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

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Abbreviations

ABCA1	ATP binding cassette transporter A1
Acc1	Acetyl-CoA carboxylase 1
Acox1	Peroxisomal acyl-CoA oxidase type 1
Acta2	Alpha-actin-2
Akt	Protein kinase B
ALT	Alanine Aminotransferase
AOAA	Amino-oxyacetic acid
ApoB100	Apoliprotein B100
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BDL	Bile duct ligation
BrdU	5-bromo-2'-deoxyuridine
BMI	Body Mass Index
CBS	Cystathionine β-synthase
Cd36	Cluster of differentiation 36
ChREBP	Carbohydrate response element binding protein
CO	Carbon monoxide
Col1α1	Collagen type 1 alpha 1
Cpt1a	Carnitine palmitoyltransferase I
hs-CRP	High sensitive C-reactive protein
CTH	Cystathionine γ-lyase
DAMPs	Damage associated molecular patterns
DATS	Diallyl trisulfide
Ddit3	DNA-damage inducible transcript 3
DDR	DNA damage response
Dgat2	Diacylglycerol O-acyltransferase 2
DL-PAG	DL-Propargylglycine
DNL	*De novo* lipogenesis
ECAR	Extra-cellular acidification rate
ECM	Extracellular matrix
ER stress	Endoplasmic reticulum stress
ETC	Electron transport chain
FAS	Fatty acid synthase
FFAs	Free fatty acids
FLI	Fatty liver index
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGT	Gamma-glutamyltransferase

GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HCC	Hepatocellular carcinoma
HDL	High-Density Lipoprotein
HFD	High fat diet
HOMA-β,	Homeostatic Model Assessment of β cell function
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HSCs	Hepatic stellate cells
HIS	Hepatic Steatosis Index
LDL	Low-Density Lipoprotein
LSEC	Liver sinusoidal endothelial cells
MPST	3-mercaptopyruvate sulfur transferase
NAC	N-acetylcysteine
NaHS	Sodium hydrosulfide
NAFLD	Non-alcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NO	Nitric oxide
MCD	Methionine choline deficient diet
OA	Oleic acid
OCR	Oxygen consumption rate
OIS	Oncogene-induced senescence
PA	Palmitic acid
p38	P38 mitogen-activated protein kinases
PDGF-BB	Platelet-derived growth factor BB
PLP	Pyridoxal phosphate
PPARα	Peroxisome proliferator-activated receptor alpha
PREVEND	Prevention of Renal and Vascular End-Stage Disease
SAA	Sulfur containing amino-acids
SA-β-gal	Senescence Associated β-Galactosidase staining
SASP	Senescence Associated Secretory Phenotype
RNS	Reactive nitrogen Species
ROS	Reactive oxygen species
RSS	Reactive Sulfur Species
SREBP1c	Sterol regulatory element-binding protein
T2D	Type II diabetes
TGFβ1	Transforming growth factor beta 1
TGs	Triglycerides

TIMP1	Tissue inhibitor metalloproteinase 1
TST	Thiosulfate sulfur transferase
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein

Summary

Chapter 1 is a general introduction on non-alcoholic fatty liver disease (NAFLD) with a special emphasis on NAFLD-associated liver fibrogenesis. We discuss in detail the role of hepatocytes and hepatic stellate cells in this process. Furthermore, we present an introduction to the gasotransmitter hydrogen sulfide (H_2S) and its role in the (patho)physiology of NAFLD and fibrogenesis.

In **Chapter 2** we investigate the factors that impair endogenous production of H_2S during development of NAFLD, in particular steatosis and free fatty acids in primary rat hepatocytes. We demonstrated that free fatty acids (FFAs) and the fibrogenic cytokine TGFβ1 strongly reduced the generation of H_2S and the expression of H_2S synthesizing enzymes. In addition, we investigated the effect of reduced H_2S generation on fatty acid metabolism and steatosis. Inhibition of H_2S increased the accumulation of lipids in hepatocytes via reduced peroxisome proliferator-activated receptor-alpha (*Pparα*) activity and reduced expression of *Pparα* target genes, resulting in decreased fatty acid β-oxidation and increased triglyceride accumulation. These effects were reversed by exogenous H_2S. These results highlight the importance of H_2S in FFA metabolism in hepatocytes and identify impaired H_2S production as one of the mechanisms leading to increased lipid accumulation in hepatocytes.

In **Chapter 3** we investigated the value of measuring free thiols (R-SH) as a marker of systemic redox status in NAFLD in the general population (PREVEND database (n=5562)). Fatty liver index (FLI) and hepatic steatosis index (HSI) were used as indicators of NAFLD. We demonstrated that serum free thiols were reduced in subjects with suspected NAFLD. In multivariable linear regression, a significant association of serum free thiols with systolic blood pressure, diabetes and total cholesterol was shown. Furthermore, this association lost its significance after adjustment for high sensitive C-reactive protein, indicating the importance of free thiol status in chronic inflammation. Lastly, serum free thiols were also able to predict all-cause mortality risk.

Chapter 4-6 focus on the role of H_2S in stellate cell biology, in particular on the role of H_2S on stellate cell activation and senescence. In **Chapter 4**, we observed that the expression of the H_2S synthesizing enzyme cystathionine

γ-lyase (CTH) and the production of H_2S was increased during activation of rat primary hepatic stellate cells (HSCs). In addition, exogenous H_2S donors increased HSCs proliferation whereas inhibition of H_2S production reduced HSCs proliferation and activation. The stimulatory effect of H_2S on HSC activation is due to increased cellular bio-energetics as a result of increased mitochondrial activity. Cell specific inhibition of H_2S production could be a novel target to limit liver fibrosis.

In **Chapter 5,** we demonstrated a reciprocal relation between stellate cell activation and senescence which is mediated by H_2S. Inhibition of H_2S production reduced fibrogenic markers whereas it increased mRNA expression of the cellular senescence markers *Cdkn1α*, *p53* and *Il6* and increased the proportion of β-galactosidase positive senescent cells. The H_2S induced induction of senescence is mediated via the PI3K-Akt signaling pathway. Furthermore, exogenous H_2S was able to reverse cellular senescence.

Chapter 6 addressed the effect of the natural coumarin derivate esculetin on the activation of HSCs. We demonstrated that esculetin induced mRNA expression of the senescence markers *Cdkn1α*, *p53*, *Il6* and $P21^{cip1}$ in HSCs and reduced the expression of the fibrogenic markers *Col1a1* and *Acta2*. The induction of senescence by esculetin was mediated by the PI3K-Akt-GSK3β pathway. Based on these results, esculetin could be a potential therapeutic compound for liver fibrosis.

Nederlandse Samenvatting

Hoofdstuk 1 is een algemene introductie over niet-alcoholische leververvetting (NAFLD) met een speciale nadruk op NAFLD-geassocieerde leverfibrogenese. We bediscussiëren in detail de rol van hepatocyten en hepatische stellaatcellen in dit proces. Daarnaast presenteren we een introductie over de gastransmitter waterstofsulfide (H_2S) en zijn rol in de pathofysiologie van NAFLD en fibrogenese.

In **Hoofdstuk 2** onderzoeken we factoren die de endogene productie van H_2S aantasten tijdens de ontwikkeling van NAFLD, met name steatose en vrije vetzure in primaire rat hepatocyten. We demonstreren dat vrije vetzuren en de fibrogene cytokine TGFβ1 de productie van H_2S en de expressie van H_2S synthese enzymen sterk verminderen. Daarnaast onderzochten we het effect van gereduceerde H_2S productie op vetzuurmetabolisme en steatose. Inhibitie van H_2S verhoogde de accumulatie van lipiden in hepatocyten via reductie van peroxisome proliferator-activated receptor-alpha (*Pparα*) en gereduceerde expressie van *Pparα* target genen, resulterend in verlaagde vetzuur beta-oxidatie en toegenomen triglyceride accumulatie. Deze effecten waren omkeerbaar als exogeen H_2S werd toegevoegd. Deze resultaten onderstrepen de belangrijke rol van H_2S bij het vrije vetzuur metabolisme van hepatocyten en identificeert verminderde H_2S productie als een van de mechanismen die leidt tot verhoogde lipidenaccumulatie in hepatocyten.

In **hoofdstuk 3** onderzochten we de waarde van het meten van vrije thiolen (R-SH) als een marker voor systematische redoxstatus in NAFLD in de algemene populatie (PREVEND database (n=5562)). De fatty liver index (FLI) en hepatic steatosis index (HSI) werden gebruikt als indicatoren voor NAFLD. We demonstreerden dat vrije thiolen in serum gereduceerd waren bij mensen met NAFLD verdenking. Met multivariabele lineaire regressie werd een significante associatie tussen vrije thiolen in serum en systolische bloeddruk, diabetes en totaal cholesterol gevonden. Deze associatie verloor echter de significantie na correctie voor C-reactive protein niveaus. Dit suggereert dat het niveau van vrije thiolen in serum een rol heeft in chronische inflammatie. Tot slot waren vrije thiolen niveaus in serum ook voorspellend voor mortaliteit door alle oorzaken.

Hoofdstukken 4-6 focussen op de rol van H_2S in stellaatcelbiologie, waarbij met name de rol van H_2S op stellaatcelactivatie en -veroudering aan bod komt. In **hoofdstuk 4** observeerden we dat de expressive van H_2S synthetiserende enzym y-lyase (CTH) en de productie van H_2S waren verhoogd tijdens de activatie van primaire rat hepatische stellaatcellen (HSCs). Daarbij zorgden exogene H_2S donoren voor toegenomen HSC proliferatie terwijl de inhibitie van H_2S productie de proliferatie en activatie van HSCs juist verminderde. Het stimulerende effect van H_2S op HSC activatie wordt veroorzaakt door een toename van cellulaire bio-energetica welke het resultaat zijn van toegenomen mitochondriale activiteit. Cel specifieke inhibitie van H_2S-productie kan een nieuwe target zijn om leverfibrose te limiteren.

In **hoofdstuk 5** wordt aangetoond dat er een wederkerige relatie bestaat tussen stellaatcelactivatie en veroudering, welke door H_2S gemedieerd is. De inhibitie van H_2S productie zorgde voor een reductie in fibrogene markers terwijl de mRNA expressie van cellulaire verouderingsmarkers *Cdkn1a*, *p53* en *Il6* juist verhoogd was. Ook was de proportie van β-galactosidase-positieve verouderde cellen verhoogd. De H_2S-geinduceerde inductie van veroudering is gemedieerd via de PI3K-Akt signaleringsroute. Daarnaast was exogeen H_2S in staat om de cellulaire veroudering terug te draaien.

Hoofdstuk 6 ten slotte bediscussieerd het effect van het natuurlijke coumarinederivaat esculetine op de activatie van HSCs. Er werd aangetoond dat esculetine de mRNA expressie van de verouderingsmarkers *Cdkn1a*, *p53*, *Il6* en $P21^{cip1}$ induceerde in HSCs en juist zorgde voor een verlaagde expressie van de fibrogene markers *Col1a1* en *Acta2*. De inductie van veroudering door esculetine werd gemedieerd door de PI3K-Akt-GSK3B signaleringsroute. Gebaseerd op deze resultaten kan esculetine worden gezien als potentiele therapeutische stof om leverfibrose tegen te gaan.

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Biography

Turtushikh Damba was born on the 17th of March 1988 in Ulaanbaatar, Mongolia. He grew up in Ulaanbaatar and graduated from high school at the 1st School of Capital, Ulaanbaatar, Mongolia. He graduated as a Pharmacist at the Health Sciences University of Mongolia (MNUMS) in 2011. After his bachelor, Turtushikh started his Master of Pharmacy degree under the supervision of Prof. Dr. Enkhjargal Dorjbal and Prof. Dr. Ambaga Miyegombo at the same university. During his study, he obtained his first exposure to cutting-edge research experiences of biomedicine and molecular biology to address cancer in *in vivo* model. In 2013, he successfully graduated master thesis, titled 'Anti-cancer activity of *Salsola laricifolia (Turcz.ex Litv)*. He worked in HSUM from 2011 to 2016 as an assistant lecturer and drug analyst in the department Pharmaceutical Chemistry and Pharmacognosy until he received a grant to pursue doctoral degree from Mongolian State Training Foundation. March 2016, he joined Prof. Dr. Han Moshage, Prof. Dr. Klaas Nico Faber in the department of Gastroenterology and Hepatology, University Medical Centre Groningen, University of Groningen, Groningen, the Netherlands as a PhD student. His research is focused to effect and role of hydrogen sulfide on chronic liver diseases, including fibrosis and Non-Alcoholic Fatty Liver diseases to identify novel treatment strategies. Results of his work are described in this thesis.