The role of the gaseous signaling molecule hydrogen sulfide in chronic liver disease

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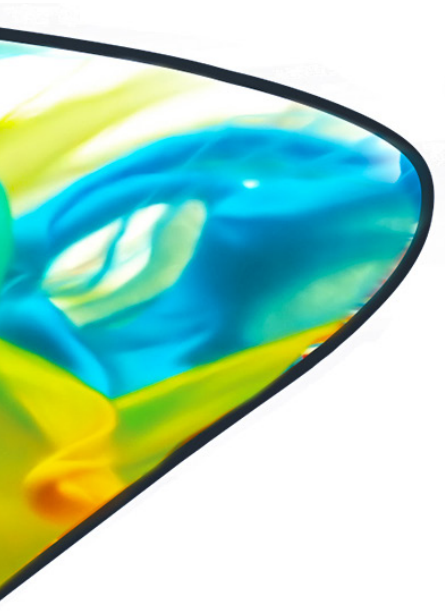

Chapter 7

General discussion and future perspectives

General discussion

Due to the high world-wide prevalence of obesity, non-alcoholic fatty liver disease (NAFLD) has become one of the major chronic liver diseases in the world. NAFLD covers a range of disease stages and in its chronic stages is always accompanied by co-morbidities, such as obesity, insulin resistance (IR), metabolic syndrome and type 2 diabetes (T2D). NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and subsequently cirrhosis and hepatocellular carcinoma (HCC) [1]. Many drugs have been proposed to treat NAFLD, however, none of them showed high efficacy, leaving a change of lifestyle in the early phase or liver transplantation at the late stages as the only effective options to treat of NAFLD [2]. It is vital to understand the complexity of the disease and to consider the underlying mechanisms in each stage. In the current thesis we used primary rat hepatocytes and hepatic stellate cells (HSCs) to study the role of hydrogen sulfide (H_2S) in NAFLD. In addition, we used a large cohort (PREVEND database, n=5562) to validate our experimental findings in a more clinical setting. We used primary rat hepatocytes to study simple steatosis and primary rat stellate cells to investigate fibrosis. In these experimental models, we investigated the role of hydrogen sulfide as well as the natural coumarin derivative esculetin. Free thiols (R-SH) were measured in serum samples of a large cohort to investigate the relation between clinical NAFLD and thiol status.

In general, H_2S is believed to be an anti-oxidant, anti-inflammatory and cytoprotective gaseous signaling molecule, as well as a mitochondrial electron donor. It is involved in many (patho)physiological processes [3]. H_2S may have both beneficial as well as detrimental effects, depending on concentration, site of release/generation and its rate of disposition. Before we discuss the role and effect of H_2S in (patho)physiological processes, we need to distinguish the dynamics of *exogenous* and *endogenous* H_2S. *Endogenous* H_2S is the by-product of enzymatic and non-enzymatic reactions of various sulfur containing amino-acids (SAA) and certain biochemical reactions, including glycolysis. Due to the high rate of catabolism or its storage as bound sulfane sulfur or acid labile sulfur, only very small amounts endogenous 'free' H_2S (~15-20 nmol/L) are present in the cells [4–6]. Furthermore, it is still not clear whether this 'free' H_2S plays an important physiological role. It is becoming increasingly clear that the exact function of endogenous H_2S depends on the cell type. Furthermore,

the effects of H_2S are dependent on the intracellular location of production as well as its metabolism and catabolism. E.g., H_2S catabolism occurs mostly in mitochondria and this reaction produces electrons that are channeled into the electron transport chain, eventually contributing to ATP synthesis, indicating that H_2S catabolism is essential for cellular bio-energetics [7,8]. Previous reports and our results confirm that endogenous H_2S is essential for cancer cells proliferation and HSCs activation and proliferation [7,9].

Thiols are a group of organosulfur compounds that are mainly found in proteins containing sulfur-based amino acids as well as in low-molecular-weight (LMW) molecules like cysteine, homocysteine and glutathione. Thiols are believed to be a marker of systemic reactive species (consisting of reactive oxygen species [ROS], reactive nitrogen species [RNS] and reactive sulfur species [RSS]). In Chapter 3, we describe that levels of serum free thiols are reduced in the population suspected with NAFLD and that it was able to predict all-cause mortality. Lastly, endogenous H_2S contributes to the protection against reactive oxygen species (ROS) due to the preservation of cellular glutathione level and its direct scavenging of ROS. The effects of H_2S on anti-oxidant status and bioenergetics are essential to maintain cellular homeostasis.

Exogenous H_2S is another source of H_2S. Usually, exogenous H_2S is generated by H_2S donors. Its effects depend on the type of donor (rate and magnitude of H_2S release) and effects may be beneficial, e.g. by reversing endogenous H_2S depletion, but may also be toxic, depending on the dose and context. For instance, NaHS is a commonly accepted H_2S fast releasing donor. However, due to its uncontrolled, rapid and high rate of H_2S release, the actual effects of exogenous H_2S may be different compared to endogenous H_2S. In addition, due to its fast evaporation (within 30 min), cells are only exposed to H_2S for a limited time (around 8 -12 h) [10,11]. On the other hand, slow-releasing H_2S donors are able to release H_2S in a controlled and stable manner for a prolonged period. Therefore, in our studies (Chapters 4 and 5) we used the slow releasing donor GYY4137 which can release H_2S up to 7 days in stable manner [11]. We compared the effect of both types of H_2S donors (GYY4137 and NaHS) in Chapter 3 with regard to HSCs proliferation. We showed that in short-term experiments, the effect of NaHS on HSC proliferation was stronger compared to the slow-releasing donor. Repeated addition of the fast-releasing donor (every 8 h) mimicked the effect of the slow-releasing donor. Another important factor that needs to be taken into consideration

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is the amount of H_2S produced and the concentration this production will result in. The production rate of H_2S is around 30-100 μmol/L from cysteine in whole tissue. Due to its catabolism and storage form (bound sulfane sulfur, acid labile sulfur) only a small amount of free H_2S (~15-20 nmol/L) exist [6]. Based on these facts, the concentration range to apply H_2S is extremely narrow and therefore the choice of H_2S donor can have great impact on the effects observed. Low concentrations of H_2S fail to induce physiological effects, whereas high(er) concentrations could be toxic. In addition, since the role and functions of H_2S are location specific, targeted H_2S donors (e.g. mitochondrial targeted H_2S donor AP39) are important tools to exactly identify the (patho)physiological roles of H_2S [12–14]. Currently, H_2S releasing sodium thiosulfates (STS, $Na_2S_2O_3$), are undergoing clinical trials (II, III) for various diseases, e.g. cardiovascular disease, calcinosis, and in cancer in combination with chemotherapy to reduce cytotoxicity on healthy cells [15,16]. Yet, it is crucial to understand the concentration and location dependency as well as the dynamics of H_2S release in order to choose the proper H_2S donor for hepatic diseases, including NAFLD.

Previous reports and our findings described in this thesis, have demonstrated dysregulation of endogenous H_2S production during simple steatosis, NASH, fibrosis, cirrhosis and hepatocellular carcinoma. In Chapter 2 we describe reduced hepatic endogenous production of H_2S and reduced expression of H_2S synthesizing enzymes during steatosis. These findings are in line with published reports [17–24]. In addition, it has been shown that inhibition of endogenous H_2S production and/or knockout of H_2S synthesizing enzymes contribute to the development of NAFLD [20,22,24–26]. Exogenous H_2S donors mitigate the development of fatty liver and fibrosis in various models, *in vivo* and *in vitro* [18,20–23,27–29]. These beneficial roles of H_2S in NAFLD may be due to its anti-oxidant, anti-inflammatory and cytoprotective properties, but may also be due to direct regulatory effects of H_2S on lipid metabolism, e.g. via *Ppar*α. In fact, there is a complicated interaction between H_2S and FFA metabolism: disturbed H_2S production leads to disturbed FFA metabolism, whereas the increased FFA influx in steatosis leads to disturbed H_2S production. In chapter 2, we observed that inhibition of H_2S synthesis impaired the mRNA expression of *Ppar*α and its target genes and increased lipid accumulation while exogenous H_2S reversed these effects. It has been described that H_2S also reduces tissue triglyceride (TG) content and cellular lipid accumulation [29–31]. Finally, it has been described that H_2S reduces portal hypertension due

to its vasodilatory effect [22]. Portal hypertension is one of the most serious complications of fibrosis and cirrhosis [26]. Taken together, our results in Chapters 2 and 3 underscore the importance of H_2S as an important gaseous signaling molecule that maintains liver homeostasis. Dysregulation of H_2S metabolism contributes to liver diseases, including NAFLD.

It has been reported that hydrogen sulfide has anti-fibrotic effects. However, in our studies (Chapter 4), we demonstrate that locally increased H_2S production and locally (i.e. in hepatic stellate cells) increased expression of the H_2S synthesizing enzyme CTH contributes to the activation of HSCs Furthermore, the inhibition of the H_2S synthesizing enzyme CTH reversed the activation of HSCs (chapter 4,5) [10]. Our results are in disagreement to some studies in which an anti-fibrotic action of H_2S was reported [28,32]. We conclude that the anti-fibrotic effects of H_2S are due to indirect effects of H_2S on hepatocytes (cytoprotective) and/or Kupffer cells (anti-inflammatory) or systemic effects (such as reduced portal hypertension by its vasorelaxant properties. Furthermore, in the reported *in vitro* studies, very high concentrations of H_2S donor (5 times higher than physiological concentrations) were used which are toxic to stellate cells or a natural H_2S donor diallyl trisulfide (DATS) was used, which could have many side effects [20,23,24,26,28,32]. Other reports support our results that H_2S, as a source of homocysteine, increases HSCs proliferation whereas platelet derived growth factor (PDGF-BB) increases CTH levels and activates fibroblastic cells [27,33]. Therefore, H_2S effects may depend on location: systematically H_2S is anti-fibrotic, while locally (i.e. in stellate cells) H_2S promotes stellate cell proliferation by increasing cellular bio-energetics as an electron donor [7]. Furthermore, we found that mRNA expression of *Cth*, *Cbs* and *Mpst* downregulated in bile duct ligated rat liver tissue as well as was downregulated by the fibrogenic cytokine TGFβ1 in primary rat hepatocytes and Kupffer cells. Thus the use of stellate cell-specific Cth knockout mice or gene silencing experiments in stellate cells would be very interesting to address these issues.

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Characteristics of cellular senescence include arrested cell proliferation, increased cytokine secretion and induction of apoptosis by triggers such as DNA damage and ROS [34]. Due to these characteristics, induction of cellular senescence has been considered a beneficial intervention for certain (patho)physiological conditions, including fibrosis and cancer [35,36]. H_2S is considered to be an anti-senescence agent due to its antioxidant capacity

via the SIRT1 and Keap1/Nrf2 pathways [37,38]. In our studies, inhibition of CTH in activated HSCs induced cellular senescence and reversed activation (Chapter 5). Furthermore, exogenous H_2S dose-dependently reversed the induced senescence in HSCs. An important regulator of cellular senescence is the PI3K-Akt pathway [39]. Indeed, we observed that inhibition of H_2S-induced cellular senescence was reversed by blocking PI3K. These results confirmed our results described in chapter 4. Taken together, increased H_2S production and CTH expression contributes to HSC activation via increased cellular bio-energetics. Inhibition of H_2S in activated HSCs is anti-fibrotic and induced cellular senescence. Accumulating evidence suggest that some natural compounds are anti-fibrotic via induction of cellular senescence. For instance, curcumin and tetramethylpyrazine induce cellular senescence in HSC and limit fibrogenesis [40,41]. In line with these reports, the natural coumarin derivate esculetin induces cellular senescence in HSCs (Chapter 6) via the PI3K-Akt-GSK3β pathway. Indeed esculetin has been reported as a hepatoprotective compound against hepatic steatosis, inflammation and fibrosis [42,43]. In conclusion, cellular senescence is a promising strategy to limit fibrogenesis. However, currently it is still not clear yet what happens to senescent stellate cells in the long-term, after inhibition of endogenous H_2S generation. Induction of apoptosis may be one way of removing senescent hepatic stellate cells [44]. Hydrogen sulfide has been shown to modulate apoptosis in a dose-dependent and cell-specific manner. At high concentration, H_2S induces apoptosis whereas at physiological or low concentration, H_2S protects against apoptosis in various cell types [45]. These reports suggest that H_2S is an anti-apoptotic agent and its inhibition could increase cellular apoptosis. In our studies we did not investigate whether inhibition of endogenous H_2S production increases apoptosis in senescent HSCs. The increased presence of senescent cells contribute to ageing and can be a risk factor for many diseases. Accumulating evidence supports the notion that selective removal of senescent cells by senolytics is a beneficial treatment strategy against ageing [46]. However, current knowledge about the effect of H_2S on senolytics (or vice versa) of senescent cells still limited. Interestingly, Latorre et al conclude that H_2S has a senostatic role in senescent cells, and is able modulate the secretary phenotype of senescent cells [47]. Furthermore, the natural polyphenolic compound quercetin has been described as senolytic and to be able to remove senescent fibroblasts via the AMPK pathway [48]. Taken together, it will be an important area of research to understand the fate of senescent HSCs.

Clinical application of H_2S is still limited due to its toxicity and gaseous characteristics. Also, the delivery of H_2S to its target site remains an important issue. So far, many H_2S prodrugs have been developed for use in clinical trials [49]. Currently, there are 32 H_2S-related observational and interventional clinical trials registered at *ClinicalTrails.gov*. Interestingly, most of them (24) consider using endogenous H_2S as a biomarker for particular diseases or as a novel tool for diagnostics and detection. Seven clinical trials utilized H_2S-releasing donors, e.g. SG1002 (sodium polysulthionate), *NAC*, STS and GIC-1001 (trimebutine 3-thiocarbomoylbenzenesulfonate) in cardiovascular disease, chronic kidney disease, colonic disease or visceral pain. Two trials applied gaseous H_2S for treatment of asthma, septic shock and stroke. Furthermore, STS is reported that a beneficial treatment for vascular calcification due to its cation-chelating and antioxidant properties. STS also approved from Food and Drug Administration for the treatment of cyanide intoxication [15,16,50]. Currently, the most advanced application of H_2S treatment is H_2S-releasing non-steroidal anti-inflammatory drug (S-NSAID). S-NSAIDs are used to reduce gastrointestinal ulceration and bleeding side effects and these compounds showed significant beneficial effects such as anti-inflammatory and anti-oxidant effects [51]. Another interesting clinical application of H_2S is its use in the preservation of donor organs. H_2S reduces the metabolic rate of the donor organ, inducing a state of hibernation and reducing ischemia-reperfusion injury [52].

Future studies on H_2S targeted therapy and clinical utility are required, including the use of H_2S targeted therapy in NAFLD.

Future perspectives

Existing knowledge and the studies described in this thesis highlight the prominent role of H_2S and its derivatives in maintaining liver homeostasis. Dysregulation of endogenous production of H_2S occurred at various stages of NAFLD, e.g. steatosis, NASH and fibrosis. Exogenous H_2S partially corrected the detrimental effects of reduced H_2S generation.

In our experiments we focused mainly on hepatic stellate cells and hepatocytes to address fibrogenesis and steatosis. However, other hepatic cell types, for example Kupffer cells, the hepatic resident macrophages,

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liver sinusoidal endothelial cells (LSEC) and bile duct epithelial cells or cholangiocytes also play an important role in the pathogenesis of NAFLD. Kupffer cells and monocyte-derived macrophages are involved in insulin resistance, fibrogenesis and inflammation associated with NAFLD [53]. We observed that the mRNA expression of endogenous H_2S synthesizing enzymes (*Cth*, *Cbs*, *Mpst*) in Kupffer cells is higher than in activated HSCs. This suggests that H_2S is produced in Kupffer cells. However, there is still a lack of knowledge about the function of H_2S produced by Kupffer cells, e.g. anti-inflammatory effects and/or effects on Kupffer cell polarization. LSECs are crucial in the maintenance of liver homeostasis and have anti-inflammatory and anti-fibrotic properties. However, in the development of NAFLD, LSECs lose their specific phenotype and functions and contribute to liver injury and promote HSCs activation [54]. However, it is not known whether H_2S is produced by LSECs and, if so, what its function is. Likewise, almost nothing is known about the role of H_2S in cholangiocytes in NAFLD. It is therefore crucial to elucidate the role of H_2S in all liver cell types in order to generate an integral picture of the role of H_2S in the pathogenesis of NAFLD.

In our studies we have shown that inhibition of H_2S production impairs β-oxidation and increases lipid accumulation. However, the mechanism of the impaired β-oxidation upon inhibition of H_2S and the mechanism of reduced H_2S production by free fatty acids remains to be elucidated. We propose 3 possible explanations: 1) Our results show that Pparα, the master regulator of lipid (FFA) metabolism and β-oxidation, is strongly reduced upon inhibition of H_2S. Therefore, we propose a cross-talk between Pparα and H_2S. 2) Our results show that inhibition of H_2S production by FFAs increases ER stress significantly. However, it remains to be elucidated whether the ER stress is cause or consequence of increased lipid accumulation and it is not clear yet how H_2S affects ER stress (and lipid metabolism). ER stress also triggers inflammation and mitochondrial dysregulation, limiting β-oxidation and thus aggravating lipid accumulation during development of NAFLD [55]. 3) As an electron donor, H_2S may also contribute to mitochondrial homeostasis and impairment of H_2S metabolism may contribute to mitochondrial dysfunction, resulting in oxidative stress and inflammation.

Contrary to reports in the literature, we reported that H_2S promotes stellate cell activation (Chapters 4,5). The reason for this apparent contradiction is

that we focused on the direct effects of H_2S on hepatic stellate cells. We demonstrate that the dominant effect of H_2S (an electron donor) on HSCs is to promote activation via increasing cellular bioenergetics in HSCs. Whether similar effects are operative *in vivo* remains to be elucidated, e.g. using cell-specific CTH knockout models.

We did not address the role of H_2S in advanced stages of NAFLD, like cirrhosis or hepatocellular carcinoma. Wei et al. observed that plasma H_2S level is reduced in cirrhotic rats and inhibition of H_2S even contributed to the severity of the disease [24]. In addition, exogenous H_2S promotes the proliferation of hepatocellular carcinoma cells via various pathways, including NF-κB and PTEN/Akt signaling pathways [56,57]. These reports indicate the importance of H_2S in the more advanced stages of NAFLD. Based on these reports, H_2S interventional strategy should also be considered for more advanced stages of NAFLD beyond the stage of steatosis and steatohepatitis. Clinical application of H_2S is still limited, although there are 32 trials related to H_2S and its derivatives being investigated in clinical interventional and observational studies ongoing or completed (May, 2020), according to the *ClinicalTrials.gov*. Unfortunately, none of them addressed liver diseases and NAFLD.

The use of H_2S in clinical practice is still limited. H_2S is toxic and difficult to handle and dose (gaseous, donors). Therefore, H_2S releasing donors or triggers to induce production of endogenous H_2S are believed to be more promising ways to deliver H_2S. In this regard, synthetic H_2S releasing donors (STS, mitochondrial targeted AP39, slow releasing, ADTOH), H_2S releasing natural compounds (garlic derived DATS) and amino acids involved in the synthesis of endogenous H_2S (*NAC*; L-cysteine) may prove to be promising option for clinical practice. However, the effects of these compounds remain systemic and many organs will be exposed to H_2S due to its gaseous nature. In addition, there is still a lack of specific H_2S delivery tools to target organs, tissues and/or specific cells.

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