulfide in the pathogenesis of vascular dysfunction in septic shock. In: *Current vascular pharmacology* 11 (2), S. 208–221.

Cortese-Krott, Miriam M.; Kuhnle, Gunter G. C.; Dyson, Alex; Fernandez, Bernadette O.; Grman, Marian; DuMond, Jenna F. et al. (2015): Key bioactive reaction products of the NO/H2S interaction are S/N-hybrid species, polysulfides, and nitroxyl. In: *Proceedings of the National Academy of Sciences of the United States of America* 112 (34), E4651-60. DOI: 10.1073/pnas.1509277112.

Cuevasanta, Ernesto; Möller, Matías N.; Alvarez, Beatriz (2017): Biological chemistry of hydrogen sulfide and persulfides. In: *Archives of Biochemistry and Biophysics* 617, S. 9–25. DOI: 10.1016/j.abb.2016.09.018.

Curtis, C. G.; Bartholomew, T. C.; Rose, F. A.; Dodgson, K. S. (1972): Detoxication of sodium 35 S-sulphide in the rat. In: *Biochemical pharmacology* 21 (17), S. 2313–2321.

Dalgliesh, Gillian L.; Furge, Kyle; Greenman, Chris; Chen, Lina; Bignell, Graham; Butler, Adam et al. (2010): Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. In: *Nature* 463 (7279), S. 360–363. DOI: 10.1038/nature08672.

Das, Sajal Kumar; Lim, Chang Su; Yang, Sun Young; Han, Ji Hee; Cho, Bong Rae (2012): A small molecule two-photon probe for hydrogen sulfide in live tissues. In: *Chemical communications (Cambridge, England)* 48 (67), S. 8395–8397. DOI: 10.1039/c2cc33909a.

Doeller, Jeannette E.; Isbell, T. Scott; Benavides, Gloria; Koenitzer, Jeffrey; Patel, Hetal; Patel, Rakesh P. et al. (2005): Polarographic measurement of hydrogen sulfide production and consumption by mammalian tissues. In: *Analytical biochemistry* 341 (1), S. 40–51. DOI: 10.1016/j.ab.2005.03.024.

Dóka, Éva; Pader, Irina; Bíró, Adrienn; Johansson, Katarina; Cheng, Qing; Ballagó, Krisztina et al. (2016): A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems. In: *Science advances* 2 (1), e1500968. DOI: 10.1126/sciadv.1500968.

Doujaiji, Bassam; Al-Tawfiq, Jaffar A. (2010): Hydrogen sulfide exposure in an adult male. In: *Annals of Saudi medicine* 30 (1), S. 76–80. DOI: 10.4103/0256-4947.59379.

Eberhardt, Mirjam; Dux, Maria; Namer, Barbara; Miljkovic, Jan; Cordasic, Nada; Will, Christine et al. (2014): H2S and NO cooperatively regulate vascular tone by activating a neuroendocrine HNO-TRPA1-CGRP signalling pathway. In: *Nature communications* 5, S. 4381. DOI: 10.1038/ncomms5381.

Enokido, Yasushi; Suzuki, Eri; Iwasawa, Kazu; Namekata, Kazuhiko; Okazawa, Hitoshi; Kimura, Hideo (2005): Cystathionine beta-synthase, a key enzyme for homocysteine

metabolism, is preferentially expressed in the radial glia/astrocyte lineage of developing mouse CNS. In: *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 19 (13), S. 1854–1856. DOI: 10.1096/fj.05-3724fje.

Filipovic, Milos R. (2015): Persulfidation (S-sulfhydration) and H2S. In: *Handbook of experimental pharmacology* 230, S. 29–59. DOI: 10.1007/978-3-319-18144-8_2.

Fischer, Emil (1883): Bildung von Methylenblau als Reaktion auf Schwefelwasserstoff. In: *Ber. Dtsch. Chem. Ges.* 16 (2), S. 2234–2236. DOI: 10.1002/cber.188301602138.

Fontana, Luigi; Partridge, Linda; Longo, Valter D. (2010): Extending healthy life span--from yeast to humans. In: *Science (New York, N.Y.)* 328 (5976), S. 321–326. DOI: 10.1126/science.1172539.

Forster, M. M. (1994): Mineral springs and miracles. In: *Canadian family physician Medecin de famille canadien* 40, S. 729–737.

Gadalla, Moataz M.; Snyder, Solomon H. (2010): Hydrogen sulfide as a gasotransmitter. In: *Journal of neurochemistry* 113 (1), S. 14–26. DOI: 10.1111/j.1471-4159.2010.06580.x.

Goubern, Marc; Andriamihaja, Mireille; Nübel, Tobias; Blachier, François; Bouillaud, Frédéric (2007): Sulfide, the first inorganic substrate for human cells. In: *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 21 (8), S. 1699–1706. DOI: 10.1096/fj.06-7407com.

Grandison, Richard C.; Piper, Matthew D. W.; Partridge, Linda (2009): Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. In: *Nature* 462 (7276), S. 1061–1064. DOI: 10.1038/nature08619.

Greiner, Romy; Pálinkás, Zoltán; Bäsell, Katrin; Becher, Dörte; Antelmann, Haike; Nagy, Péter; Dick, Tobias P. (2013): Polysulfides link H2S to protein thiol oxidation. In: *Antioxidants & redox signaling* 19 (15), S. 1749–1765. DOI: 10.1089/ars.2012.5041.

Guidotti, T. L. (1996): Hydrogen sulphide. In: *Occupational medicine (Oxford, England)* 46 (5), S. 367–371.

Guidotti, Tee L. (2015): Hydrogen sulfide intoxication. In: *Handbook of clinical neurology* 131, S. 111–133. DOI: 10.1016/B978-0-444-62627-1.00008-1.

Guo, Zhi; Chen, Guiqiu; Zeng, Guangming; Li, Zhongwu; Chen, Anwei; Wang, Jiajia; Jiang, Longbo (2015): Fluorescence chemosensors for hydrogen sulfide detection in biological systems. In: *The Analyst* 140 (6), S. 1772–1786. DOI: 10.1039/c4an01909a.

Han, J. C.; Han, G. Y. (1994): A procedure for quantitative determination of tris(2-carboxyethyl)phosphine, an odorless reducing agent more stable and effective than dithiothreitol. In: *Analytical biochemistry* 220 (1), S. 5–10. DOI: 10.1006/abio.1994.1290.

Hill, F. B. (1973): Atmospheric sulfur and its links to the biota. In: *Brookhaven symposia in biology* (30), S. 159–181.

Hine, Christopher; Harputlugil, Eylul; Zhang, Yue; Ruckenstuhl, Christoph; Lee, Byung Cheon; Brace, Lear et al. (2015): Endogenous hydrogen sulfide production is essential for dietary restriction benefits. In: *Cell* 160 (1-2), S. 132–144. DOI: 10.1016/j.cell.2014.11.048.

Hine, Christopher; Mitchell, James R. (2015): Calorie restriction and methionine restriction in control of endogenous hydrogen sulfide production by the transsulfuration pathway. In: *Experimental gerontology* 68, S. 26–32. DOI: 10.1016/j.exger.2014.12.010.

Hosoki, R.; Matsuki, N.; Kimura, H. (1997): The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. In: *Biochemical and biophysical research communications* 237 (3), S. 527–531. DOI: 10.1006/bbrc.1997.6878.

Hu, Li-Fang; Lu, Ming; Tiong, Chi Xin; Dawe, Gavin S.; Hu, Gang; Bian, Jin-Song (2010): Neuroprotective effects of hydrogen sulfide on Parkinson's disease rat models. In: *Aging cell* 9 (2), S. 135–146. DOI: 10.1111/j.1474-9726.2009.00543.x.

Hu, Li-Fang; Lu, Ming; Wu, Zhi-Yuan; Wong, Peter T-H; Bian, Jin-Song (2009): Hydrogen sulfide inhibits rotenone-induced apoptosis via preservation of mitochondrial function. In: *Molecular pharmacology* 75 (1), S. 27–34. DOI: 10.1124/mol.108.047985.

Huang, P. L.; Dawson, T. M.; Bredt, D. S.; Snyder, S. H.; Fishman, M. C. (1993): Targeted disruption of the neuronal nitric oxide synthase gene. In: *Cell* 75 (7), S. 1273–1286.

Ikeda, Mayumi; Ishima, Yu; Shibata, Akitomo; Chuang, Victor T. G.; Sawa, Tomohiro; Ihara, Hideshi et al. (2017): Quantitative determination of polysulfide in albumins, plasma proteins and biological fluid samples using a novel combined assays approach. In: *Analytica chimica acta* 969, S. 18–25. DOI: 10.1016/j.aca.2017.03.027.

Ishigami, Mari; Hiraki, Keiko; Umemura, Ken; Ogasawara, Yuki; Ishii, Kazuyuki; Kimura, Hideo (2009): A source of hydrogen sulfide and a mechanism of its release in the brain. In: *Antioxidants & redox signaling* 11 (2), S. 205–214. DOI: 10.1089/ars.2008.2132.

Isobe, Chiaki; Murata, Takahiko; Sato, Chigumi; Terayama, Yasuo (2005): Increase of total homocysteine concentration in cerebrospinal fluid in patients with Alzheimer's disease and Parkinson's disease. In: *Life sciences* 77 (15), S. 1836–1843. DOI: 10.1016/j.lfs.2005.02.014.

Jackson, Michael R.; Melideo, Scott L.; Jorns, Marilyn Schuman (2012): Human sulfide:quinone oxidoreductase catalyzes the first step in hydrogen sulfide metabolism and produces a sulfane sulfur metabolite. In: *Biochemistry* 51 (34), S. 6804–6815. DOI: 10.1021/bi300778t.

Jain, Sushil K.; Bull, Rebeca; Rains, Justin L.; Bass, Pat F.; Levine, Steven N.; Reddy, Sudha et al. (2010): Low levels of hydrogen sulfide in the blood of diabetes patients and streptozotocin-treated rats causes vascular inflammation? In: *Antioxidants & redox signaling* 12 (11), S. 1333–1337. DOI: 10.1089/ars.2009.2956.

Jain, Sushil K.; Kahlon, Gunjan; Morehead, Lester; Lieblong, Benjamin; Stapleton, Tommie; Hoeldtke, Robert et al. (2012): The effect of sleep apnea and insomnia on blood levels of leptin, insulin resistance, IP-10, and hydrogen sulfide in type 2 diabetic patients. In: *Metabolic syndrome and related disorders* 10 (5), S. 331–336. DOI: 10.1089/met.2012.0045.

Jhee, Kwang-Hwan; Kruger, Warren D. (2005): The role of cystathionine beta-synthase in homocysteine metabolism. In: *Antioxidants & redox signaling* 7 (5-6), S. 813–822. DOI: 10.1089/ars.2005.7.813.

Kabil, Hadise; Kabil, Omer; Banerjee, Ruma; Harshman, Lawrence G.; Pletcher, Scott D. (2011): Increased transsulfuration mediates longevity and dietary restriction in

Drosophila. In: *Proceedings of the National Academy of Sciences of the United States of America* 108 (40), S. 16831–16836. DOI: 10.1073/pnas.1102008108.

Kabil, Omer; Banerjee, Ruma (2014): Enzymology of H2S biogenesis, decay and signaling. In: *Antioxidants & redox signaling* 20 (5), S. 770–782. DOI: 10.1089/ars.2013.5339.

Kabil, Omer; Zhou, You; Banerjee, Ruma (2006): Human cystathionine beta-synthase is a target for sumoylation. In: *Biochemistry* 45 (45), S. 13528–13536. DOI: 10.1021/bi0615644.

Kanagy, Nancy L.; Szabo, Csaba; Papapetropoulos, Andreas (2017): Vascular biology of hydrogen sulfide. In: *American journal of physiology. Cell physiology* 312 (5), C537-C549. DOI: 10.1152/ajpcell.00329.2016.

Karsten, William E.; Cook, Paul F. (2002): Detection of intermediates in reactions catalyzed by PLP-dependent enzymes: O-acetylserine sulfhydrylase and serine-glyoxalate aminotransferase. In: *Methods in enzymology* 354, S. 223–237.

Katewa, Subhash D.; Kapahi, Pankaj (2010): Dietary restriction and aging, 2009. In: *Aging cell* 9 (2), S. 105–112. DOI: 10.1111/j.1474-9726.2010.00552.x.

Kery, V.; Bukovska, G.; Kraus, J. P. (1994): Transsulfuration depends on heme in addition to pyridoxal 5'-phosphate. Cystathionine beta-synthase is a heme protein. In: *The Journal of biological chemistry* 269 (41), S. 25283–25288.

Kimura, Hideo (2016): Hydrogen polysulfide (H2S n) signaling along with hydrogen sulfide (H2S) and nitric oxide (NO). In: *Journal of Neural Transmission* 123 (11), S. 1235–1245. DOI: 10.1007/s00702-016-1600-z.

Kimura, Yuka; Mikami, Yoshinori; Osumi, Kimiko; Tsugane, Mamiko; Oka, Jun-ichiro; Kimura, Hideo (2013): Polysulfides are possible H2S-derived signaling molecules in rat brain. In: *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 27 (6), S. 2451–2457. DOI: 10.1096/fj.12-226415.

Kimura, Yuka; Toyofuku, Yukiko; Koike, Shin; Shibuya, Norihiro; Nagahara, Noriyuki; Lefer, David et al. (2015): Identification of H2S3 and H2S produced by 3-

mercaptopyruvate sulfurtransferase in the brain. In: *Scientific reports* 5, S. 14774. DOI: 10.1038/srep14774.

Koike, Shin; Shibuya, Norihiro; Kimura, Hideo; Ishii, Kazuyuki; Ogasawara, Yuki (2015): Polysulfide promotes neuroblastoma cell differentiation by accelerating calcium influx. In: *Biochemical and biophysical research communications* 459 (3), S. 488–492. DOI: 10.1016/j.bbrc.2015.02.133.

Kolluru, Gopi K.; Shen, Xinggui; Bir, Shyamal C.; Kevil, Christopher G. (2013): Hydrogen sulfide chemical biology: pathophysiological roles and detection. In: *Nitric oxide : biology and chemistry* 35, S. 5–20. DOI: 10.1016/j.niox.2013.07.002.

Koutmos, Markos; Kabil, Omer; Smith, Janet L.; Banerjee, Ruma (2010): Structural basis for substrate activation and regulation by cystathionine beta-synthase (CBS) domains in cystathionine {beta}-synthase. In: *Proceedings of the National Academy of Sciences of the United States of America* 107 (49), S. 20958–20963. DOI: 10.1073/pnas.1011448107.

Kovačić, Dragan; Glavnik, Nina; Marinšek, Matej; Zagožen, Petra; Rovan, Ksenija; Goslar, Tomaž et al. (2012): Total plasma sulfide in congestive heart failure. In: *Journal of cardiac failure* 18 (7), S. 541–548. DOI: 10.1016/j.cardfail.2012.04.011.

Krijt, Jakub; Kopecká, Jana; Hnízda, Aleš; Moat, Stuart; Kluijtmans, Leo A. J.; Mayne, Philip; Kožich, Viktor (2011): Determination of cystathionine beta-synthase activity in human plasma by LC-MS/MS: potential use in diagnosis of CBS deficiency. In: *Journal of inherited metabolic disease* 34 (1), S. 49–55. DOI: 10.1007/s10545-010-9178-3.

Krishnan, Navasona; Fu, Cexiong; Pappin, Darryl J.; Tonks, Nicholas K. (2011): H2S-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. In: *Science signaling* 4 (203), ra86. DOI: 10.1126/scisignal.2002329.

Kruger, W. D.; Evans, A. A.; Wang, L.; Malinow, M. R.; Duell, P. B.; Anderson, P. H. et al. (2000): Polymorphisms in the CBS gene associated with decreased risk of coronary artery disease and increased responsiveness to total homocysteine lowering by folic acid. In: *Molecular genetics and metabolism* 70 (1), S. 53–60. DOI: 10.1006/mgme.2000.2993.

Levonen, A. L.; Lapatto, R.; Saksela, M.; Raivio, K. O. (2000): Human cystathionine gamma-lyase: developmental and in vitro expression of two isoforms. In: *The Biochemical journal* 347 Pt 1, S. 291–295.

Li, Ling; Bhatia, Madhav; Zhu, Yi Zhun; Zhu, Yi Chun; Ramnath, Raina Devi; Wang, Zhong Jing et al. (2005): Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. In: *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 19 (9), S. 1196–1198. DOI: 10.1096/fj.04-3583fje.

Libiad, Marouane; Yadav, Pramod Kumar; Vitvitsky, Victor; Martinov, Michael; Banerjee, Ruma (2014): Organization of the human mitochondrial hydrogen sulfide oxidation pathway. In: *The Journal of biological chemistry* 289 (45), S. 30901–30910. DOI: 10.1074/jbc.M114.602664.

Linden, David R. (2014): Hydrogen sulfide signaling in the gastrointestinal tract. In: *Antioxidants & redox signaling* 20 (5), S. 818–830. DOI: 10.1089/ars.2013.5312.

Liu, Xiang-qin; Liu, Xiao-qi; Jiang, Ping; Huang, Hua; Yan, Yong (2008): Plasma levels of endogenous hydrogen sulfide and homocysteine in patients with Alzheimer's disease and vascular dementia and the significance thereof. In: *Zhonghua yi xue za zhi* 88 (32), S. 2246–2249.

Lloyd, David (2006): Hydrogen sulfide: clandestine microbial messenger? In: *Trends in microbiology* 14 (10), S. 456–462. DOI: 10.1016/j.tim.2006.08.003.

Mani, Sarathi; Li, Hongzhu; Untereiner, Ashley; Wu, Lingyun; Yang, Guangdong; Austin, Richard C. et al. (2013): Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. In: *Circulation* 127 (25), S. 2523–2534. DOI: 10.1161/CIRCULATIONAHA.113.002208.

Matoba, T.; Shimokawa, H.; Nakashima, M.; Hirakawa, Y.; Mukai, Y.; Hirano, K. et al. (2000): Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. In: *The Journal of clinical investigation* 106 (12), S. 1521–1530. DOI: 10.1172/JCI10506.

Melideo, Scott L.; Jackson, Michael R.; Jorns, Marilyn Schuman (2014): Biosynthesis of a Central Intermediate in Hydrogen Sulfide Metabolism by a Novel Human

Sulfurtransferase and Its Yeast Ortholog. In: *Biochemistry* 53 (28), S. 4739–4753. DOI: 10.1021/bi500650h.

Meng, Guoliang; Ma, Yan; Xie, Liping; Ferro, Albert; Ji, Yong (2015): Emerging role of hydrogen sulfide in hypertension and related cardiovascular diseases. In: *British journal of pharmacology* 172 (23), S. 5501–5511. DOI: 10.1111/bph.12900.

Meng, Guoliang; Zhao, Shuang; Xie, Liping; Han, Yi; Ji, Yong (2017): Protein S-sulfhydration by hydrogen sulfide in cardiovascular system. In: *British journal of pharmacology. DOI:* 10.1111/bph.13825.

Mikami, Yoshinori; Shibuya, Norihiro; Kimura, Yuka; Nagahara, Noriyuki; Ogasawara, Yuki; Kimura, Hideo (2011): Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. In: *The Biochemical journal* 439 (3), S. 479–485. DOI: 10.1042/BJ20110841.

Miller, Dana L.; Roth, Mark B. (2007): Hydrogen sulfide increases thermotolerance and lifespan in Caenorhabditis elegans. In: *Proceedings of the National Academy of Sciences of the United States of America* 104 (51), S. 20618–20622. DOI: 10.1073/pnas.0710191104.

Miller, Richard A.; Buehner, Gretchen; Chang, Yayi; Harper, James M.; Sigler, Robert; Smith-Wheelock, Michael (2005): Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. In: *Aging cell* 4 (3), S. 119–125. DOI: 10.1111/j.1474-9726.2005.00152.x.

Miyamoto, Ryo; Koike, Shin; Takano, Yoko; Shibuya, Norihiro; Kimura, Yuka; Hanaoka, Kenjiro et al. (2017): Polysulfides (H2Sn) produced from the interaction of hydrogen sulfide (H2S) and nitric oxide (NO) activate TRPA1 channels. In: *Scientific reports* 7, S. 45995. DOI: 10.1038/srep45995.

Módis, Katalin; Panopoulos, Panagiotis; Coletta, Ciro; Papapetropoulos, Andreas; Szabo, Csaba (2013): Hydrogen sulfide-mediated stimulation of mitochondrial electron transport involves inhibition of the mitochondrial phosphodiesterase 2A, elevation of cAMP and activation of protein kinase A. In: *Biochemical pharmacology* 86 (9), S. 1311–1319. DOI: 10.1016/j.bcp.2013.08.064.

Mok, Ying-Yuan Pamela; Atan, Mohammed Shirhan Bin Mohammed; Yoke Ping, Cheong; Zhong Jing, Wang; Bhatia, Madhav; Moochhala, Shabbir; Moore, Philip K. (2004): Role of hydrogen sulphide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulphide biosynthesis. In: *British journal of pharmacology* 143 (7), S. 881–889. DOI: 10.1038/sj.bjp.0706014.

Montoya, Leticia A.; Pluth, Michael D. (2012): Selective turn-on fluorescent probes for imaging hydrogen sulfide in living cells. In: *Chemical communications (Cambridge, England)* 48 (39), S. 4767–4769. DOI: 10.1039/c2cc30730h.

Mustafa, Asif K.; Gadalla, Moataz M.; Sen, Nilkantha; Kim, Seyun; Mu, Weitong; Gazi, Sadia K. et al. (2009): H2S signals through protein S-sulfhydration. In: *Science signaling* 2 (96), ra72. DOI: 10.1126/scisignal.2000464.

Nagahara, N.; Ito, Takaaki; Kitamura, Hitoshi; Nishino, Takeshi (1998): Tissue and subcellular distribution of mercaptopyruvate sulfurtransferase in the rat. Confocal laser fluorescence and immunoelectron microscopic studies combined with biochemical analysis. In: *Histochemistry and Cell Biology* 110 (3), S. 243–250. DOI: 10.1007/s004180050286.

Nagai, Yasuo; Tsugane, Mamiko; Oka, Jun-ichiro; Kimura, Hideo (2004): Hydrogen sulfide induces calcium waves in astrocytes. In: *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 18 (3), S. 557–559. DOI: 10.1096/fj.03-1052fje.

O'Brien, Dennis J.; Birkner, Francis B. (1977): Kinetics of oxygenation of reduced sulfur species in aqueous solution. In: *Environ. Sci. Technol.* 11 (12), S. 1114–1120. DOI: 10.1021/es60135a009.

Ogasawara, Y.; Isoda, S.; Tanabe, S. (1994): Tissue and subcellular distribution of bound and acid-labile sulfur, and the enzymic capacity for sulfide production in the rat. In: *Biological & pharmaceutical bulletin* 17 (12), S. 1535–1542.

Olson, Kenneth R. (2009): Is hydrogen sulfide a circulating "gasotransmitter" in vertebrate blood? In: *Biochimica et biophysica acta* 1787 (7), S. 856–863. DOI: 10.1016/j.bbabio.2009.03.019.

Olson, Kenneth R.; Straub, Karl D. (2016): The Role of Hydrogen Sulfide in Evolution and the Evolution of Hydrogen Sulfide in Metabolism and Signaling. In: *Physiology (Bethesda, Md.)* 31 (1), S. 60–72. DOI: 10.1152/physiol.00024.2015.

Olson, Kenneth R.; Whitfield, Nathan L.; Bearden, Shawn E.; St Leger, Judy; Nilson, Erika; Gao, Yan; Madden, Jane A. (2010): Hypoxic pulmonary vasodilation: a paradigm shift with a hydrogen sulfide mechanism. In: *American journal of physiology. Regulatory, integrative and comparative physiology* 298 (1), R51-60. DOI: 10.1152/ajpregu.00576.2009.

Panthi, Sandesh; Chung, Hyung-Joo; Jung, Junyang; Jeong, Na Young (2016): Physiological Importance of Hydrogen Sulfide: Emerging Potent Neuroprotector and Neuromodulator. In: *Oxidative medicine and cellular longevity* 2016, S. 9049782. DOI: 10.1155/2016/9049782.

Paul, Bindu D.; Snyder, Solomon H. (2015): Protein sulfhydration. In: *Methods in enzymology* 555, S. 79–90. DOI: 10.1016/bs.mie.2014.11.021.

Peng, Bo; Liu, Chunrong; Li, Zhen; Day, Jacob J.; Lu, Yun; Lefer, David J.; Xian, Ming (2017): Slow generation of hydrogen sulfide from sulfane sulfurs and NADH models. In: *Bioorganic & medicinal chemistry letters* 27 (3), S. 542–545. DOI: 10.1016/j.bmcl.2016.12.023.

Peter, Elvis A.; Shen, Xinggui; Shah, Shivang H.; Pardue, Sibile; Glawe, John D.; Zhang, Wayne W. et al. (2013): Plasma free H2S levels are elevated in patients with cardiovascular disease. In: *Journal of the American Heart Association* 2 (5), e000387. DOI: 10.1161/JAHA.113.000387.

Petersen, L. C. (1977): The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase. In: *Biochimica et biophysica acta* 460 (2), S. 299–307.

Renga, Barbara (2011): Hydrogen sulfide generation in mammals: the molecular biology of cystathionine-β- synthase (CBS) and cystathionine-γ-lyase (CSE). In: *Inflammation & allergy drug targets* 10 (2), S. 85–91.

Robert, Karine; Vialard, François; Thiery, Eric; Toyama, Kiyoko; Sinet, Pierre-Marie; Janel, Nathalie; London, Jacqueline (2003): Expression of the cystathionine beta

synthase (CBS) gene during mouse development and immunolocalization in adult brain. In: *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 51 (3), S. 363–371. DOI: 10.1177/002215540305100311.

Rose, Peter; Moore, Philip K.; Zhu, Yi Zhun (2017): H2S biosynthesis and catabolism: new insights from molecular studies. In: *Cellular and molecular life sciences : CMLS* 74 (8), S. 1391–1412. DOI: 10.1007/s00018-016-2406-8.

Ruckenstuhl, Christoph; Netzberger, Christine; Entfellner, Iryna; Carmona-Gutierrez, Didac; Kickenweiz, Thomas; Stekovic, Slaven et al. (2014): Lifespan extension by methionine restriction requires autophagy-dependent vacuolar acidification. In: *PLoS genetics* 10 (5), e1004347. DOI: 10.1371/journal.pgen.1004347.

Searcy, Dennis G.; Lee, So Hyung (1998): Sulfur reduction by human erythrocytes. In: *J. Exp. Zool.* 282 (3), S. 310–322. DOI: 10.1002/(SICI)1097-010X(19981015)282:3<310::AID-JEZ4>3.0.CO;2-P.

Shen, Xinggui; Pattillo, Christopher B.; Pardue, Sibile; Bir, Shyamal C.; Wang, Rui; Kevil, Christopher G. (2011): Measurement of plasma hydrogen sulfide in vivo and in vitro. In: *Free radical biology & medicine* 50 (9), S. 1021–1031. DOI: 10.1016/j.freeradbiomed.2011.01.025.

Shen, Xinggui; Peter, Elvis A.; Bir, Shyamal; Wang, Rui; Kevil, Christopher G. (2012): Analytical measurement of discrete hydrogen sulfide pools in biological specimens. In: *Free radical biology & medicine* 52 (11-12), S. 2276–2283. DOI: 10.1016/j.freeradbiomed.2012.04.007.

Shibuya, Norihiro; Koike, Shin; Tanaka, Makiko; Ishigami-Yuasa, Mari; Kimura, Yuka; Ogasawara, Yuki et al. (2013): A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. In: *Nature communications* 4, S. 1366. DOI: 10.1038/ncomms2371.

Shibuya, Norihiro; Tanaka, Makiko; Yoshida, Mikiharu; Ogasawara, Yuki; Togawa, Tadayasu; Ishii, Kazuyuki; Kimura, Hideo (2009): 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. In: *Antioxidants & redox signaling* 11 (4), S. 703–714. DOI: 10.1089/ars.2008.2253.

Singh, Sangita; Padovani, Dominique; Leslie, Rachel A.; Chiku, Taurai; Banerjee, Ruma (2009): Relative contributions of cystathionine beta-synthase and gamma-cystathionase to H2S biogenesis via alternative trans-sulfuration reactions. In: *The Journal of biological chemistry* 284 (33), S. 22457–22466. DOI: 10.1074/jbc.M109.010868.

Singha, Subhankar; Kim, Dokyoung; Moon, Hyunsoo; Wang, Taejun; Kim, Ki Hean; Shin, Youn Ho et al. (2015): Toward a selective, sensitive, fast-responsive, and biocompatible two-photon probe for hydrogen sulfide in live cells. In: *Analytical chemistry* 87 (2), S. 1188–1195. DOI: 10.1021/ac503806w.

Speakman, J. R.; Mitchell, S. E.; Mazidi, M. (2016): Calories or protein? The effect of dietary restriction on lifespan in rodents is explained by calories alone. In: *Experimental gerontology* 86, S. 28–38. DOI: 10.1016/j.exger.2016.03.011.

Srilatha, Balasubramanian; Adaikan, P. Ganesan; Li, Ling; Moore, Philip K. (2007): Hydrogen sulphide: a novel endogenous gasotransmitter facilitates erectile function. In: *The journal of sexual medicine* 4 (5), S. 1304–1311. DOI: 10.1111/j.1743-6109.2007.00561.x.

Stipanuk, M. H.; Beck, P. W. (1982): Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat. In: *The Biochemical journal* 206 (2), S. 267–277.

Stipanuk, Martha H. (2004): Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. In: *Annual review of nutrition* 24, S. 539–577. DOI: 10.1146/annurev.nutr.24.012003.132418.

Sun, Qingxiang; Collins, Ruairi; Huang, Shufen; Holmberg-Schiavone, Lovisa; Anand, Ganesh S.; Tan, Choon-Hong et al. (2009): Structural basis for the inhibition mechanism of human cystathionine gamma-lyase, an enzyme responsible for the production of H(2)S. In: *The Journal of biological chemistry* 284 (5), S. 3076–3085. DOI: 10.1074/jbc.M805459200.

Suzuki, Kunihiro; Sagara, Masaaki; Aoki, Chie; Tanaka, Seiichi; Aso, Yoshimasa (2017): Clinical Implication of Plasma Hydrogen Sulfide Levels in Japanese Patients with Type 2 Diabetes. In: *Internal medicine (Tokyo, Japan)* 56 (1), S. 17–21. DOI: 10.2169/internalmedicine.56.7403.

TAGUCHI, Tetsuya; AWATA, Shiro; NISHIOKA, Michiko; ARAKAWA, Yukiko; SHIRAISHI, Noriyuki; RYU, Shuhei et al. (1995): Elevation of Cystathionine.GAMMA.-lyase Activity in the Serum of Rats Treated with a Single Dose of Carbon Tetrachloride. In: *INDUSTRIAL HEALTH* 33 (4), S. 199–205. DOI: 10.2486/indhealth.33.199.

Takano, Yoko; Shimamoto, Kazuhito; Hanaoka, Kenjiro (2016): Chemical tools for the study of hydrogen sulfide (H2S) and sulfane sulfur and their applications to biological studies. In: *Journal of clinical biochemistry and nutrition* 58 (1), S. 7–15. DOI: 10.3164/jcbn.15-91.

Talaei, Fatemeh; Bouma, Hjalmar R.; Hylkema, Machteld N.; Strijkstra, Arjen M.; Boerema, Ate S.; Schmidt, Martina; Henning, Rob H. (2012): The role of endogenous H2S formation in reversible remodeling of lung tissue during hibernation in the Syrian hamster. In: *The Journal of experimental biology* 215 (Pt 16), S. 2912–2919. DOI: 10.1242/jeb.067363.

Teague, B.; Asiedu, S.; Moore, P. K. (2002): The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. In: *British journal of pharmacology* 137 (2), S. 139–145. DOI: 10.1038/sj.bjp.0704858.

Teraoka, T.; Ohta, J.; Abe, T.; Inoue, H.; Ubuka, T. (1993): Inhibition of sulfate-forming activity in rat liver mitochondria by (aminooxy)acetate. In: *Amino Acids* 5 (2), S. 245–251. DOI: 10.1007/BF00805987.

Tiranti, Valeria; Viscomi, Carlo; Hildebrandt, Tatjana; Di Meo, Ivano; Mineri, Rossana; Tiveron, Cecilia et al. (2009): Loss of ETHE1, a mitochondrial dioxygenase, causes fatal sulfide toxicity in ethylmalonic encephalopathy. In: *Nature medicine* 15 (2), S. 200–205. DOI: 10.1038/nm.1907.

Togawa, T.; Ogawa, M.; Nawata, M.; Ogasawara, Y.; Kawanabe, K.; Tanabe, S. (1992): High performance liquid chromatographic determination of bound sulfide and sulfite and thiosulfate at their low levels in human serum by pre-column fluorescence derivatization with monobromobimane. In: *Chemical & pharmaceutical bulletin* 40 (11), S. 3000–3004.

Vandiver, M. Scott; Snyder, Solomon H. (2012): Hydrogen sulfide: a gasotransmitter of clinical relevance. In: *Journal of molecular medicine (Berlin, Germany)* 90 (3), S. 255–263. DOI: 10.1007/s00109-012-0873-4.

Verma, A.; Hirsch, D. J.; Glatt, C. E.; Ronnett, G. V.; Snyder, S. H. (1993): Carbon monoxide: a putative neural messenger. In: *Science (New York, N.Y.)* 259 (5093), S. 381–384.

Vitvitsky, Victor; Yadav, Pramod K.; Kurthen, Angelika; Banerjee, Ruma (2015): Sulfide oxidation by a noncanonical pathway in red blood cells generates thiosulfate and polysulfides. In: *The Journal of biological chemistry* 290 (13), S. 8310–8320. DOI: 10.1074/jbc.M115.639831.

Wang, Rui (2002): Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter? In: *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 16 (13), S. 1792–1798. DOI: 10.1096/fj.02-0211hyp.

Wang, Rui (2012): Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. In: *Physiological reviews* 92 (2), S. 791–896. DOI: 10.1152/physrev.00017.2011.

Wang, Rui; Szabo, Csaba; Ichinose, Fumito; Ahmed, Asif; Whiteman, Matthew; Papapetropoulos, Andreas (2015): The role of H2S bioavailability in endothelial dysfunction. In: *Trends in pharmacological sciences* 36 (9), S. 568–578. DOI: 10.1016/j.tips.2015.05.007.

Wang, Wen-Juan; Cai, Guang-Yan; Ning, Yi-Chun; Cui, Jing; Hong, Quan; Bai, Xue-Yuan et al. (2016): Hydrogen sulfide mediates the protection of dietary restriction against renal senescence in aged F344 rats. In: *Scientific reports* 6, S. 30292. DOI: 10.1038/srep30292.

Watanabe, M.; Osada, J.; Aratani, Y.; Kluckman, K.; Reddick, R.; Malinow, M. R.; Maeda, N. (1995): Mice deficient in cystathionine beta-synthase. Animal models for mild and severe homocyst(e)inemia. In: *Proceedings of the National Academy of Sciences* 92 (5), S. 1585–1589. DOI: 10.1073/pnas.92.5.1585.

Whiteman, Matthew; Cheung, Nam Sang; Zhu, Yi-Zhun; Chu, Siew Hwa; Siau, Jia Ling; Wong, Boon Seng et al. (2005): Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? In: *Biochemical and biophysical research communications* 326 (4), S. 794–798. DOI: 10.1016/j.bbrc.2004.11.110.

Whiteman, Matthew; Le Trionnaire, Sophie; Chopra, Mohit; Fox, Bridget; Whatmore, Jacqueline (2011): Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools. In: *Clinical science (London, England : 1979)* 121 (11), S. 459–488. DOI: 10.1042/CS20110267.

Whiteman, Matthew; Winyard, Paul G. (2011): Hydrogen sulfide and inflammation: the good, the bad, the ugly and the promising. In: *Expert review of clinical pharmacology* 4 (1), S. 13–32. DOI: 10.1586/ecp.10.134.

Whitfield, Nathan L.; Kreimier, Edward L.; Verdial, Francys C.; Skovgaard, Nini; Olson, Kenneth R. (2008): Reappraisal of H2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. In: *American journal of physiology. Regulatory, integrative and comparative physiology* 294 (6), R1930-7. DOI: 10.1152/ajpregu.00025.2008.

Wintner, Edward A.; Deckwerth, Thomas L.; Langston, William; Bengtsson, Asa; Leviten, Dina; Hill, Paul et al. (2010): A monobromobimane-based assay to measure the pharmacokinetic profile of reactive sulphide species in blood. In: *British journal of pharmacology* 160 (4), S. 941–957. DOI: 10.1111/j.1476-5381.2010.00704.x.

Wu, Dan; Hu, Qingxun; Liu, Xinhua; Pan, Lilong; Xiong, Qinghui; Zhu, Yi Zhun (2015): Hydrogen sulfide protects against apoptosis under oxidative stress through SIRT1 pathway in H9c2 cardiomyocytes. In: *Nitric oxide : biology and chemistry* 46, S. 204–212. DOI: 10.1016/j.niox.2014.11.006.

Yadav, Pramod Kumar; Yamada, Kazuhiro; Chiku, Taurai; Koutmos, Markos; Banerjee, Ruma (2013): Structure and kinetic analysis of H2S production by human mercaptopyruvate sulfurtransferase. In: *The Journal of biological chemistry* 288 (27), S. 20002–20013. DOI: 10.1074/jbc.M113.466177.

Yang, Guangdong; Wu, Lingyun; Jiang, Bo; Yang, Wei; Qi, Jiansong; Cao, Kun et al. (2008): H2S as a physiologic vasorelaxant: hypertension in mice with deletion of

cystathionine gamma-lyase. In: *Science (New York, N.Y.)* 322 (5901), S. 587–590. DOI: 10.1126/science.1162667.

Yu, Fabiao; Li, Peng; Song, Ping; Wang, Bingshuai; Zhao, Jianzhang; Han, Keli (2012): An ICT-based strategy to a colorimetric and ratiometric fluorescence probe for hydrogen sulfide in living cells. In: *Chemical communications (Cambridge, England)* 48 (23), S. 2852–2854. DOI: 10.1039/c2cc17658k.

Zhang, Dehui; Macinkovic, Igor; Devarie-Baez, Nelmi O.; Pan, Jia; Park, Chung-Min; Carroll, Kate S. et al. (2014): Detection of protein S-sulfhydration by a tag-switch technique. In: *Angewandte Chemie (International ed. in English)* 53 (2), S. 575–581. DOI: 10.1002/anie.201305876.

Zhao, W.; Zhang, J.; Lu, Y.; Wang, R. (2001): The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. In: *The EMBO journal* 20 (21), S. 6008–6016. DOI: 10.1093/emboj/20.21.6008.

Zhao, Weimin; Ndisang, Joseph Fomusi; Wang, Rui (2003): Modulation of endogenous production of H2S in rat tissues. In: *Canadian journal of physiology and pharmacology* 81 (9), S. 848–853. DOI: 10.1139/y03-077.

Zhu, Weidong; Lin, Alexander; Banerjee, Ruma (2008): Kinetic properties of polymorphic variants and pathogenic mutants in human cystathionine gamma-lyase. In: *Biochemistry* 47 (23), S. 6226–6232. DOI: 10.1021/bi800351a.

Zhu, Xiao-Yan; Gu, Hang; Ni, Xin (2011): Hydrogen sulfide in the endocrine and reproductive systems. In: *Expert review of clinical pharmacology* 4 (1), S. 75–82. DOI: 10.1586/ecp.10.125.