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

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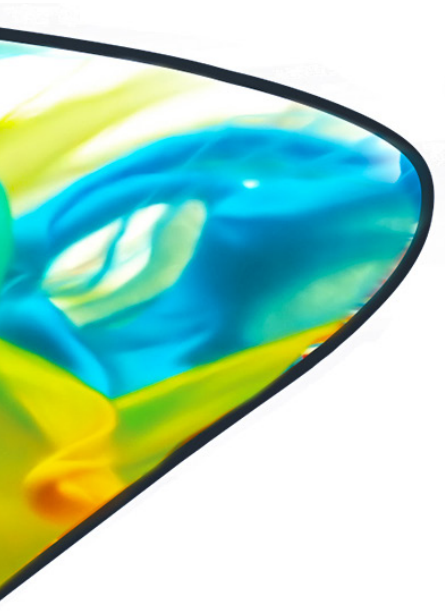

Chapter 1

General introduction and scope of the thesis

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PART 1. Non-alcoholic fatty liver disease (NAFLD) and fibrogenesis

1. General introduction to NAFLD and free fatty acid metabolism

a. Epidemiology, clinical aspects and pathophysiology of NAFLD

Non-alcoholic fatty liver disease (NAFLD) is defined as excessive accumulation of lipids in hepatocytes in the absence of excessive an alcohol consumption [1]. Excessive alcohol use is defined as the intake of alcohol more than 20g/day in men and more than 10g/day for women [2]. NAFLD includes the spectrum of diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and eventually cirrhosis and hepatocellular carcinoma (HCC). Many co-morbidities coincide with the development of NAFLD, such as obesity, insulin resistance (IR) and the metabolic syndrome (MetS), including type 2 diabetes (T2D) [3].

Currently, NAFLD is considered one of the most prevalent chronic liver diseases worldwide (25% of the global adult population) [4]. A recent meta-analysis estimated the NAFLD incidence in the Western world to be 28 per 1000 person-years and 50-52 per 1000 person-years in Asia. The high and increasing incidence is related to changes in life-style in the industrialized (Western) society, including increased nutritional intake, in particular carbohydrates, and a sedentary life-style leading to obesity and the increased incidence and poor outcome of hepatocellular carcinoma [5,6]. Patients with NAFLD have an increased overall mortality compared to a matched control population without NAFLD. As the epidemic of obesity expands, the incidence of NAFLD will also increase [7]. Therapeutic options are still limited due to the heterogeneity of the clinical manifestations of NAFLD, leaving changes in life style the only viable option for prevention. Current drug therapies are targeted to reverse or treat the comorbidities of the NAFLD [8]. For example, some natural compounds like vitamin E, curcumin and esculetin attenuate hepatic steatosis by improving antioxidant status, while metformin reduces body-weight and serum levels of cholesterol and glucose in patients [9–11]. Thus, it is crucial to understand the underlying mechanisms leading to the development of NAFLD and to identify potential targets for intervention in basic research for future therapeutic application.

b. The metabolism of free fatty acids (FFA) and its dysregulation in NAFLD

The liver plays a major role in lipid metabolism. Free fatty acids (FFA) are an important energy source and play a central role in lipid metabolism. As shown in Figure 1, under normal physiological conditions circulating FFAs are bound to the serum protein albumin and are taken up by fatty acid translocase (CD36) and fatty acid transport proteins (FATPs) into liver cells like hepatocytes. FFAs can also be synthesized in the liver via *de novo*

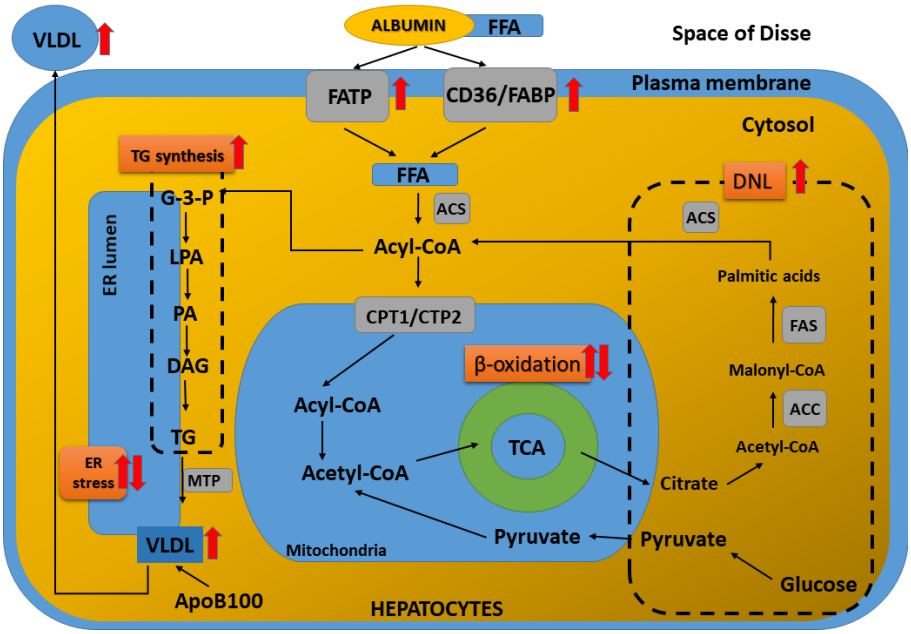

Figure 1. Free fatty acid (FFA) metabolism and its dysregulation in NAFLD. Free fatty acid uptake is mediated by fatty acid transport protein (FATP) and CD36/FABP. Inside the hepatocyte, FFAs are activated to form Acyl-CoA by fatty acyl-CoA synthetase (ACSs). De novo lipogenesis can be increased during NAFLD due to overconsumption of glucose via Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Cytosolic Acyl-CoA is transferred into mitochondria for β-oxidation via carnitine palmitoyl transferase (CPT1/2). In the mitochondria, acyl-CoA is converted into acetyl-CoA which enters the citric acid cycle, eventually yielding ATP. Initially, β-oxidation increases in NAFLD due to excessive FFA intake. However, it eventually levels off and may even decrease because of the sustained FFA accumulation during the progression of the disease. Excessive cytosolic Acyl-CoA is esterified by glycerol-3-phosphate (G-3-P) acyltransferase and esterified again to form triglyceride (TG) by acyl-CoA:diacylglycerol acyltransferase (DGAT) in the ER lumen. Prolonged accumulation of FFA and TG synthesis can therefore lead to ER stress. During NAFLD, production of very low density lipoprotein is increased due to increased supply of FFAs via microsomal triglyceride transport protein (MTP).

lipogenesis (DNL). This occurs in the hepatocytes via acetyl-CoA carboxylase I (ACC1) and fatty acid synthase (FAS). In NAFLD, DNL and FFA uptake are increased due to high nutritional intake into the hepatocytes. FFAs are converted in the cell into acyl-CoA by acyl-CoA synthetase (ACS) and subsequently transported into the mitochondria via carnitine palmitoyl transferase I (CPT1) for β-oxidation. Excess FFAs are converted into the neutral lipid storage form, termed triglycerides (TGs), by enzymatic conversion involving a group of enzymes, including diacyltransferase 2 (DGAT2). In normal conditions, cellular TG levels are tightly regulated and the normal content (wet weight) of TG is 5.5-8% of total liver weight [12]. Excess TGs are secreted from the liver into the blood as very low density lipoprotein (VLDL). Biosynthesis of VLDL is highly dependent on the regulation of the structural component apoliprotein B100 (apoB100) and the microsomal triglyceride transfer protein (Mtp) in the endoplasmic reticulum (ER) [13].

The most abundant fatty acids in the diet and in the steatotic liver are saturated palmitic acid (PA, C16:0) and monounsaturated oleic acid (OA, C18:1) [15]. Both PA and OA can act as steatogenic agents, however, their actual effects are dependent on concentration, target cell type and species [16]. The toxic effect of FFAs on cells is termed lipotoxicity. Initially, PA was termed a lipotoxic FFA, whereas OA was termed a non-toxic FFA. However, toxicity also depends on concentration and cell type. Furthermore, combining FFAs may attenuate the toxicity of FFAs. E.g. it has been shown that OA protects hepatocytes from PA-induced toxicity [17,18]. TG accumulation in hepatocytes has been postulated to contribute to the development of NAFLD. However, recent studies demonstrate that TG accumulation is insufficient to cause IR. Furthermore, we and others have shown that TG accumulation actually correlates with the absence of FFA toxicity [17,19,20]. Interestingly, the rate-limiting enzyme in TG hydrolysis, PNPLA3 is increased in patients with NAFLD and correlates with increased lipid toxicity [21].

FFA metabolism is connected to major metabolic pathways in the liver. In the fed state, excess carbohydrates in the liver are converted into FFA via DNL. In fact, DNL contributes to about 25% of total liver lipids in patients with NAFLD [22]. This process is regulated via transcription factors, like sterol regulatory binding protein I (SREBP1) and carbohydrate response element binding protein (ChREBP). During fasting, ChREBP is phosphorylated in a glucagon-dependent manner by protein kinase A (PKA) and AMP activated protein kinase (AMPK) to decrease ChREBP ac-

tivity [23]. Another important regulator of FFA metabolism is Peroxisome Proliferator-Activated Receptor α (PPARα). PPARα is a nuclear receptor that regulates FAA uptake, β-oxidation, ketogenesis, bile acid synthesis and TG turnover [24]. There are 3 PPAR isoforms, alpha (α), beta/delta (β/δ) and gamma (γ). PPAR isoforms form heterodimers with retinoid X receptors (RXR) [25]. PPARα expression is decreased in the liver of patients with NAFLD. This suggests that PPARα may be a prominent target for intervention in the treatment of NAFLD. Several fibrate drugs and pan-agonists for PPAR isotypes are currently in clinical trials to treat NASH [26].

In NAFLD, FFA influx in the liver and endogenous FFA synthesis is increased due to the high nutritional intake and stimulation of DNL. The excess supply of FFAs surpasses the capacity for β-oxidation and FFA export, resulting in accumulation of lipids in the liver. This results in lipid toxicity and compensatory fatty acid oxidation inducing oxidative stress, mitochondrial dysfunction, ER stress and ultimately cell death. As a result, pro-inflammatory signaling pathways are activated that promote the secretion of inflammatory cytokines (TNF-α, IL-6, IL-10) and the generation of reactive oxygen species (ROS) leading to activation of hepatic stellate cells (HSCs) and fibrogenesis. At this stage, NAFLD has evolved into non-alcoholic steatohepatitis (NASH) [27].

2. *Fibrogenesis: pathophysiological mechanisms and treatment options*

a. Biology of hepatic stellate cells and their role in NAFLD

The development of fibrosis in NAFLD starts at the stage of chronic inflammation, NASH. Recent studies report that early-stage hepatic fibrosis is an independent and strong predictor of mortality for NAFLD patients [6]. Hepatic fibrogenesis is the continuous, dysregulated, but still reversible wound-healing response characterized by excessive synthesis of extracellular matrix (ECM) components by activated hepatic stellate cells (HSCs). When the injurious trigger persists, the inflammation becomes chronic and the continuous production of large amounts of ECM leads to progressive fibrosis and disruption of normal liver architecture accompanied by portal hypertension. Ultimately, cirrhosis develops which has a poor outcome and high mortality. Depending on the risk factors, the development of advanced fibrosis and cirrhosis may take as long as 20 to 40 years [28,29]. Currently, there is no approved drug to treat liver fibrosis, leaving liver

transplantation as the only viable clinical treatment option. Therefore, it is important to understand the mechanisms that control liver fibrogenesis and stellate cell activation in NAFLD [30].

Hepatic stellate cells, also known as perisinusoidal cells or Ito cells, are non-parenchymal cells located in the perisinusoidal space of Disse, between hepatocytes and liver sinusoidal endothelial cells (LSEC). In healthy liver, HSCs are in a quiescent state and store vitamin A as retinyl esters (70% of total retinoid content) in lipid droplets. HSCs represent around 8% of the total number of liver cells. In acute and chronic liver injury, quiescent HSCs (qHSCs) transdifferentiate into myofibroblast-like cells, termed activated HSCs (aHSCs). Once activated, HSCs lose their vitamin A droplets and become the major source of ECM. aHSCs are proliferative, contractile, inflammatory and chemotactic cells which make them key players in fibrogenesis. Collagen (type I, III and IV) and fibronectin are the main components of ECM in the liver [31].

HSCs can be activated by a wide variety of triggers and mediators. For instance, transforming growth factor-β (TGFβ), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) are potent inducers of HSCs via their receptors on HSCs and downstream signaling pathways like SMAD, MAPK and ERK. In addition, cytokines play an important role in the activation and reversion of HSCs. IL-15 signaling via its receptor IL15α has an anti-fibrotic effect. HSCs isolated from IL-15α-KO mice have increased expression of the fibrosis marker collagen type I, whereas IL-15α-KO mice have a deficiency in natural killer (NK) cells [32]. Additional cytokines, like IL-17, IL-20, IL-13 and IL-33, have been reported as pro-fibrotic cytokines to promote activation and proliferation of HSCs [33]. In the development of NAFLD, several pro-inflammatory cytokines have been implicated to promote fibrogenesis, in particular TNFα, IL-1β, IL-6, IL-8 [33,34].

In addition to growth factors and cytokines, oxidative stress can also promote the activation of HSCs. Oxidative stress is defined as unbalanced redox homeostasis leading to excessive exposure to reactive oxygen species (ROS). In NASH, ROS production is increased due to increased FFA metabolism and toxicity and inflammation and this may contribute to the activation of HSCs [35,36]. Another important factor in the activation of HSCs is the cellular bio-energetic state [37]. Recent studies report that upon activation of

HSCs, their bioenergetic state is increased, as demonstrated by increased mitochondrial oxidative phosphorylation as well as increased cytosolic glycolysis [38]. Furthermore, inhibition of cellular bioenergetics reduces HSCs activation [37].

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Various important nuclear receptors regulate HSC homeostasis, including LXR, FXR and PPARγ/PPARδ. These nuclear receptors regulate glucose and lipid metabolism and negatively modulate HSC activation and fibrogenesis [39]. PPARγ (re)activation reverses activation of HSCs to a quiescent phenotype and inhibits expression of fibrogenic markers like αSMA (Acta2) and Collagen type I (Col1α1) [40]. PPARγ and lecithin:retinol acyltransferase (LRAT) are highly expressed in quiescent HSCs, but they are rapidly downregulated after isolation and culture. Many signaling pathways are involved in the activation of HSCs. For example, the phosphoinositide-3-kinase (PI3K), protein kinase B (Akt), extracellular signal-regulated kinase-1 and 2 (ERK1/2) and c-Jun N-terminal kinase (JNK) pathways have been shown to be involved in HSC activation, governing different aspects like proliferation, differentiation, matrix synthesis and response to various stressors [41,42].

In the development of NASH, HSCs can be activated by various mediators including ROS, hedgehog signaling (Hh), damage associated molecular patterns (DAMPs), growth factors, lipid peroxides, inflammatory cytokines and cell death signals. Most of them directly promote activation of HSCs. Furthermore, engulfment of hepatocyte apoptotic bodies by HSCs also promotes their activation [43,44]. Kupffer cells, the hepatic resident macrophages, are involved in immune homeostasis in the liver and also play an important role in the activation of HSCs. Cytokines and chemokines produced by Kupffer cells (and infiltrating macrophages) during inflammation promote HSCs activation, e.g. TGFβ, PDGF, TNFα, IL-1β, MCP1, CCL3 and CCL5 [45.] Macrophages have also been postulated as the key cell type involved in the resolution of fibrosis. E.g. matrix metalloproteinases 9,12,13 (MMPs 9/12/13) play an important role in the resolution of fibrosis and are produced by macrophages [46,47]. In addition, other liver cell types, e.g. NK cells, LSECs and cholangiocytes have been reported to play a role in HSC homeostasis and fibrogenesis as well, in particular in NAFLD [48,49].

Overall, liver fibrosis is the most accurate predictor of morbidity in patients with NAFLD. This means that simple steatosis is considered rela-

tively benign until the development of (chronic) inflammation and fibrosis. Therefore, it is vital to understand the mechanisms that control the trans-differentiation of quiescent HSCs into myofibroblast-like activated HSCs during NAFLD.

b. Resolution of hepatic fibrosis and cellular senescence

Liver fibrosis is the result of a continuous wound healing and/or tissue repair response. When the damaging stimulus is removed, the wound healing response also stops. However, in chronic liver diseases, the injurious trigger is persistent and the wound healing response becomes self-sustaining, leading to progressive fibrogenesis. Recovery of liver fibrosis therefore involves the removal of the injurious trigger. However, it should also involve the removal and/or reversal to quiescence of already activated HSCs. Several anti-fibrotic therapies are being considered or tested: PPARγ agonists like pioglitazone, dual agonists of PPARα and PPAR δ like elafibranor, dual CCR2-CCR5 receptor antagonists like Cenicriviroc and Simituzamab, a neutralizing antibody of lysyl-oxidase 2, the enzyme that is responsible for cross-linking of collagen [50–52]. In general, there are three strategies to resolve and/or remove aHSCs: induction of apoptosis, reversion of activation and induction of senescence [39]. *Apoptosis* of aHSCs: pro-inflammatory cytokines including TNFα and IL-1β promote the resistance to apoptosis of aHSCs. In addition, pro-fibrogenic agents like TGFβ1 and tissue inhibitor metalloproteinase 1 (TIMP1) act as anti-apoptotic agents and promote survival of aHSCs [45,53]. Therefore, promoting apoptosis of aHSCs involves the removal of these cytokines and/or pro-fibrogenic molecules. In addition, compounds like gliotoxin, sulfasalazine, benzodiazepine ligands and the natural compounds curcumin and tanshinone I induce HSCs apoptosis [54–58]. The coumarin derivative esculetin also ameliorates hepatic fibrosis via Akt/PI3K/FoxO1 signaling in a high fat diet (HFD) rat model of NASH [59].

Reversion or deactivation of activated HSCs has been reported during regression of liver fibrosis. Using Cre-LoxP-based fluorescently labelled HSCs in mice it was shown that about 50% of aHSCs escape from apoptosis and downregulate the activation markers *Col1α1, Acta2, Tgf*β*1* and *Timp1*. However, these inactivated cells do not completely reverse to truly quiescent cells, since they fail to (re)express some quiescence phenotype markers. In addition, they do not restore lipid storage [60,61].

Cellular senescence is characterized by irreversible cell-cycle arrest, accompanied by increased cytokine secretion, termed the Senescence-Associated Secretory Phenotypes (SASP) [62]. In physiological conditions, senescent cells are removed by immune cells to facilitate wound healing and tumor suppression. However, persistent cellular senescence contributes to aging and promotes pathological conditions including, in the long term, cancer. There are certain triggers that promote cellular senescence. The first described trigger of cellular senescence was telomere shortening or dysfunction. Telomeres represent the capacity of cells to divide and shortening of telomeres limits this capacity due to the inability of replicative polymerases to synthesize DNA at chromosome ends [63]. Furthermore, if telomeres are shortened, they can no longer protect against DNA damage via the DNA damage response (DDR). This condition is termed replication stress (RS) induced senescence [63]. Apart from telomere length, other stimuli can trigger senescence as well. E.g. the activation of oncogenes like *RAS* and *RAF* trigger senescence in normal cells via the Raf/MEK/MAP kinase cascade, which is termed oncogene-induced senescence (OIS). In the short term OIS may provide a defense against the transition of a normal cell into a tumor cell [64,65]. Therefore, cell senescence acts as a potent tumor suppressor mechanism [66–68]. Numerous findings reported that OIS might be mediated by DNA damage, often associated with ROS. In fact, ionizing radiation, UV light, chemotherapeutic drugs and oxidative stress can all activate cell senescence, referred to as stress-induced premature senescence (SIPS) [69]. Importantly, all these triggers of senescence (RS, OIS, SIPS) are mediated via DDR. Excessive DDR leads to apoptotic cell death, whereas mild and persistent DDR can induce cell cycle arrest and senescence [70]. Moreover, high levels of ROS trigger apoptosis, while lower concentrations appear to favor senescence [71]. In fact, DDR and ROS are interrelated factors that dose-dependently trigger cellular senescence.

The induction of DDR and ROS generation initially activate the p53-p21 pathway to cell cycle arrest. This is the main driving force for induction of the senescence program [72]. If DNA damage cannot be resolved, p16(INK4a) appears to regulate the long-term maintenance of cell cycle arrest via the retinoblastoma tumor suppressor (Rb) pathway [73]. The induction of the DDR also promotes the secretion of soluble factors, including pro-inflammatory cytokines and growth factors in senescent cells via the p38 and NF-κB pathways [74,75]. In fact, the stimulation of the innate immune response, triggered by senescent cells, has been described as an important mech-

anism for the elimination and clearance of fibroblast-like cells, including HSCs. Previous studies reported that senescent HSCs can be cleared by M1-type macrophages during liver damage [76]. Another study reported that the natural compound curcumin shows anti-fibrotic effects via induction of senescence in HSCs. NK cells play an important role in the clearance of senescent HSCs. Curcumin increases the clearance of NK cell via activator ligands major histocompatibility complex class I chain-related genes A (MICA) and UL-16-binding protein 2 (ULBP2)[77].

The standard method to detect senescent cells is senescence associated β-galactosidase (SA β-gal) staining. This method detects lysosomal β-gal protein that is regulated by the GLB1 gene in senescent cells at pH 6.0 [78]. The SASP in senescent cells acts as a double-edged sword. On the one hand, it can recruit immune cells to facilitate HSCs clearance. On the other hand, the increased expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8 can also transmit senescence signals to neighboring cells. This latter phenomenon is termed paracrine senescence [79]. Although cellular senescence appears to be a consequence of a pathophysiological process, it could also be a protective mechanism against mild pathogenic stimuli, such as ROS and the activation of fibroblast-like cells, including HSCs. Induction of senescence in HSCs could be a promising therapeutic strategy to intervene in the development of fibrosis during NAFLD.

PART 2. The gasotransmitter hydrogen sulfide and its role in liver (patho)physiology

1. General introduction to hydrogen sulfide: synthesis, metabolism and (patho)biological functions

Currently, the family of gasotransmitters consists of three distinct gaseous signaling molecules that are synthesized in a variety of living organisms (bacteria to human) and in a variety of different cells. These gasotransmitters are hydrogen sulfide (H_2S), nitric oxide (NO) and carbon monoxide (CO). Gasotransmitters are involved in many physiological and pathophysiological processes as signaling molecules, transmitters or post-translational modifying agents. The distinctive characteristic of these molecules is that they are gases, which allows them to penetrate barriers (e.g. membranes) and to diffuse easily into and through the cytosol. Moreover, they can have both paracrine and autocrine functions and in this way regulate

many processes [80]. The range of action of gasotransmitters is limited by the fact that they are reactive and have a short half-life (e.g. NO) and/or avidly bind to carrier molecules like proteins (e.g. CO binds hemoglobin), sometimes limiting, sometimes enhancing their 'range of action' [81]. Among these gasotransmitters, H_2S is the most recently discovered gaseous signaling molecule which is increasingly recognized as an important mediator in a wide range of cellular functions in health and in disease.

Hydrogen sulfide is known as a toxic gas with the smell of rotten eggs. H_2S was initially identified as a neuromodulator and smooth muscle relaxant, however, many additional effects have subsequently been reported and H_2S is now considered as a critical and versatile signaling molecule as well as an anti-senescence agent. Biosynthesis of H_2S can occur via two different pathways: enzymatic and non-enzymatic. Enzymatic synthesis of H_2S occurs via three enzymes in living organisms (Figure 2): cystathionine β-synthase (CBS), cystathionine γ-lyase (CTH) and 3-mercaptopyruvate sulfur transferase (MPST) [82]. These enzymes use sulfur-containing amino acids (SAA) like L-cysteine and homocysteine as substrates to produce H_2S as a by-product. CTH, CBS, and MPST are differentially expressed and/or regulated in different cell types allowing the cell and organ specific production of H_2S. The actual concentration of H_2S synthesized from L-cysteine can be around 100 μmol/L in tissue. However, the catabolic rate of H_2S in liver and brain can exceed the rate of synthesis. Thus, the steady stage H_2S concentration is estimated to be around 15 nmol/L in most tissues, including liver [83]. Non-enzymatic H_2S production occurs via glucose, glutathione, inorganic and organic thiocystine, thiosulfate, polysulfides (garlic) and elemental sulfur. H_2S can be generated from glucose either via glycolysis or from phosphogluconate via NADPH oxidase. Glucose reacts with methionine, homocysteine or cysteine to produce methanethiol and H_2S. H_2S is produced directly from glutathione or elemental sulfur via reduction using NADPH and NADH [84–86]. In addition, iron in red blood cells and tissues together with vitamin B6 catalyzes the production of H_2S from SAAs [87].

Endogenous H_2S is present in cells and serum in different forms. In general, H_2S species are categorized as acid-labile bound sulfur and bound-sulfane sulfur as well as free H_2S. Free or unbound sulfide exists as S^{2-}, HS^- or H_2S. Acid-labile sulfide is mainly in the form of iron-sulfur (Fe-S) complexes and persulfides, which play a critical role in redox reactions in cytoplasm, mitochondria and serum. Bound-sulfane sulfur exists as compounds containing

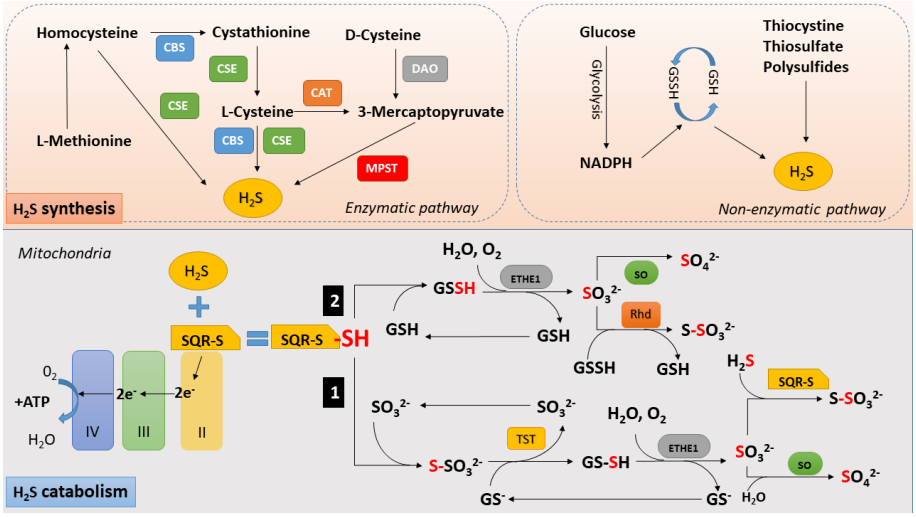

Figure 2. Synthesis and catabolism of H_2S in the cell. H_2S synthesis: Enzymatic pathway starts with L-methionine, homocysteine and cysteine as substrates for H_2S production via CBS, CSE (CTH in rat) and 3MST (MPST in rat; transsulfuration pathway). Under stress conditions, CBS and CSE can be translocated into mitochondria to produce H_2S in addition to 3MST and cysteine aminotransferase (CAT). D-Amino acid oxidase (DAO) in brain and kidney peroxisomes can generate H_2S via 3MST. In the non-enzymatic pathway, H_2S production mediated via NADPH and NADH which are supplied by glycolysis of glucose. Reactive sulfur species like persulfides, thiosulfate, and polysulfides are reduced into H_2S and other metabolites. H_2S catabolism: In mitochondria, H_2S binds to the enzyme sulfur quinone oxidoreductase (SQR) transferring 2 electrons for ATP synthesis and oxidizing persulfide (SQRS-S). Persulfide loses 2 more electrons forming SQR-sulfane sulfur (SQR-SH). Pathway 1: SQR-SH is converted to sulfite ($S_2O_3^{2-}$) and then to glutathione (GSH) forming glutathione persulfide (GSSH) catalyzed by thiosulfate sulfur transferase (TST). Mitochondrial sulfur dioxygenase (ETHE1) oxidizes GS-SH to sulfite, which can then be further oxidized to sulfate (SO_4^{2-}) by sulfite oxidase (SO). Pathway 2: Rhodanase (Rhd) catalyzes the formation of thiolsulfate from sulfite and GSSH.

sulfur-bonded sulfur (R-S-SH). These include compounds like thiols, polysulfides, thiosulfate and elemental sulfur. Bound-sulfane sulfur compounds release H_2S in reducing condition, indicating that redox homeostasis is important for H_2S bioavailability. However, the release of H_2S equivalents from these pools and the (patho)physiological conditions in which they are biochemically converted remain poorly understood [88–91].

Catabolism of H_2S occurs in mitochondria through oxidation (Figure 2). In the first step, H_2S is enzymatically converted into protein-bound persulfide (SQRS-S) by sulfur quinone oxireductase (SQR). In this process two elec-

trons are transferred into the electron transport chain (ETC) via quinone and contribute to the production of ATP. Persulfide (SQSR-S) is then further metabolized via 2 different pathways. In the first pathway, the persulfide is transferred to the carrier sulfite ($S_2O_3^{2-}$), forming thiosulfate ($S_2O_3^{2-}$) and then to glutathione (GSH) by thiosulfate sulfur transferase (TST), yielding glutathione persulfide (GS-SH). Mitochondrial sulfur dioxygenase (ETHE1 or SDO) oxidizes GS-SH to form sulfite (SO_3^{2-}), which can then be further oxidized by sulfite oxidase (SO) to sulfate (SO_4^{2-}), producing electrons that are delivered to cytochrome c (Cyt-c) or receive another H_2S and form thiosulfate ($S_2O_3^{2-}$). The second pathway is similar except that GSH is the initial carrier and TST (rhodanase (Rhd)) catalyzes the formation of thiosulfate from sulfite and GSSH. Sulfate comprises 77-92% of total urinary sulfur [92,93].

The biological functions of H_2S are diverse. It is important to note that the effects of H_2S are dependent on their concentration and the type of donor used. For instance, at low concentrations H_2S is anti-inflammatory, while at higher concentrations it is pro-inflammatory or toxic. Therefore, it is important to investigate the effects of H_2S in the proper concentration range. This is especially important when using H_2S-releasing donors. These donors differ in the rate and magnitude of H_2S release, resulting often in contradictory results. In general, H_2S releasing donors can be divided in slow (GYY4137, ADT-OH) and fast releasing donors (NaHS, Na_2S), as well as targeted donors (AP39, mitochondria targeted) and natural donors like N-acetylcysteine (NAC), diallyl disulfide and trisulfide (DADS, DATS) from garlic [94]. H_2S is able to modulate a wide range of physiological responses. E.g. it has been shown to be anti-inflammatory and to reduce oxidative stress. Moreover, it is involved in the modulation of neurotransmission, vasodilatation, protection against reperfusion injury and inhibition of insulin resistance [95,96]. Another area of growing interest is the role of H_2S as an anti-senescent agent. Several studies have indicated that H_2S protects cells from senescence via SIRT1 and Keap1-dependent activation of Nrf2, leading to antioxidant and cytoprotective responses [86,97]. In addition, it has been demonstrated that CSE deficiency in mouse embryonic fibroblasts induces early development of cellular senescence. Given the importance of H_2S in many (patho)physiological processes and the ongoing discovery of novel actions, additional research on this interesting molecule is still warranted [86].

2. Dysregulation of hydrogen sulfide in chronic liver diseases

The liver is the major organ for endogenous H_2S production and clearance due to abundant expression of CSE, CBS and MPST. There is increasing evidence that H_2S plays a significant role in normal liver function and in the pathogenesis of liver diseases [98]. H_2S affects glucose, increased insulin sensitivity, lipid metabolism and mitochondrial bioenergetics in the liver. Dysregulation of hepatic H_2S metabolism is involved in the pathogenesis of many liver diseases, including NAFLD. Recent results suggest that H_2S deficiency is detrimental in the liver. H_2S synthesis is impaired in NAFLD and may therefore contribute, in part, to the pathogenesis of NAFLD. This suggestion is supported by observations in CBS-KO mice, having impaired H_2S biosynthesis. These mice display increased oxidative stress, fibrosis and hepatic steatosis in dietary NAFLD/NASH models (high fat diet (HFD) and methionine and choline deficient-diet (MCD) diet). In addition, CBS deficiency induces dysregulation of genes involved in lipid homeostasis and increased levels of serum TG, non-esterified cholesterol and fatty acids, whereas serum level of high density lipoprotein (HDL) is decreased. Also, cellular β-oxidation is impaired in CBS deficient mice. Conversely, exogenous H_2S prevented NASH by abating oxidative stress and suppressing inflammation in these models [99,100]. Exogenous H_2S also protects against hepatic ischemia-reperfusion injury and carbon tetrachloride-induced liver injury in animals [101,102]. Furthermore, MPST expression is increased in hepatocytes by FFAs and in the high fat diet mediated via NF-κB/p65. Inhibition of MPST reduced FFA accumulation in L02 cells and increased expression of CSE and production of H_2S via SREBP1c, c-Jun N-terminal kinase phosphorylation and oxidative stress [103]. Interestingly, statins which are used to treat hypercholesterolemia increase endogenous production of H_2S in Wistar rats [104]. Diallyl trisulfide/disulfide (DATS/DADS), a garlic derived organic polysulfide compound acts as an H_2S donor. DATS and DADS ameliorate ethanol-induced hepatic steatosis via modulating SREBP1, PPARα and cytochrome p450 2E1 (CYP2E1) in mice [105]. However, the role of H_2S in NAFLD remains to be elucidated in more detail. In particular, it is important to explore the role of H_2S in the metabolism of FFAs, lipogenesis and lipotoxicity in different stages of NAFLD.

Scope and outline of the thesis

The general aim of this thesis is to explore the role of the gasotransmitter H_2S in the context of NAFLD. Specifically, we aim to clarify its role in lipid metabolism, lipotoxicity and fibrosis. We also aim to determine the role of H_2S in senescence of hepatic stellate cells. In that regard, we have investigated its role in senescence of hepatic stellate cells induced by the coumarin derivative esculetin.

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In **chapter 1,** we provide a general introduction to this thesis as well as an outline of the thesis. In **chapter 2**, we investigated the role of H_2S in lipid metabolism, specifically free fatty acids, in primary rat hepatocytes. In **chapter 3,** we investigated serum free thiols, a prominent systemic redox proxy in the general population (PREVEND database, n=5562) and related these levels to different scores of NAFLD, including the fatty liver index (FLI) and hepatic steatosis index (HSC). In **chapter 4** we addressed the role of H_2S in hepatic fibrogenesis, particularly the homeostasis of endogenous H_2S in activation of HSCs. Furthermore, we researched how exogenous H_2S donor effects on HSCs activation. In **chapter 5**, we further researched the reason of inactivated HSCs by the endogenous H_2S inhibition in hepatic stellate cells and possible relationship with cellular senescence. In **chapter 6** we focused on the natural coumarin derivative esculetin induced senescence via the PI3K-Akt-GSK3β pathway in HSCs. Finally, **in chapter 7**, we summarize all our results and provide a general discussion and perspective for future studies.

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