**CHARLES UNIVERSITY**

**FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ**

**DEPARTMENT OF BIOLOGICAL AND MEDICAL SCIENCES**


# DOCTORAL THESIS

**Title:** Modulation of cholesterol and bile acid metabolism via soluble endoglin and pharmacotherapy

**Ivone Cristina Igreja e Sá**

**Hradec Králové, 2022**

## DECLARATION

I hereby declare that I am the sole author of this doctoral thesis and that this thesis is my original work generated independently under the orientation of my supervisor. All the sources of information and literature I have used for the redaction of this doctoral thesis are cited in the text and properly listed in the "References" section of this thesis. This work has not been used to obtain another or the same degree.

I agree to make my thesis available through the information system of Charles University.

July 2022

Rochester, MN, USA

# ABSTRACT

Charles University, Faculty of Pharmacy in Hradec Králové

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| Training Workplace | Department of Biological and Medical Sciences |
| Doctoral Degree Program | Pharmacology and Toxicology |
| **Candidate** | M.Sc. Ivone Cristina Igreja e Sá |
| Supervisor | prof. PharmD. Petr Nachtigal, Ph.D. |
| **Title of Doctoral Thesis** | Modulation of cholesterol and bile acid metabolism via soluble endoglin and pharmacotherapy |


Endoglin (Eng, CD105) is a transmembrane glycoprotein and co-receptor of the Transforming growth factor-β (TGFβ) receptor complex. Upregulated expression of Eng has been implicated in endothelial dysfunction, liver impairment, and hepatic fibrosis development. When Eng is cleaved by the matrix metalloproteinase-14 (MMP14), its soluble form termed soluble endoglin (sEng, sCD105) is released into the circulation. Increased plasma levels of sEng have not only been observed in patients with cardiovascular and metabolic diseases associated with hypercholesterolemia (e.g., atherosclerosis) but have also been reported to promote conditions of the metabolic syndrome (e.g., hypertension). However, the direct role of sEng in the modulation of hepatic metabolism and liver functions under physiologic or pathological conditions has not been previously explored. Therefore, this doctoral thesis aimed to test the hypothesis that sEng plays a role in the modulation of cholesterol and bile acids (BA) metabolism and entero-hepatic turnover in healthy liver and hepatic liver disease and that sEng levels in circulation may be modulated by pharmacotherapy.

The results obtained in the thesis showed that healthy mice overexpressing human sEng present increased hepatic accumulation of cholesterol and increased hepatic availability of BA, as well as increased plasma concentration of BA by upregulated ileal reabsorption into enterohepatic circulation, as a consequence of complex changes in the expression of responsible liver and ileal transporters. Moreover, increased sEng levels were observed upon nonalcoholic steatohepatitis (NASH) development, and an additional rise in circulating sEng levels results in increased hepatic accumulation of cholesterol and triglycerides. This suggests the possibility of sEng being not only a biomarker of NASH but also that sEng may have a role in impairing essential mechanisms against cholesterol and triglycerides accumulation.

In conclusion, this thesis contributed to the understanding of sEng's unfavorable role in the modulation of cholesterol and BA homeostasis in the liver. Thus, we may suggest that sEng may be a possible biomarker of liver damage with a direct role in liver impairment and that sEng levels should be taken into account in patients prone to develop hepatic pathologies.

# ABSTRAKT

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Školicí pracoviště: Katedra biologických a lékařských věd

Doktorský studijní program: Farmakologie a toxikologie

**Kandidátka / kandidát:** M.Sc. Ivone Cristina Igreja e Sá

Školitelka / školitel: prof. Pharm.Dr. Petr Nachtigal, Ph.D.

**Název disertační práce:** Modulace metabolismu cholesterolu a žlučových kyselin prostřednictvím solubilního endoglinu a farmakoterapie

Endoglin (Eng, CD105) je transmembránový glykoprotein a koreceptor receptorového komplexu pro transformující růstový faktor β (TGFβ). Zvýšená exprese Eng se podílí na endotelové dysfunkci, poškození jater a rozvoji jaterní fibrózy. Když je Eng odštěpen metaloproteinázou-14 (MMP14), uvolňuje se do oběhu jeho rozpustná forma označovaná jako solubilní endoglin (sEng, sCD105). Zvýšené plazmatické hladiny sEng byly pozorovány nejen u pacientů s kardiovaskulárními a metabolickými onemocněními spojenými s hypercholesterolemií (např. aterosklerózou), ale byly dány do souvislosti s rozvojem metabolického syndromu (např. arteriální hypertenzí). Přímá role sEng v ovlivnění jaterního metabolismu a jaterních funkcí za fyziologických nebo patologických podmínek však dosud nebyla zkoumána. Cílem této disertační práce bylo proto ověřit hypotézu, že sEng hraje roli v modulaci metabolismu cholesterolu a žlučových kyselin (ŽK) a enterohepatálního obratu ve zdravých játrech a při jaterním onemocnění a že hladiny sEng v cirkulaci mohou být ovlivněny farmakoterapií.

Výsledky získané v této práci ukazují, že u zdravých myší s vysokou expresí lidského sEng dochází ke zvýšené jaterní akumulaci cholesterolu a zvýšené jaterní dostupnosti ŽK, jakož i ke zvýšené plazmatické koncentraci ŽK v důsledku zvýšené ileální reabsorpce do enterohepatálního oběhu, což je důsledkem komplexních změn v expresi odpovědných jaterních a ileálních transportérů. Kromě toho byly při rozvoji nealkoholické steatohepatitidy (NASH) pozorovány zvýšené hladiny sEng přičemž další zvýšení cirkulujících hladin sEng má za následek zvýšenou jaterní akumulaci cholesterolu a triglyceridů. To naznačuje možnost, že sEng je nejen biomarkerem NASH, ale že sEng může mít také roli v narušení základních mechanismů, které brání akumulaci cholesterolu a triglyceridů v játrech.

Závěrem lze říci, že tato disertační práce přispěla k pochopení nepříznivé role sEng v modulaci homeostázy cholesterolu a ŽK v játrech. Z tohoto důvodu tvrdíme, že sEng může být možným biomarkerem jaterního poškození a induktorem dalších patologických změn, a že hladiny sEng by měly být brány v úvahu u pacientů náchylných k rozvoji jaterních onemocnění.

“

*Para ser grande, sê inteiro: nada*
*Teu exagera ou exclui.*
*Sê todo em cada coisa. Põe quanto és*
*No mínimo que fazes.*
*Assim em cada lago a lua toda*
*Brilha, porque alta vive.*

”

Fernando Pessoa
(Odes de Ricardo Reis[1])

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[1] ***Translation:*** *To be great, be whole: nothing / of yours exaggerates or excludes. / Be all in everything. Put what you are / in the least you do. So, in every lake the whole moon / shines because high lives.*

## ACKNOWLEDGMENTS

I would like to thank the people without whom this work would not have been possible. Firstly, I must express my gratitude to Prof. PharmD. Petr Nachtigal, Ph.D., my supervisor, for the opportunity to pursue my postdoctoral training under his outstanding mentorship. Thank you for providing me wisdom and guidance on how to proceed with my Ph.D. projects since the very first day until the writing of this dissertation. Thank you for being an inspiring mentor and for all the great support in my career development.

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Additionally, I would like to express my appreciation to PharmD. Petra Hiršova, Ph.D., for her mentoring during my internship at the Liver Disease Immunobiology laboratory at Mayo Clinic and for her infinite patience during brainstorming and scientific debates on whether things make sense or not. Thank you for teaching me brain gymnastics and active critical thinking skills and how to look at a research project from different perspectives.

I would like to acknowledge the stupendous research teams that had my back during this journey and taught me almost everything I know: the research team at the Department of Biological and Medical Sciences at the Faculty of Pharmacy, for the supportive and collaborative environment (with a special shout out to the very productive coffee breaks in the office) that made me grow not only as a scientist and a "teacher" but also at a personal level; the research team of the Department of Pharmacology of the Faculty of Pharmacy in Hradec Králové, with whom I've learned most of the lab skillset that I own. Thank you for the assistance with troubleshooting experiments and during all the tissue harvest marathons; the research team from the Liver disease immunobiology lab at Mayo Clinic, for teaching me sophisticated lab skillset and respective troubleshooting approaches. And, a very special thanks to the vivarium's team for the assistance with the breeding, daily care of my beloved animals, and aid during drug administration season.

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## ABBREVIATIONS[2]

| | |
|---|---|
| Abca1 | ATP-binding cassette subfamily A member 1 |
| Abcg5 | ATP Binding Cassette Subfamily G Member 5 |
| Abcg8 | ATP Binding Cassette Subfamily G Member 8 |
| Acat | Acyl-coenzyme A cholesterol acyltransferase |
| ACC | Acetyl-CoA carboxylase |
| Acetyl-CoA | Acetyl-coenzyme A |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| ApoB | Apolipoprotein B |
| Asbt | Na–dependent bile acid transporter |
| AST | Aspartate aminotransferase |
| BA | Bile acids |
| Bsep | Bile salts export pump |
| CA | Cholic acid |
| Cd36 | Cluster differentiation protein 36 |
| CDCA | Chenodeoxycholic acid |
| Cpt1 | Carnitine palmitoyl transferase 1 |
| CYP | Cytochromes P450 |
| Cyp27a1 | Sterol 27-hydroxylase |
| Cyp7a1 | Cholesterol 7α-hydroxylase |
| Cyp7b1 | Oxysterol 7α-hydroxylase |
| Cyp8b1 | Sterol 12α-hydroxylase |
| DAG | Diacylglycerols |
| DCA | Deoxycholic acid |
| DGAT | Diacylglycerol acyltransferase |
| EASL | European Association for the Study of the Liver |
| EMA | European Medicines Agency |
| Eng, CD105 | Endoglin |
| FAS | Fatty acid synthase |

[2] ***Nomenclature note:*** *in this thesis, human gene and protein symbols are all capitalized. Rodent gene and protein symbols are in lower case with an initial capital.*

| | |
|---|---|
| FDA | Federal Drug Administration |
| FFA | Free fatty acids |
| FFC diet | High fat, high fructose, and high cholesterol diet |
| GPAT | Glycerol-3-phosphate acyltransferase |
| GSH | Glutathione |
| HDL | High-density lipoprotein |
| HLDA | Human Leukocyte Differentiation Antigen |
| Hmgcr | 3-hydroxy-3-methylglutaryl coenzyme A reductase |
| LCA | Lithocholic acid |
| Lce | Long-chain fatty acyl elongase |
| LDL | Low-density lipoprotein |
| MMP14 | Metalloprotease-14 |
| Mrp2 | Multidrug resistance-associated protein 2 |
| Mrp4 | Multidrug resistance-associated protein 4 |
| Mttp | Microsomal triglyceride transfer protein |
| NAFLD | Nonalcoholic fatty liver disease |
| NASH | Nonalcoholic steatohepatitis |
| Ntcp | Na+-taurocholate co-transporting polypeptide |
| Ostα/ Ostβ | Organic solute transporter α and β |
| Scd1 | Stearoyl–CoA desaturase |
| sEng, sCD105 | Soluble endoglin |
| Sr-b1 | Scavenger receptor, class B type 1 |
| TAG | triacylglycerols |
| TCA | Taurocholic acid |
| TCDCA | Taurochenodeoxycholic acid |
| TGFβ | Transforming growth factor-β |
| UDCA | Ursodeoxycholic acid |
| VLDL | Very-low-density lipoproteins |
| ZP | Zona pellucida |
| αMCA | α-Muricholic acid |
| β-MCA | β-Muricholic acid |
| γGT | Gamma-glutamyl transferase |

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[3] *All figures present in this thesis are original and were created by the author. Diagram of liver histology and graphical representation of synthetic and metabolic pathways were created using PowerPoint software. Microscopic photos of human histology sections stained with hematoxylin-eosin were taken by the author during her internship at the Mayo Clinic, Rochester, NM, USA.*

# 1 INTRODUCTION

## 1.1 LIVER

Comprising approximately 2.5% of the body weight of an adult human being, the liver can weigh between 968 to1860 grams, which makes it the largest gland of the human body (1). The liver is a major metabolic organ, and it is responsible for the extraction and metabolism of nutrients, synthesis of serum proteins (e.g., albumin) and hormones, as well as detoxification of the organism from xenobiotics and systemic waste products (2). It also performs a wide variety of functions that are essential for the preservation of homeostasis in the organism. Some of the hepatic metabolic functions, such as the metabolism of lipids and lipoproteins (cholesterol and triacylglycerols (TAG)), as well as the metabolism of bile acids and bile formation, will be explored in detail during the following subchapters.

### 1.1.1 Liver histology

The classic structural unit of the liver is a hexagonal hepatic lobule with an efferent venule at the center (the central vein) and the portal tracts at the periphery, formed by bile ducts, branch of hepatic arteries, and portal venules (**Figure 1**) (2). The major feature of the liver structure is a functional tissue composed of distinct cell types: liver parenchymal cells (hepatocytes) correspond to 80% of the liver mass (i.e., 60% of the total amount of cells), and the remaining 20% represent non-parenchymal liver cells such as sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, cholangiocytes, and a variety of resident and transient immune cells (e.g., lymphocytes) (3, 4). The different cell types are responsible for a variety of functions:

Hepatocytes are the major component of liver structure, and they are responsible for the hepatic metabolism of cholesterol and bile acids (detailed overview in the upcoming subchapter) (5, 6), protein synthesis (e.g., albumin), detoxification and drug elimination (7, 8). They are also involved in the activation of the innate immunity against invading microorganisms (9).

Endothelial cells represent roughly 30% of overall liver cells and correspond to approximately 3% of hepatic volume (10, 11). Hepatic endothelial cells contribute actively to multiple processes within the liver, such as metabolite transport, maintenance of vascular tone and homeostasis during vascular damage, as well as intervening in inflammation and angiogenesis (12, 13).

Hepatic resident macrophages called Kupffer cells are considered to be the first line of defense of the organism (3). They represent about 2% of the liver volume and are mostly located in the portal areas and within the lumen of sinusoids, where they usually keep their position due to light attachment to the sinusoidal endothelium (14). Their main function in the healthy liver is to phagocytose microorganisms, and toxic agents transported through the hepatic circulation and serve as antigen-presenting cells (4, 15). However, Kupffer cells participate in the crosstalk between the resident liver cells and the cells that are recruited to the liver (16). Indeed, Kupffer cells can secrete cytokines and chemokines contributing to the recruitment of cells, such as neutrophils, lymphocytes, and blood monocyte-derived macrophages to the liver (3, 16).

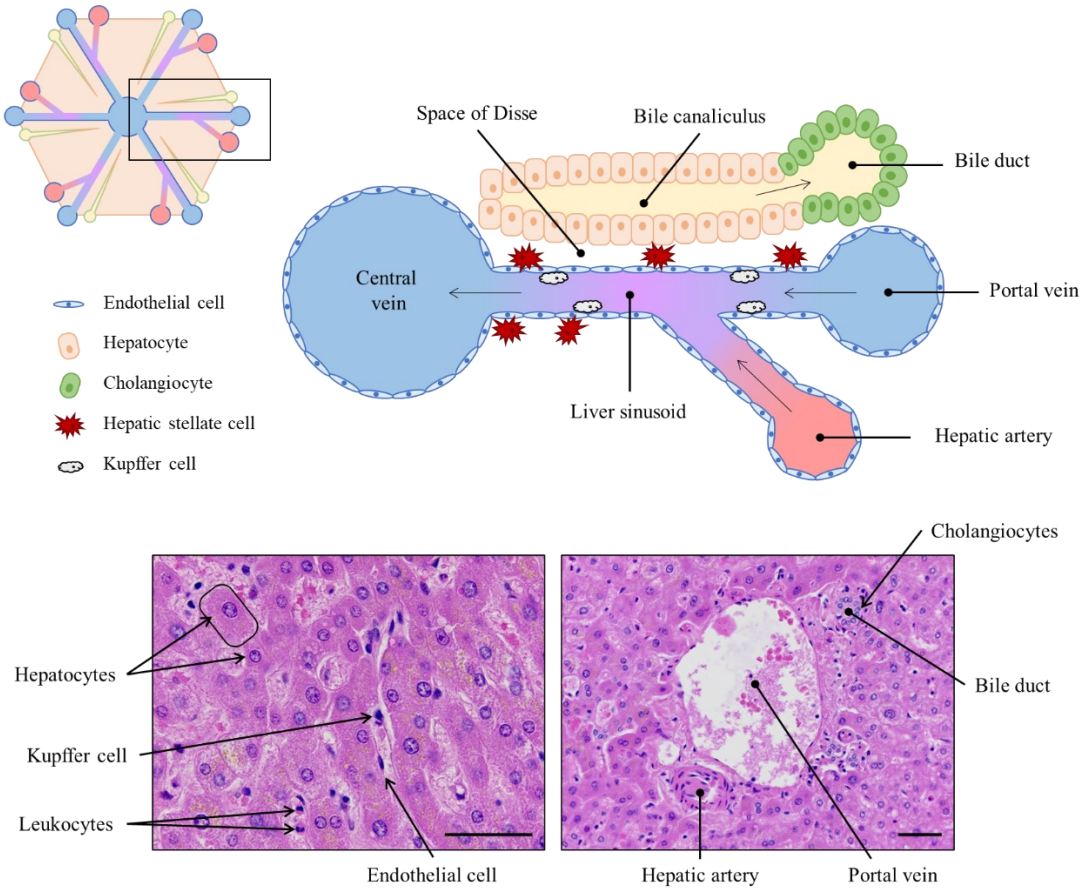


**Figure 1.** Liver structure and its different cell types. Schematic representation of the liver structure (top, drawing not-to-scale) Direction of bile flow and blood flow (black arrows). Identification of the distinct structures and different cellular populations in the histologic section of the human liver stained with hematoxylin-eosin (bottom).

Hepatic stellate cells comprise about 1.5% of the hepatic volume and are located in the space of Disse and surrounding sinusoids (3, 17). Hepatic stellate cells are the cell type responsible for the storage of vitamin A and maintenance of the homeostasis of the extracellular matrix, and preservation of hepatocyte mass by the production of hepatocyte growth factor. During liver injury, the hepatic stellate cells can differentiate into alpha-smooth muscle actin-expressing contractile myofibroblasts. Therefore, they are considered one of the major cell types involved in hepatic fibrogenesis driven by connective tissue growth factor and transforming growth factor-β (TGFβ) (17).

The biliary tree begins from the bile canaliculi, those are regions delimited by tight junctions in the cell membrane of adjacent hepatocytes and continues with structures called bile ducts (18, 19). Cholangiocytes are biliary epithelial cells that line bile ducts and are responsible for the final composition and volume of bile secretion by basal and hormone-regulated events (18, 19). These cells are the specific target of a class of human diseases termed cholangiopathies (e.g., Primary sclerosing cholangitis and Primary biliary cirrhosis) (18, 19).

In addition, the liver vascular compartment contains several circulating and resident immune cells that accumulate preferentially around portal tracts. These include dendritic cells and B cells, as well as a variety of T cells (4). Even though the immune cell subsets in the healthy liver are restricted to a specific region outside of the hepatocyte region, this is breached during inflammation where immune cells infiltrate the parenchyma (4).

### 1.1.2 Liver function

Due to its strategic placement in the body, the liver has access to a large supply of nutrients such as amino acids, lipids, carbohydrates, and vitamins, as well as xenobiotics that are absorbed from the diet in the gastrointestinal tract (20). This makes the liver a major metabolic organ with vital metabolic functions, such as the metabolism of lipids and lipoproteins (cholesterol and triacylglycerols (TAG)), as well as the metabolism of bile acids and bile formation.

#### *1.1.2.1 Cholesterol metabolism in the liver*

Cholesterol is predominantly synthesized in the liver from acetyl-coenzyme A (acetyl-CoA) via the rate-limiting enzyme Hmgcr (3-hydroxy-3-methylglutaryl coenzyme A reductase) (**Figure 2**) (21). In addition to *de novo* biosynthesis, the cholesterol carried by low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particles can be removed from

the circulation by the hepatocyte's basal surface receptors: LDL receptor (Ldlr) and Sr-b1 (scavenger receptor, class B type 1) (22), respectively.

Once in the cytoplasm of hepatocytes, cholesterol can be transformed into cholesteryl ester by the exzyme acyl-coenzyme A cholesterol acyltransferase (Acat) in order to be stored in lipid droplets (e.g., steatosis) or to form lipoproteins to be released into circulation such as very-low-density lipoproteins (VLDL) (23). Excess cholesterol can be eliminated from the hepatocytes via ATP-binding cassette subfamily A member 1 (Abca1) efflux transporter to the blood or by the Abcg5 and Abcg8 heterodimer into bile in the bile ducts to be later delivered into the intestinal lumen (5). Furthermore, cholesterol can also be used for the "*de novo*" synthesis of bile acids in the liver.

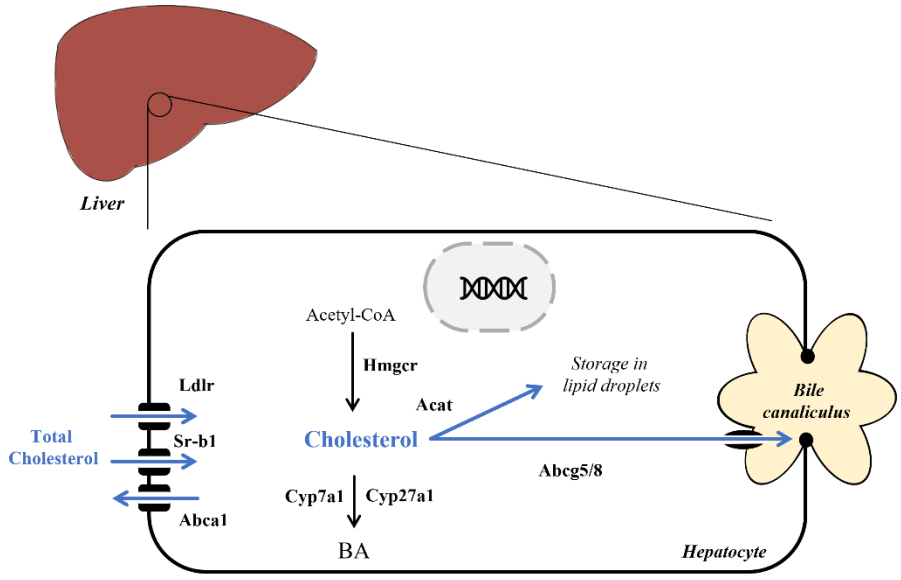


**Figure 2**. Cholesterol metabolism in the liver. Schematic representation of cholesterol metabolism in the liver from the *de novo* synthesis of cholesterol via Hmgcr to its conversion into bile acids (BA) via Cyp7a1 or Cyp27a1 (black arrows). Circulation of cholesterol (blue arrows). The influx of cholesterol from the circulation into the hepatocyte occurs via Ldlr and Sr-b1, and its storage into lipid droplets is promoted by Acat. Biliary elimination of cholesterol is promoted by Abcg5/8. Hmgcr, 3-hydroxy-3-methylglutaryl coenzyme A reductase; Acat, acyl-coenzyme A cholesterol acyltransferase; Cyp7a1, Cholesterol 7α-hydroxylase; Cyp27a1, Sterol 27-hydroxylase; Ldlr, low-density lipoprotein receptor; SR-b1, scavenger receptor, class B type 1; Abca1, ATP-binding cassette subfamily A member 1; Abcg5/8, ATP Binding Cassette Subfamily G Member 5 and 8.

#### *1.1.2.2 Bile acids metabolism in the liver and entero-hepatic turnover*

Bile acids (BA) are metabolites derived from cholesterol that aid the intestinal absorption and transport of dietary lipids (24, 25). The conversion of cholesterol into BA is exclusively a hepatic function and is an important pathway for cholesterol elimination as well as bile acid *de novo* synthesis (**Figure 3**) (24, 26). In humans and mice, primary BA are synthesized in the liver by the involvement of at least fourteen enzymes (i.e., cytochromes P450 (CYPs)) and can be divided into two major pathways: the classic (neutral) pathway and the alternative (acidic) pathway (25). Cholesterol 7α-hydroxylase (Cyp7a1) in the endoplasmic reticulum is the initiating and rate-limiting enzyme of the classic synthetic pathway (27, 28). This pathway, with the intervention of sterol 27-hydroxylase (Cyp27a1), leads to the formation of chenodeoxycholic acid (CDCA) and the additional involvement of sterol 12α-hydroxylase (Cyp8b1) is required for the synthesis of cholic acid (CA) (26). On the other hand, the alternative pathway is initiated by mitochondrial Cyp27a1 and followed by oxysterol 7α-hydroxylase (Cyp7b1), which is nonspecific and catalyzes the hydroxylation of 27-hydroxycholesterol into CDCA (26). Both CA and CDCA may be conjugated with taurine into taurocholic acid (TCA) and taurochenodeoxycholic acid (TCDCA), respectively. Interestingly, CDCA is abundant in human bile but not in mice because, in rodents, CDCA is 6β-hydroxylated to α-muricholic acid (αMCA) and ursodeoxycholic acid (UDCA) to form β-MCA by Cyp2c70 (29, 30). Both BA can then be conjugated with taurine into TαMCA and TβMCA, respectively.

Bile is an essential fluid indispensable for intestinal digestion and absorption of lipids. Bile is mainly formed by water (95%), inorganic and organic solutes are only approximately 5% of its composition (31). Hepatocytes produce bile by secreting BA, cholesterol, conjugated bilirubin, phospholipids, proteins (e.g., glutathione (GSH)), ions, and water into their canaliculi (31). BA are the main component of the bile and are the major driving force of the bile production (i.e., bile acid-dependent bile flow) (32). When BA are transported from periportal hepatocytes predominately by Bile salts export pump (Bsep, encoded by *Abcb11* gene) into the lumen of the biliary canaliculus, the water follows by osmosis, most likely through aquaporin water channels (24). Cholesterol is transported through the canalicular membrane of hepatocytes via Abcg5/8 (5). A phospholipid lipase (Mdr2 in rodents/MDR3 in humans) is mainly responsible for the biliary secretion of phospholipids (24). On the other hand, bile salt–independent bile flow can be stimulated by certain solutes, such as GSH, which is released into bile by the canalicular Multidrug resistance-associated protein 2 (Mrp2, encoded by *Abcc2*) (24). Canalicular bile is then directed to the bile ducts and stored in the gallbladder, where it is further concentrated (up to 10-fold) before reaching the intestine. Once in the intestine, secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid (LCA),

ωMCA, HCA, HDCA, and MDCA, are produced by gut microbiota from the primary bile acids.

Enterohepatic circulation of BA from the liver to the intestine and back to the liver plays a central role in nutrient absorption, metabolic regulation, and bile acid metabolism homeostasis (28). Nearly 95% of BA is reabsorbed at the level of the distal ileum and returns to the liver via entero-hepatic recirculation; meanwhile, the rest is eliminated in the feces (33). The reabsorption into the portal circulation happens in two distinct steps firstly BA is transported into the enterocyte through the apical membrane Na–dependent bile acid transporter (Asbt, encoded by *Slc10a2* gene), followed by BA efflux from the enterocyte into the portal circulation via heterodimeric Organic solute transporter α and β (Ostα/ Ostβ) on the basolateral pole of ileal enterocytes (33). The hepatocytes from the periportal region are the main responsible for the absorption of bile acids from the entero-hepatic circulation by the sodium-dependent uptake of BA from the sinusoidal blood by Na+-taurocholate co-transporting polypeptide (Ntcp, encoded by *Slc10a1* gene).

The efflux of BA from the hepatocytes into the circulation can also occur via Multidrug resistance-associated protein 4 (Mrp4 encoded by *Abcc4* gene) efflux transport. Even though weakly expressed on the basolateral membrane of hepatocytes in normal liver, it is highly upregulated in cholestatic liver disease (34, 35). Mrp4 upregulation in cholestasis represents hepatoprotective feedback because it generates an alternative elimination pathway of excess BA from the hepatocytes (35).

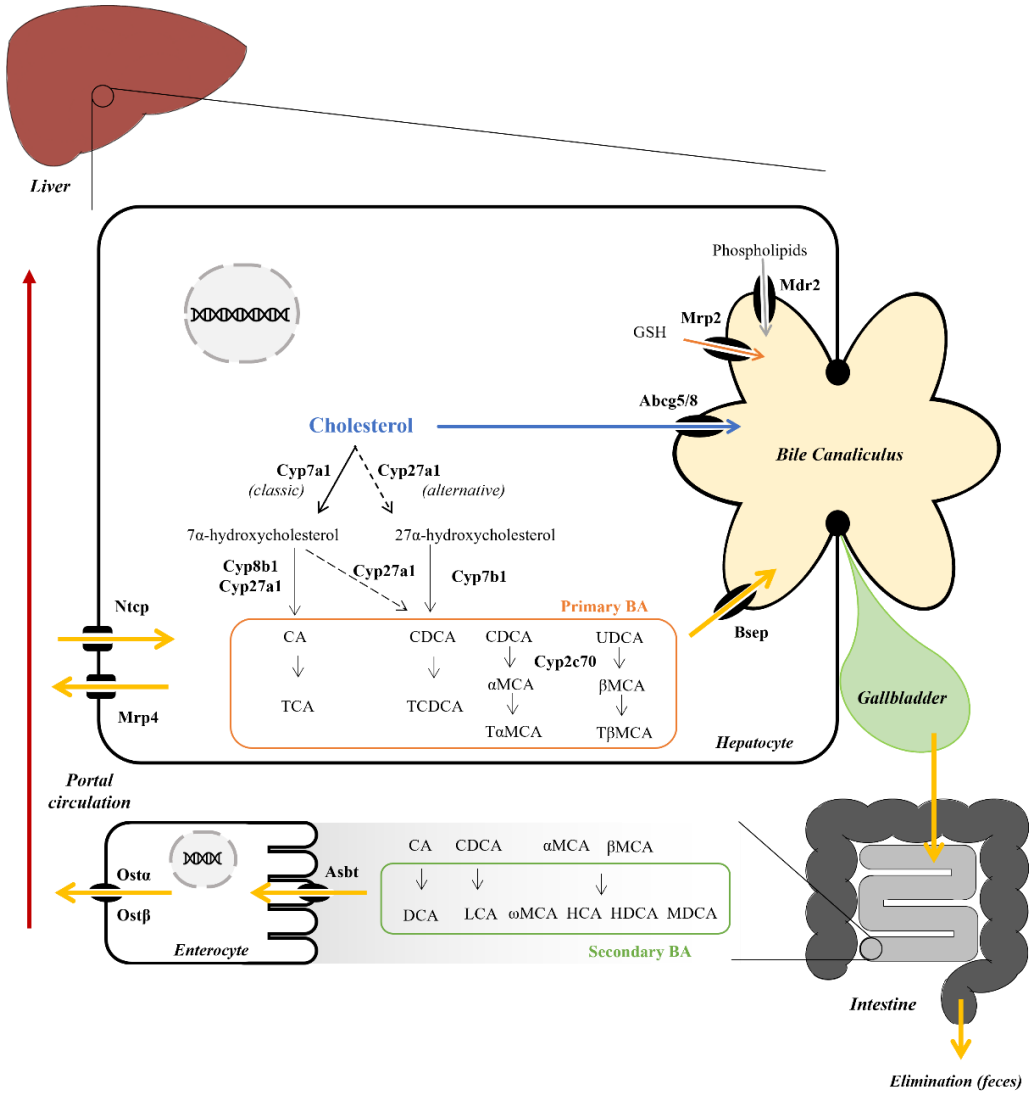


**Figure 3.** Bile acid synthesis in the liver and entero hepatic turnover of bile acids (BA). The synthesis of BA in the liver occurs by conversion of cholesterol in BA by two distinct pathways: the classic (neutral) pathway or via the alternative (acidic) pathway. Cyp7a1 initiates the classic pathway producing 7α-hydroxycholesterol that is further metabolized into cholic acid (CA) by Cyp8b1 and Cyp27a1. The alternative pathway initiates with the production of 27α-hydroxycholesterol via Cyp27a1, which is converted into chenodeoxycholic acid (CDCA) by Cyp7b1. In mice, Cyp2c70 is responsible for the synthesis of muricholic acid (MCA). Biliary elimination of BA is promoted by Bsep, and it leads to bile salt-dependent bile flow. Once in the intestine, BA are altered by the gut microbiome and converted into secondary BA. BA are eliminated in the feces or can be reabsorbed into entero-hepatic circulation via Asbt and Ostα/β in the enterocyte. Hepatocytes retrieve BA from the circulation through Ntcp. Excess BA in the hepatocyte is released in the circulation by Mrp4. Bsep, Bile salts export pump; Asbt, Na–dependent bile acid transporter; Ostα/Ostβ, Organic solute transporter α and β; Ntcp, $Na^{+}$-taurocholate co-transporting polypeptide; Mrp4, Multidrug resistance-associated protein 4.

#### *1.1.2.3 Triacylglycerol metabolism in the liver*

The synthesis of triacylglycerols (TAG) is an important hepatic metabolic pathway for the control of lipid metabolism and maintenance of energy homeostasis (**Figure 4**). It is estimated that about one-third of the free fatty acids (FFA) removed from the bloodstream is taken up by the liver via Cd36 (cluster differentiation protein 36) and converted to TAG, which are either stored in hepatic lipid droplets or released into the circulation as VLDL (36).

TAG are formed by esterification of three fatty acids with the three alcohol groups of a glycerol molecule. In the liver, glycerol-3-phosphate (G-3-P) can be generated by the hepatocytes during glycolysis by the intervention of G3P-dehydrogenase or by direct phosphorylation of glycerol by glycerokinase (37). Glycerol-3-phosphate acyltransferase (GPAT) is the rate-limiting enzyme for the *de novo* synthesis of glycerophospholipids and catalyzes the first step of the synthesis of TAG by promoting the esterification of FFA with the G-3-P into diacylglycerols (DAG). DAG are then converted into TAG by acylation catalyzed by diacylglycerol acyltransferase (DGAT) (38).

In addition to this synthetic pathway, in hepatocytes, glucose can also be used for glycogen synthesis or metabolized to generate pyruvate through glycolysis. Pyruvate is transported into the mitochondria and oxidized through the Krebs cycle and oxidative phosphorylation to generate energy. Acetyl-CoA, which originated from mitochondrial metabolism, can be used to synthesize cholesterol via Hmgcr or redirected into fatty acids through lipogenesis. Indeed, a previous study reported that stimulation of cholesterol and BA *de novo* synthesis by upregulation of Hmgcr (rate-limiting enzyme for the synthesis of cholesterol) and Cyp7a1 (rate-limiting enzyme for BA synthesis) results in reduced the *de novo* synthesis of TAG by limiting the availability of acetyl-CoA by directing it to sterol synthesis (39). This suggests the existence of a shared pathway where cholesterol and TAG *de novo* syntheses regulate each other via the subtract availability thus, reduced use of Acetyl-CoA by cholesterol synthesis may promote its redirection to the TAG *de novo* synthesis.

Fatty acids are synthesized *de novo* from acetyl-CoA by acetyl-CoA carboxylase (ACC), which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. Malonyl-CoA is then converted to to long-chain fatty acids by fatty acid synthase (FAS) (40). When in excess, malonyl-CoA inhibits Cpt1 (carnitine palmitoyl transferase 1), which is the shuttle transporter of fatty acids into the mitochondria for β-oxidation (41, 42). The inhibition of Cpt1 and consequent fatty acid β-oxidation results in increased availability of fatty acids for TAG synthesis and storage or secretion (43).

Even though in mice, the principal fatty acid produced by FAS is palmitic acid (16:0), the hepatic fatty acid accumulation is composed of 18 carbon-long fatty acids rather than 16

carbon-long fatty acids (44). This additional elongation by two carbons from palmitic to stearic acid is catalyzed by a long-chain fatty acyl elongase (Lce) (44, 45). The difference between the saturated to the monounsaturated fatty acid synthetic pathway is the catalytic activity of Stearoyl–CoA desaturase (Scd1), which converts palmitoyl–CoA and stearoyl–CoA into palmitoyl–CoA (16:1) and oleoyl–CoA (18:1), respectively (46). Variations in the phospholipid composition can affect cell characteristics and functions; for example, alteration in fatty acids chain length and degree of un/saturation is responsible for membrane fluidity.

The hepatic synthesis of TAG is usually coordinated with their secretion in the form of VLDL (47). In aberrant situations, such as steatosis development in fatty liver disease, TAG accumulation occurs when the rate of import or synthesis of fatty acids by hepatocytes exceeds the rate of export or catabolism (48, 49). Microsomal triglyceride transfer protein (Mttp) plays an essential role in the transfer of lipids to apolipoprotein B (ApoB) during the early stages of lipoprotein assembly, being therefore essential for the assembly and secretion of ApoB-containing proteins (50, 51). Inhibition of ApoB-Mttp results in increased intracellular TAG accumulation in the liver (52, 53).

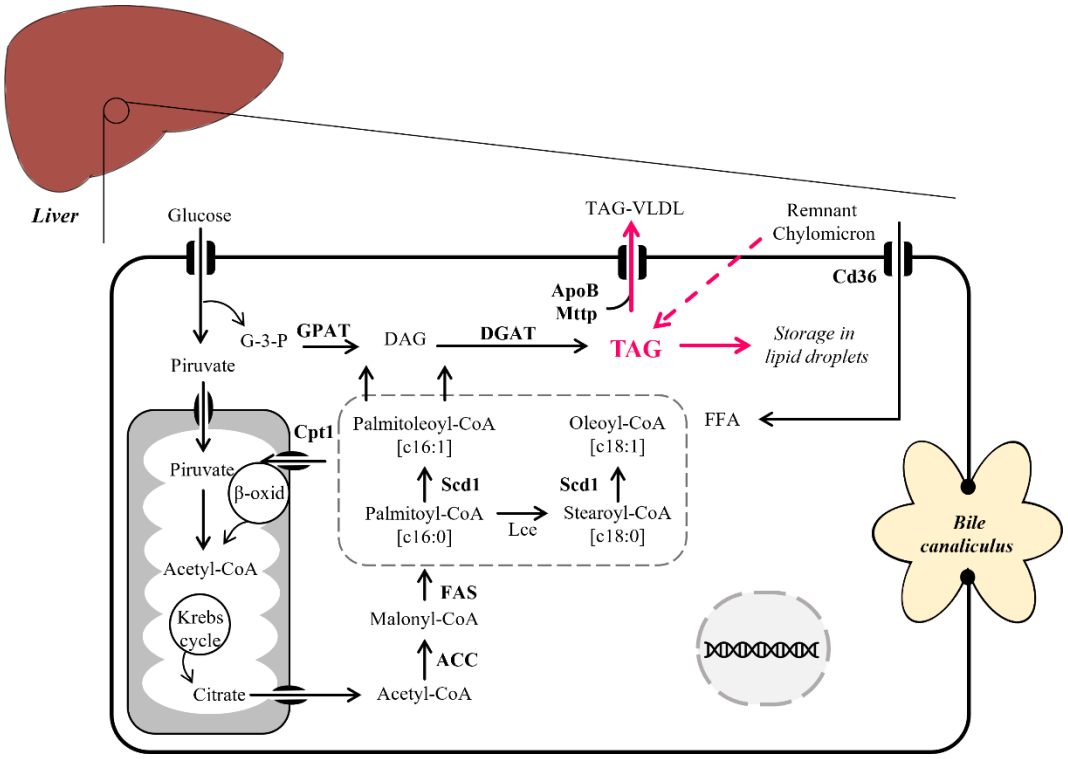


**Figure 4.** Triacylglycerols (TAG) metabolism in the liver. TAG are synthesized by esterification of three free fatty acids (FFA) to the three alcohol groups of a glycerol molecule (G-3-P) by GPAT and DGAT. FFA may be synthesized in the liver via ACC, FAS, and Scd1 or obtained from the circulation via Cd36 influx transporter or from the remnant chylomicron that enter the hepatocyte by endocytosis. Excess FFA can be imported into the mitochondria via Cpt for β-oxidation. The synthesis of TAG in the liver is usually matched with their secretion in the form of VLDL. Assembly of TAG-VLDL assembly requires Mttp and ApoB. ACC, Acetyl-CoA carboxylase; FAS, Fatty acid synthase; Scd1, Stearoyl–CoA desaturase; Lce, Long-chain fatty acyl elongase; Cpt1, Carnitine palmitoyl transferase 1; GPAT, Glycerol-3-phosphate acyltransferase; DAG, Diacylglycerols; DGAT, Diacylglycerol acyltransferase; ApoB, Apolipoprotein B; Mttp, Microsomal triglyceride transfer protein; Cd36, Cluster differentiation protein 36.

The previous chapters described the individual metabolic pathways of cholesterol, BA, and TAG. However, it is of interest to mention that metabolic pathways are interconnected, as illustrated in the following figure (**Figure 5**).

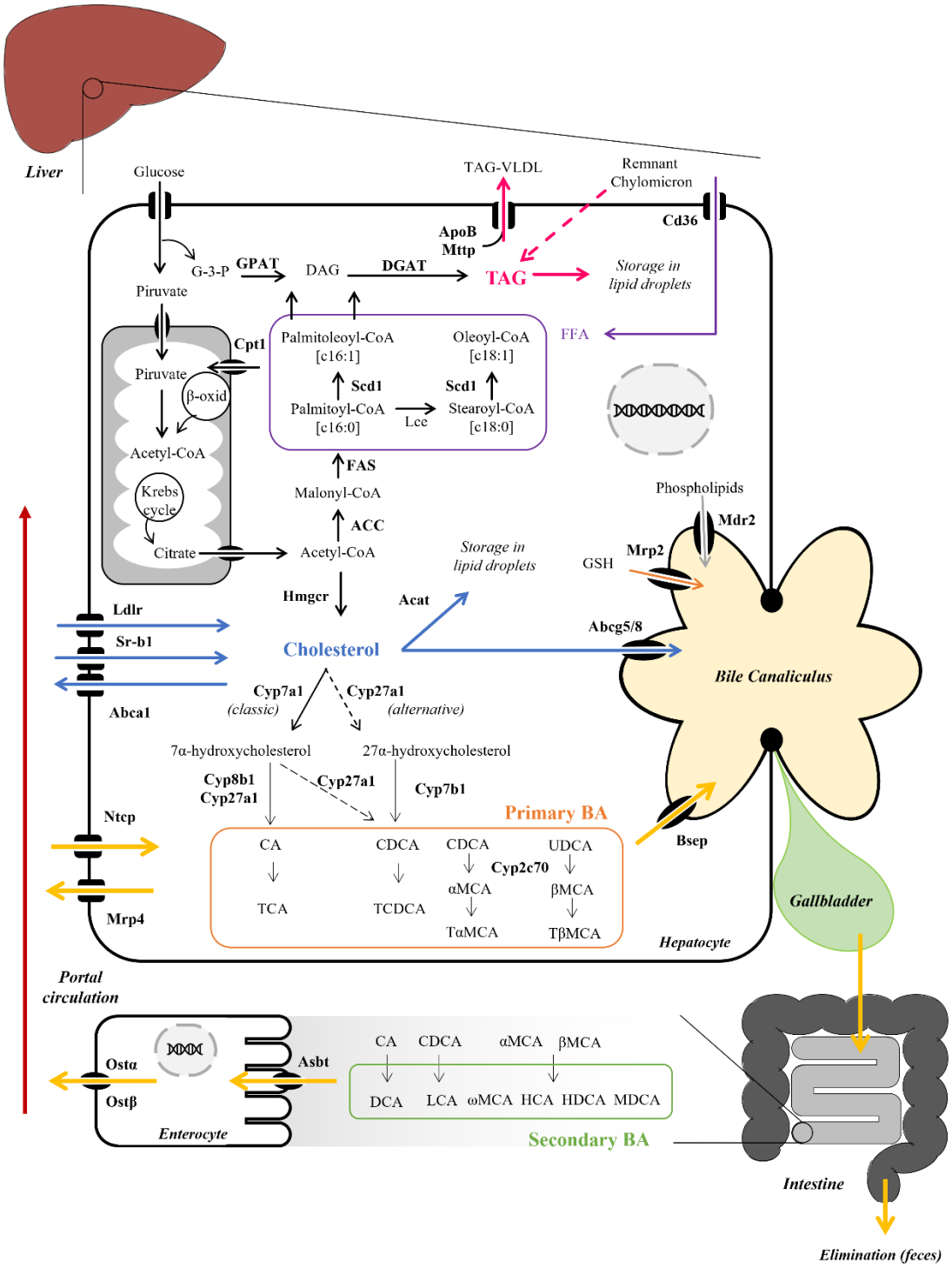


**Figure 5.** Schematic representation of interconnection between cholesterol, bile acid, and triacylglycerol metabolism in the hepatocyte, bile production, and entero-hepatic circulation. Entero-hepatic circulation of bile acids (BA) (yellow arrows). Influx, efflux, and storage of cholesterol in hepatocyte (blue arrows). Triacylglycerols (TAG) storage and efflux into circulation (pink arrows). Metabolic pathways (black arrows).

## 1.2 LIVER PATHOLOGY

As described above, the liver is responsible for many vital functions, including nutrient metabolism carried out by hepatocytes such as cholesterol synthesis and bile production, which is involved in the digestive system homeostasis. Hepatocytes are also key regulators of lipid metabolism since they are able to take up fatty acids from the blood and assemble them with glycerol to produce TAG. Dysregulation of these metabolic pathways may lead to pathological conditions.

In clinical practice, an alteration in the normal liver function or changes in tissue and cell integrity during liver impairment/injury may be detected by biochemical evaluation of serum biomarkers such as serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (γGT) (54). Another biomarker, mostly in cholestatic liver disease, may be serum levels of total bilirubin, which, even though it is a diagnostic sign of liver injury, is most commonly used as the criteria of severity (55). Furthermore, biochemical analysis of total bile acids, cholesterol, and TAG in patients' serum, may also provide useful information about hallmarks and risk factors of pathologies.

### 1.2.1 Nonalcoholic steatohepatitis (NASH)

Nonalcoholic fatty liver disease (NAFLD) is traditionally known as the hepatic manifestation of metabolic syndrome, whose main hallmark is the increased storage of fat in the liver (56). As the name suggests, it affects individuals with low to no alcohol consumption (57-59). NAFLD is the most common form of chronic liver disease in most regions around the world, especially in Western nations, affecting about 25% of the population of the United States (57).

NAFLD is an umbrella term that includes distinct stages of progressive liver impairment such as nonalcoholic fatty liver and nonalcoholic steatohepatitis (NASH). Meanwhile, nonalcoholic fatty liver presents isolated steatosis, defined as an abnormal accumulation of fat in the hepatocytes, individuals with associated metabolic disorders such as insulin resistance, type 2 diabetes, and obesity are particularly at high risk of progressing to NASH (60). NASH is an aggressive form of fatty liver disease defined by liver inflammation which may progress to advanced scarring (cirrhosis) and liver failure (**Figure 6**) (61). Besides the liver damage directly associated with NASH, the pro-atherogenic lipid profile of NASH patients has been shown to increase the risk of life-threatening cardiovascular events (62, 63).

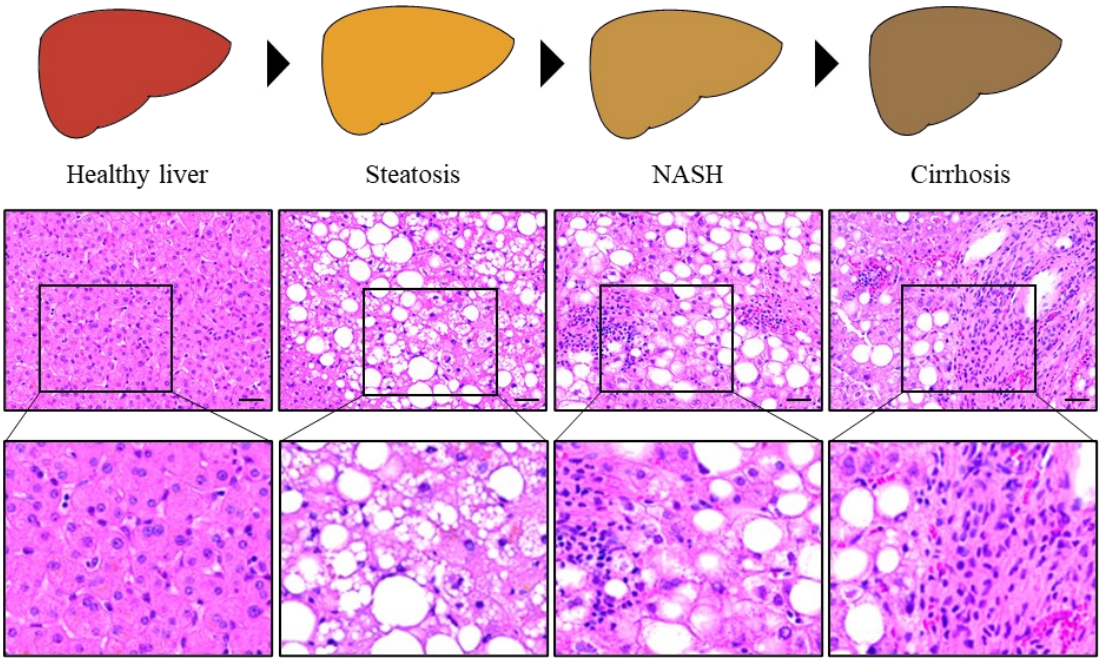


**Figure 6.** Histologic hallmarks of progression of liver damage in humans. Histologic sections of human biopsies stained with hematoxylin-eosin with the main hallmarks of the different stages of liver damage. 200X magnification (top), 400X magnification (bottom).

NASH is a concerning progressive illness that affects approximately 5% of the US population (57-59) with a lifetime cost of 222.6 billion dollars for all NASH patients (as of 2017) (64). Currently, there are no therapies approved by European Medicines Agency (EMA) or Federal Drug Administration (FDA) for NASH patients' treatment, and the results of recent clinical trials have been suboptimal (65, 66). The lack of efficient pharmacotherapies is likely due to our poor understanding of the underlying molecular mechanisms of NASH pathogenesis, which makes it a research topic in demand.

The understanding of the pathophysiology of NASH has evolved substantially from the original two-hit hypothesis where a first hit, such as insulin resistance, resulted in hepatic steatosis, and a subsequent second hit, such as oxidative stress, was required to develop NASH (67, 68). Currently, the two-hit hypothesis is no longer sufficient to describe the multiple pathways that may be interconnected and contribute to NASH; thus, a multi-hit model has been proposed more recently for the pathophysiology of NASH (60). With this model, other hits include, for example, the impairment of liver homeostasis due to the accumulation of toxic lipids (6, 69, 70); or the involvement of the gut-liver axis, where increased gut permeability may permit bacteria-derived products to reach the liver, where they can activate immune responses (71, 72). Indeed, T lymphocytes have also been reported to be involved in the NASH complex pathogenic immune response (73, 74). NASH is also associated with increased recruitment and infiltration of monocyte-derived macrophages in response to activation of the

liver resident macrophages (i.e., Kupffer cells) that contribute to local inflammation of hepatic tissue and hepatocytes' death (15, 69, 75). Hepatocytes undergoing apoptosis or inflammation can release signaling molecules contributing to the activation of hepatic stellate cells and the formation of scar tissue leading to hepatic fibrosis.

NAFLD/NASH is generally considered an underdiagnosed condition. Due to most patients having no obvious symptoms and the lack of non-invasive biomarkers for regular screening, the illness is frequently discovered during a routine blood test or a screening for another medical condition (76). For example, if the biochemical analysis of a patient reveals high serum levels of liver enzymes, in association with the presence of risk factors (e.g., metabolic syndrome), and the medical evaluation excludes alternative causes for liver disease, NASH may be suspected (76, 77). A definitive diagnosis of NASH is performed only by hepatic biopsy (78, 79). Histologically, NASH hallmarks are steatosis, inflammation, hepatocyte ballooning (a form of hepatocellular injury), and fibrosis (77-80).

## 1.3 MANAGEMENT AND CURRENT PHARMACOTHERAPY APPROACHES FOR NAFLD/NASH PATIENTS

Currently, there are no EMA or FDA-approved pharmacotherapies for NASH, despite the high volume of research and the clinical trials performed. NASH has been managed primarily by the change in habits, such as an unhealthy diet and sedentarism, to achieve a healthier lifestyle. Indeed, adopting a balanced diet and reducing body weight by 7% to 10% resulted in histological improvement of NASH in terms of steatosis, ballooning injury, lobular inflammation, and improvement in ALT levels (81, 82). Moreover, a minimum of 15 minutes of daily physical activity have been reported to reduce hepatic steatosis, apoptosis, and free fatty acids in plasma and visceral adipose tissue in NAFLD patients, as well as leading to a reduced risk of progression to NASH and advanced fibrosis (83, 84). Thus, a combination of a hypocaloric diet and moderate-intensity exercise is likely to provide the best probability of sustaining weight loss over time (78, 79). Another strategy for reducing weight loss mentioned in the guidelines is the surgical approaches, such as bariatric surgery, which is known to improve obesity and diabetes, as well as reduce the fat deposition in the liver and improve all histological lesions of NASH, thus likely reducing NASH progression (78, 79). Even though all guidelines for NASH management agree that lifestyle changes bring promising benefits, those behaviors are sometimes hard to implement, and there is a risk that the patients will fail to maintain them long-term (78, 79, 85). Therefore, pharmacotherapies are needed to complement the lifestyle modifications and to prevent the progression of the disease to end-

stage liver disease, such as in NASH patients with liver failure and/or hepatocellular carcinoma, for which the only available treatment is liver transplantation (78, 79).

In general, the pharmacologic approach for the management of NAFLD should consist of treating underlying liver disease, as well as the associated metabolic comorbidities such as obesity, hyperlipidemia, insulin resistance, and type II diabetes mellitus in order to prevent NASH progression to cirrhosis, liver failure or hepatocellular carcinoma (79). According to the current guidelines, patients with NAFLD without NASH or fibrosis have a very good prognosis, and therefore pharmacological treatments aimed at liver disease should be reserved for patients with biopsy-proven NASH and fibrosis. (79).

Historically, with the two-hit hypothesis of NASH pathophysiology (i.e., first-hit to be insulin resistance, and oxidative stress as a second hit) (67), it is not surprising that the first drug tested for NASH aimed at exactly both hits, insulin resistance and oxidative stress (86). Indeed, the antioxidant activity of vitamin E was demonstrated to be able to normalize the levels of liver enzymes in NAFLD patients, as well as improved histologic hallmarks of NASH (i.e., steatosis, inflammation, and hepatocyte ballooning) in biopsy-proven NASH (86, 87). Since then, further studies have been published to compare the effects of vitamin E to placebo and assess the association between vitamin E and antidiabetic drugs, such as pioglitazone and metformin (88, 89). Pioglitazone is a thiazolidinedione derivative and a peroxisome proliferator-activated receptor γ (PPARγ) agonist that improves glucose and lipid metabolism, as well as insulin resistance in type 2 diabetes mellitus (90). Moreover, a recent meta-analysis has suggested that pioglitazone treatment can lead to the improvement of liver histology and plasma liver enzymes (AST and ALT) in patients with prediabetes or type 2 diabetes mellitus combined with NAFLD (91). Metformin, a liver-specific insulin sensitizer, is the first-line drug for type 2 diabetes mellitus management in most clinical practice guidelines; however, the direct mechanism of action is still not fully understood (92).

Even though current FDA guidelines still recommend administration of vitamin E (daily dose of 800 IU/day) in nondiabetic adults with biopsy-proven NASH, as well as pioglitazone administration to patients with and without diabetes with biopsy-proven NASH due to improvements in liver histology (79); the European Association for the Study of the Liver (EASL) guidelines allow their use (even in combination), but without a firm recommendation, due to the concerns about the long-term safety of both drugs (78). Although some studies have shown an improvement in serum aminotransferases and insulin resistance after metformin administration, this drug has not shown improvement in liver histology, which is the reason why it is not recommended for NASH treatment in either of the guidelines (78, 79).

Another important concern in NASH patients is their pro-atherogenic lipid profile and the increased risk of life-threatening cardiovascular events (62, 63). Notwithstanding claiming that statins may not have direct benefits on liver disease, EASL and FDA guidelines still recommend that they may be used to reduce LDL cholesterol since they are generally considered safe and also promote a greater benefit in the prevention of cardiovascular morbidity and mortality (78, 79, 93).

### 1.3.1 Statins

As previously mentioned, NAFLD is known as the hepatic manifestation of metabolic syndrome, where NAFLD patients usually present at least three out of the five conditions (56, 94). The metabolic syndrome is defined as a cluster of conditions that usually occur simultaneously: arterial hypertension, hyperglycemia, abdominal obesity-related insulin resistance with hyperglycemia, and alterations in lipoprotein metabolism such as hypercholesterolemia (elevated LDL but low HDL cholesterol) and hyperlipidemia (increased circulating levels of TAG) (94). The increased risk of life-threatening cardiovascular events is a major issue in NAFLD patients due to their pro-atherogenic lipid profile (62, 63). These include atherosclerosis, myocardial infarction (by obstruction of coronary arteries), as well as stroke (95). Indeed, high circulating and/or intracellular levels of cholesterol are toxic and are associated with adverse effects such as oxidative damage, impaired function of signaling proteins, and cardiovascular diseases. Thus, it is not surprising that antihyperlipidemic therapies are recommended in the management of NAFLD and the prevention of cardiovascular events (78, 79).

Interestingly, when it comes to the management of cardiovascular disease, according to the new guidelines from the European Society of Cardiology published in 2021, the current lipid-lowering drugs range beyond the direct inhibitors of cholesterol biosynthesis (HMGCR inhibitors, i.e., statins), also including fibrates, selective cholesterol absorption inhibitors (e.g., ezetimibe), bile acid sequestrants, and PCSK9 inhibitors (96). However, statins are still the first-line treatment for the primary prevention of atherosclerotic cardiovascular disease in patients with elevated LDL cholesterol levels (according to the guidelines for Primary Prevention of Cardiovascular Disease from the American College of Cardiology and American Heart Association) (97).

Results from clinical trials indicate that treatment with statins and other lipid-lowering drugs results in significant reductions in major vascular events (98). However, recent data suggests that statins may not only be beneficial due to their cholesterol-lowering effects but

that they may also have cholesterol-independent effects (pleiotropic effects) (99). Examples of these beneficial pleiotropic effects are the improvement of endothelial function and attenuation of vascular remodeling, as well as inhibition of the inflammatory response of vessels and stabilization of atherosclerotic plaque (100).

Even though statins are generally well-tolerated, there is a possibility that adverse effects may occur in some patients. The most frequent adverse effect is myopathy; however, some patients may develop a more severe form of rhabdomyolysis. Less common adverse effects include hepatotoxicity, peripheral neuropathy, and impaired myocardial contractility (101). Notwithstanding the possibility of hepatoxicity promoted by statins, they are still considered a safe approach even in NAFLD patients (102, 103).

In the NAFLD/NASH management guidelines, statins are recommended as a preventive measure of cardiovascular-related events in these patients, despite the possibility that they may not have direct benefits on liver disease (78, 79). Even though the use of statins in liver disease has increased over time, statins are persistently underutilized in individuals with NAFLD/NASH (104). Whereas improving statin utilization in NAFLD is primarily important for the cardiovascular protection of NAFLD/NASH patients, recent evidence has suggested that in addition to lipid-lowering properties, the pleiotropic effects of statins may also be beneficial for a reduction of NAFLD/NASH-associated hepatic lipotoxicity, oxidative stress, inflammatory responses, and fibrosis (104, 105).

Atorvastatin and rosuvastatin are mainly used in NAFLD/NASH; therefore, the data with other statins are limited (106, 107). Indeed, studies have reported that in addition to the lipid-lowering properties, atorvastatin was found to significantly decrease levels of hepatic damage enzymes (ALT, AST, ALP, γGT) as well as NASH hepatic features such as steatosis, inflammation, and oxidative stress (108-110). Other animal studies have reported that also simvastatin treatment leads to improvement in hepatic damage enzymes, NASH hepatic features such as ballooning, hepatic lipid deposition, and decreased leukocyte recruitment to hepatic and fat microcirculation, and improved hepatic capillary network (111, 112). Simvastatin has also been associated with the activation of antioxidant enzymes and decreased lipid peroxidation, as well as decreased hepatic stellate cell activation (111, 113).

Despite the established statins' benefits in cardiovascular prevention and recently demonstrated benefits in direct alleviation of liver impairment, the inclusion of statins as a treatment for NAFLD/NASH in the recommendation guidelines may require further confirmation by larger clinical trials (114).

### 1.3.2 Future approaches in the therapy of NASH

As previously described, NASH results from the accumulation of fat in the liver (steatosis), which induces hepatocyte stress and apoptosis, with inflammatory infiltrates and progressive fibrogenic remodeling, which can ultimately lead to cirrhosis. Unsurprisingly, the emerging therapies with the intent to treat NASH currently under development aim to target the hepatic mechanisms underlying these elements, such as targeting lipid and carbohydrate metabolism, lipotoxic hepatocyte injury with inflammation, and reversal of fibrosis (115). In addition to liver targets, also gut-liver axis has become a desirable target, such as aiming to alter the gut microbiome or to modulate BA enterohepatic circulation and signaling. Indeed, BA are also known to regulate lipid and glucose homeostasis and promote insulin sensitivity (116, 117).

Despite the suboptimal results obtained in previous clinical trials, NAFLD/NASH is still a very fast-moving field of research in hepatology, with plenty of clinical trials currently investigating potentially effective drugs. Indeed, as of May 2022, there are around 200 active and/or recruiting NASH-related clinical trials, of which 16 are currently in phase III, according to the website ClinicalTrials.gov (118).

Obeticholic acid is a semi-synthetic bile acid analogue and a potent and selective FXR agonist that modulates bile acid metabolism (117). An ongoing phase 3 REGENERATE trial with obeticholic acid was intended to confirm the improvement in histologic NASH features by modulation of lipid metabolism, improvement of insulin sensitivity, and potential reduction of liver fibrosis observed in the successful phase 2 FLINT trial (119, 120). The results from the FLINT trial include the histological improvement of steatosis, lobular inflammation, and hepatocellular ballooning, as well as improvement of fibrosis by at least one stage (121). However, despite the seemingly promising results, obeticholic acid treatment was linked to the development of dyslipidemia and pruritus in patients (122, 123). These side effects raised awareness not only of the possibility of patient's lack of compliance due to the impaired quality of life but also safety concerns for long-term treatment.

Another example of a promising drug under the phase 2 GOLDEN-505 trial is elafibranor, a dual peroxisome proliferator-activated receptor-alpha, and delta (PPARα–PPARδ) agonist. PPARα being responsible for lipid oxidation, thus predicting a reduction in steatosis, and PPARδ targeting inflammatory activity in macrophages and Kupffer cells, therefore predicted to reduce the inflammation. However, the phase 3 (RESOLVE-IT) trial was terminated early because the study did not meet the predefined primary efficacy endpoint; however, no safety concerns were reported (124). Another PPAR agonist, Lanifibranor is able to modulate key metabolic, inflammatory, and fibrogenic pathways in NASH pathogenesis.

After its successful phase 2 clinical trial that resulted in reduced ballooning and inflammation without worsening of fibrosis (125), it is now in phase 3 NATiV3 trial (118). NATiV3 trial started in August 2021, is still recruiting, and aims to evaluate the long-term efficacy and safety of lanifibranor in adult patients with different fibrotic stages of liver fibrosis in NASH. Its primary outcome, the resolution of NASH and improvement of fibrosis, is predicted to be available by February 2024 (126).

In a similar manner to how statins are used in the management of hypercholesterolemia due to their capacity to inhibit cholesterol *de novo* synthesis, new drugs aim to reduce steatosis by inhibiting enzymes from *de novo* lipogenesis, namely Aramchol, a partial inhibitor of hepatic SCD1, and Firsocostat (GS-0976), an inhibitor of ACC (127). Aramchol was considered safe and well-tolerated in phase 2; however, despite the study did not meet the significance level for the primary end point of reducing liver fat, the observed safety and promising changes in liver histology and enzymes provided the rationale that inhibition of SCD1 may still be a promising therapy for NASH and fibrosis, thus is currently being evaluated in an ongoing phase 3 program (126, 128). The phase 3 ARMOR study started in September 2019 and is still recruiting patients. The aimed primary outcome is to reduce fibrosis without worsening steatohepatitis and to resolve NASH without worsening fibrosis, and it is predicted to be achieved by December 2024 (126). Phase 2 clinical trial with firsocostat (GS-0976) administration to NASH patients resulted in a 30% or greater reduction in hepatic steatosis in 50% of the patients, which was associated with histologic improvements (129). Additionally, results reported improvement in selected markers of fibrosis and liver biochemistry (129). Although the treatment was considered safe and well-tolerated, 16% of the patients presented asymptomatic and dose-independent hypertriglyceridemia that was treated by fibrate administration (129). Despite the results of the study being announced in 2018, to the date, there is no ongoing phase 3 trial (118).

The new evidence from the ongoing clinical trials is expected to change the current status of knowledge and hopefully find suitable therapeutic agents for NASH treatment and their possible use in combination therapy.

## 1.4 ENDOGLIN AND SOLUBLE ENDOGLIN

### 1.4.1 Structure, function, and signaling

Endoglin (Eng, CD105) is a type I integral membrane glycoprotein and part of the Transforming growth factor-β (TGFβ) receptor complex. Eng is a 180kDa homodimer with an

extracellular region of 561 amino acids, a hydrophobic transmembrane domain, and a 47-residue serine/threonine-rich cytoplasmic tail (**Figure 7**) (130). Eng extracellular part is composed of an orphan domain and the zona pellucida (ZP), containing an RGD tripeptide (aa399-401, arginyl-glycyl-aspartic acid) (131). Eng's RGD domain was the first identified on a surface protein of endothelium, RGD is a fundamental recognition structure that suggests a key role for Eng in the binding of integrins and/or other RGD receptors (130, 131). Upon its discovery, Eng's distribution was thought to be restricted to endothelial cells of the vasculature (i.e., capillaries, arterioles, and venules) of a variety of tissues, thus considered as a specific marker of the endothelium (130). So, at the Fifth edition of the International Workshop on Human Leukocyte Differentiation Antigen (HLDA), during the classification of molecules into clusters of differentiation, Eng was assigned as the Cluster Differentiation 105 (CD105) (132). Curiously, Eng was one of the first three endothelial clusters of differentiation to be assigned, together with CD106 (VCAM-1) and CDw109 (132, 133).

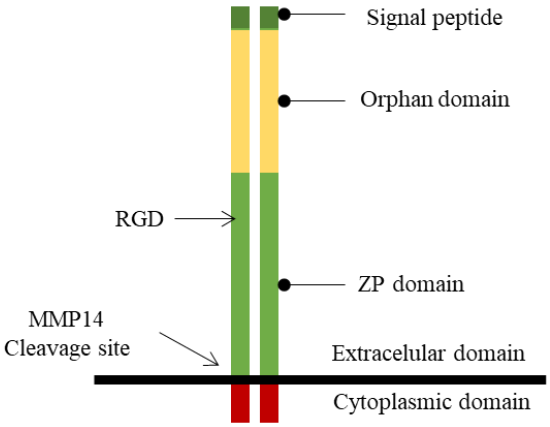


**Figure 7.** Schematic representation of endoglin (Eng). Eng is composed of a cytoplasmic and an extracellular domain. The extracellular domain is composed of a Zona pellucida (ZP) domain with an RGD motive, an orphan domain, and a signal peptide. Eng's extracellular domain can be cleaved by matrix metalloproteinase-14 (MMP14) at the interface between the extracellular domain and the cellular membrane.

According to its cytoplasmic domain, Eng can be classified into two different isoforms (i.e., alternative splicing variants), namely long Eng (L-Eng), the isoform containing 47 amino acid cytoplasmic tail discovered by Gougos and Letarte (130), or a short Eng (S-Eng), which cytoplasmic domain is composed by only 14 amino acid tail (134). It has been reported that both variants may interact with the TGFβ complex, however, they may promote distinct signaling pathways. L-Eng, the predominant form, has been reported to act as a co-receptor of TGFβ by interaction with ALK1, meanwhile, S-Eng interacts with ALK5 (135). However,

since L-Eng is the predominant form, and due to the lack of knowledge of the specific functions of S-Eng, for the purpose of this dissertation, we will refer to the membrane-bound protein as Eng. Thus, we can say that Eng acts as a co-receptor of the TFG-β complex, mediating the signaling with ALK1 and ALK5 and regulating critical aspects of cellular and biological responses via Smad phosphorylated-activation pathways (**Figure 8**). Two distinct Smad pathways can be activated, either the ALK1-Smad1/5/8-ID1 or ALK5-Smad2/3-PAI1. Association with Smad4 and subsequent translocation of Smad complex into the nucleus modulates transcription of different genes, which results in biological responses such as activation of endothelial cells, endothelial cell proliferation, migration, and angiogenesis (i.e., ALK1-Smad1/5/8) or endothelial cell senescence (i.e., ALK5-Smad2/3) (136-138).

In addition to the membrane-anchored form of Eng, a soluble form of Eng (sEng, sCD105) can be found in the bloodstream or cell culture media (139). sEng is a result of proteolytic cleavage of Eng's extracellular domain by the action of matrix metalloproteinase-14 (MMP14) (140). sEng has been recognized as a biomarker and an aggravation factor for preeclampsia, and it is also involved in other pathologies (141-144).

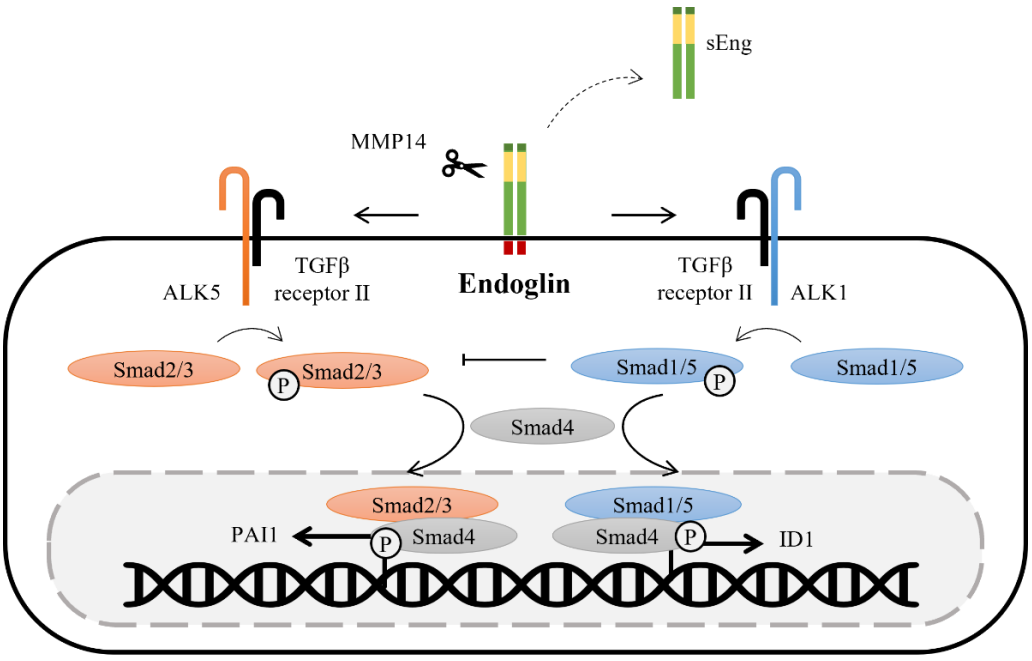


**Figure 8.** Endoglin (Eng) and soluble endoglin (sEng) signaling pathway. Eng as a TGFβ co-receptor mediated the activation of two distinct Smad pathways, either the ALK1-Smad1/5/8 or ALK5-Smad2/3. Association of phospho-Smad with Smad4 and subsequent translocation of Smad complex into the nucleus results in transcription of different genes, such as PAI1 and ID1, respectively. Cleavage of Eng via MMP14 results in the release of sEng in circulation. TGFβ, Transforming growth factor-β; MMP14, Matrix metalloproteinase-14; ID1, Inhibitor of DNA binding 1; PAI1, Plasminogen activator inhibitor type 1.

### 1.4.2 Endoglin role in liver disorders

Nowadays, there are more than 3000 published works related to Eng, however, to this date, only about 200 of them are related to the hepatic environment, according to the Pubmed database. In the liver, Eng is expressed in the plasma membrane of liver sinusoid endothelial cells, fibroblasts, hepatic stellate cells, and Kupfer cells, but not in hepatocytes (145-147).

Eng is known to play an important role in fibrosis development in the liver (148, 149). Eng expression was upregulated during activation of hepatic stellate cells and their transdiferentiation myofibroblast-like cells, in cell culture and in experimental models of liver injury in rodents ($CCl_4$ application or bile duct ligation) (146, 150). Moreover, increased expression of Eng was also reported in patients with hepatitis C virus infection with advanced fibrosis, compared to patients with early fibrosis and normal liver, as well as in patients with liver disease and hepatocellular carcinoma (151-156). Recently Meurer et al. demonstrated that exosomes isolated from primary hepatic cells contain full-length Eng. These particles participate in intercellular communication and may contribute to hepatic angiogenesis and the progression of liver fibrosis (147).

Although these studies suggest a profibrotic effect of endoglin, it is still debated whether endoglin stimulates or inhibits fibrosis, and studies have been conducted that support both hypotheses. Alsamman et al. suggested Eng as protective against fibrotic injury when found in two different murine models that deficiency of Eng significantly aggravates liver fibrosis in response to liver injury by modulating TGFβ/Smad signaling in the liver (157).

Recently, About et al. suggested that this conflict of results observed in the role of Eng in fibrosis may lie in the contribution of L- or S-Eng variants and that the differences in the cytoplasmic domain may be responsible for the different effects (158).

Interestingly, despite the limited knowledge and contradictory results regarding Eng regulation and expression concerning hepatic functions and pathology, most available studies have been focused on hepatic stellate cells and liver fibrosis (146, 148, 149). There was just one study that reported increased hepatic expression of Eng during NASH development in human patients (mainly on sinusoidal endothelial cells and fibroblast-like cells located in portal tracts), which significantly correlated with the presence of fibrosis (159).

Regarding Eng's role in liver metabolism, only one study is available. Beira et al. reported decreased hepatic triglyceride content and lower insulin levels in heterozygous deficiency in Eng expression (Eng+/-) mice compared with wild-type mice, without changes in body weight and adiposity (160), suggesting a potential role of Eng in hepatic lipid content, without exploring the detailed mechanism of this effect.

### 1.4.3 Soluble endoglin role in liver disorders

When the extracellular domain of Eng is cleaved by a metalloproteinase (i.e., MMP14), its extracellular domain named, sEng, is released into the circulation (140). Increased levels of sEng in circulation have been reported in patients with pathologies related to metabolic syndrome (i.e., hypercholesterolemia, type II diabetes mellitus, and arterial hypertension) (161-163). Increased levels of sEng were also found in patients with liver diseases (153), namely chronic hepatitis C (151), biliary atresia (164), cystic fibrosis associated with liver disease (165), liver fibrosis, and hepatic carcinoma (156, 166).

Even though a limited number of studies related to circulating levels of sEng in hepatic disorders are currently available, all published studies have reported an association between increased sEng levels and the progression of liver damage. High sEng levels were linked with poor outcomes for biliary atresia (164). In cystic fibrosis associated with liver disease, higher levels of sEng were observed in patients with cirrhosis when compared to patients with liver fibrosis but without cirrhosis (165). In patients with chronic hepatitis C and advanced hepatic fibrosis, upregulated intrahepatic expression of both Eng and sEng showed a significant correlation with histologic and serum biomarkers of hepatic fibrosis (156). Moreover, among patients with liver disease (with varying degrees of severity and etiology), levels of sEng were increased with the progression of the disease from fibrosis to cirrhosis, and patients with the most advanced cirrhosis presented the highest levels of sEng.(153).

Regarding sEng's role in liver metabolism, there was no published data prior to this dissertation.

# 2 HYPOTHESIS AND AIMS OF THE DOCTORAL THESIS

Given the knowledge that increased levels of soluble endoglin in circulation have been reported in patients with pathologies linked to metabolic syndrome accompanied with hypercholesterolemia, as well as detected in the circulation of patients with varying severity degree and etiology of liver diseases. It was interesting that the role of sEng in the hepatic metabolism of cholesterol or bile acids was unknown.

This dissertation proposed to test the hypothesis that soluble endoglin plays a role in the modulation of cholesterol and bile acid metabolism in healthy and pathologic conditions, and its levels in circulation may be modulated by pharmacotherapy.

To test this hypothesis, we used four synergistic yet independent, specific aims:

1. Explore the role of soluble endoglin in the **metabolism of cholesterol and bile acids in the liver and modulation of entero-hepatic circulation in healthy mice** by using a chow-fed transgenic mouse model with high expression of human soluble endoglin in plasma.

2. Study soluble endoglin's effect on the **modulation of cholesterol, bile acids, and triacylglycerols metabolism under liver impairment** by using a diet-induced NASH mouse model with high fidelity to human NASH in transgenic male mice overexpressing human soluble endoglin and their wild-type littermates.

3. Evaluate whether **pharmacologic treatment of liver damage would modulate sEng levels in circulation** by administration of atorvastatin to a NASH mouse model induced by high fat, high fructose, and high cholesterol diet (FFC diet).

4. Analysis of current literature on the role of membrane-bound and soluble endoglin in conditions related to metabolic syndrome and liver impairment.

# 3 LIST OF PUBLICATIONS INCLUDED IN THE DOCTORAL THESIS

The aims and hypothesis for this doctoral thesis were achieved, and the outcomes were published in peer-reviewed journals with impact factor. Therefore, this dissertation is conceived as an annotated collection of works comprising two first-authorship original (experimental) articles, as well as two co-authorship articles (one experimental and one literature review) published in journals with impact factor.

**1.** Dolezelova E*, **Igreja Sa IC***, Prasnicka A, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Cermanova J, Pericacho M, Visek J, Lasticova M, Micuda S, Nachtigal P. **High soluble endoglin levels regulate cholesterol homeostasis and bile acids turnover in the liver of transgenic mice.** Life Sci. 2019 Sep 1; 232:116643. [IF=3.647] (Q2); [AIS=0.674] (Q2) * designates shared co-first authorship

**2. Igreja Sa IC**, Tripska K, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Schreiberova J, Dolezelova E, Eissazadeh S, Vitverova B, Najmanova I, Vasinova M, Pericacho M, Micuda S, Nachtigal P. **Soluble endoglin as a potential biomarker of nonalcoholic steatohepatitis (NASH) development, participating in aggravation of NASH-related changes in mouse liver**. Int J Mol Sci. 2020 Nov 27;21(23):9021. [IF=5.924] (Q1); [AIS=1.123] (Q2)

**3.** Lastuvkova H, Faradonbeh FA, Schreiberova J, Hroch M, Mokry J, Faistova H, Nova Z, Hyspler R**, Igreja Sa IC**, Nachtigal P, Stefela A, Pavek P, Micuda S. **Atorvastatin modulates bile acid homeostasis in mice with diet-induced nonalcoholic steatohepatitis**. Int J Mol Sci. 2021 Jun 16;22(12):6468. [IF=6.208] (Q1); [AIS=1.064] (Q2)

**4**. Vicen M, **Igreja Sa IC**, Tripska K, Vitverova B, Najmanova I, Eissazadeh S, Micuda S, Nachtigal P. **Membrane and soluble endoglin role in cardiovascular and metabolic disorders related to metabolic syndrome.** Cell Mol Life Sci. 2021 Mar;78(6):2405-2418. [IF=9.207] (Q1); [AIS=2.069] (Q1)

# 4 COMMENTARY ON PUBLISHED WORKS

## 4.1 HIGH SOLUBLE ENDOGLIN LEVELS REGULATE CHOLESTEROL HOMEOSTASIS AND BILE ACIDS TURNOVER IN THE LIVER OF TRANSGENIC MICE

Dolezelova E*, **Igreja Sa IC***, Prasnicka A, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Cermanova J, Pericacho M, Visek J, Lasticova M, Micuda S, Nachtigal P. Life Sci. 2019 Sep 1; 232:116643. DOI: 10.1016/j.lfs.2019.116643. PMID: 31299237. [IF=3.647] (Q2); [AIS=0.674] (Q2) * designates shared co-first authorship

In this paper, we elucidated for the first time that increased levels of sEng in circulation are able to modulate cholesterol and BA metabolism in healthy mice via alterations in hepatic and intestinal homeostasis.

Transgenic mice overexpressing human sEng and their wild-type littermates (without detectable circulating levels of human sEng) were used in this study in order to evaluate the sEng-mediated alterations in hepatic enzymes and transporters involved in cholesterol and BA metabolism, as well as ileal BA transporters involved in the entero-hepatic turnover of BA. Human sEng and mouse sEng have a high degree of homology (167, 168). Indeed, previous experiments have shown that human sEng is able to modulate mouse Eng signaling pathway *in vivo* (162, 169-171).

Mice overexpressing human sEng presented reduced concentration of total cholesterol (mostly by reduction of LDL cholesterol) in plasma but increased concentration of cholesterol in the liver. Evaluation of the hepatic metabolic pathway of cholesterol revealed that this effect was due to the upregulation of basolateral influx transporters of cholesterol from plasma to the hepatocytes (i.e., Ldl receptor and Sr-b1). Increased cholesterol content in the liver leads to upregulation of its elimination pathways, namely upregulation of Abcg8, the canalicular efflux transporter of cholesterol to bile, which resulted in increased biliary elimination of cholesterol. In addition to this elimination pathway, sEng also leads to the increased conversion of cholesterol into BA via upregulation of Cyp7a1, the first and rate-limiting enzyme of the *de novo* synthesis of BA. Increased hepatic availability of BA resulted in increased biliary elimination of BA associated with choleretic activity (i.e., BA-dependent bile flow). However, sEng mice presented reduced fecal elimination of BA, and combination with increased ileal reabsorption of BA led to increased plasma concentration of BA.

Confirmation of sEng influence on cholesterol and BA metabolism raised the question about the significance of these findings under pathologic conditions associated with hypercholesterolemia and/or BA metabolism dysregulation and increased levels of sEng.

## 4.2 SOLUBLE ENDOGLIN AS A POTENTIAL BIOMARKER OF NONALCOHOLIC STEATOHEPATITIS (NASH) DEVELOPMENT, PARTICIPATING IN AGGRAVATION OF NASH-RELATED CHANGES IN MOUSE LIVER

**Igreja Sa IC**, Tripska K, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Schreiberova J, Dolezelova E, Eissazadeh S, Vitverova B, Najmanova I, Vasinova M, Pericacho M, Micuda S, Nachtigal P. Int J Mol Sci. 2020 Nov 27;21(23):9021. DOI: 10.3390/ijms21239021. PMID: 33261044. [IF=5.924] (Q1); [AIS=1.123] (Q2)

In this paper, we aimed to answer the questions raised in the second aim of this dissertation, namely, if increased sEng levels can affect cholesterol, BA, and TAG metabolism in a diet-induced mouse model of NASH, with high fidelity to hallmarks of human NASH.

Firstly, we reported upregulated expression of membrane Eng in NASH, which was in agreement with a previous study that reported increased Eng expression by immunohistochemistry analysis of human NASH liver biopsies (159). Additionally, for the first time to our knowledge, we demonstrated increased levels of sEng in NASH. Increased sEng levels were found to be associated with upregulated expression of both Eng and MMP14, the enzyme mediating cleavage of Eng into sEng.

Regarding sEng involvement in cholesterol and BA metabolism in a NASH mouse model, in this study, we evaluated protein expression of transporters and enzymes involved in cholesterol and BA metabolism in the liver of transgenic mice overexpressing human sEng and their wild-type littermates. Transgenic mice with high circulating levels of human sEng presented increased hepatic concentration of cholesterol and reduced biliary elimination of BA due to the reduced *de novo* synthesis of BA. Downregulation of Cyp7a1, the enzyme responsible for conversion of cholesterol into BA, resulted in the increased hepatic deposition of cholesterol because of the impaired elimination of cholesterol via conversion into BA. Additionally, overexpression of sEng resulted in increased hepatic accumulation of TAG, via reduced beta-oxidation of fatty acids, in combination with reduced hepatic efflux of TAG into plasma. Even though the only difference between the experimental groups was the presence of high plasma levels of sEng, we were not able to elucidate the direct mechanism of how sEng modulates these pathways.

This paper points out the possibility of sEng being a biomarker of NASH and that sEng may have a role in the impairment of essential mechanisms against cholesterol and TAG accumulation. Thus, we suggest that the determination of sEng levels is important, especially in patients prone to the development of NASH.

## 4.3 ATORVASTATIN MODULATES BILE ACID HOMEOSTASIS IN MICE WITH DIET-INDUCED NONALCOHOLIC STEATOHEPATITIS

Lastuvkova H, Faradonbeh FA, Schreiberova J, Hroch M, Mokry J, Faistova H, Nova Z, Hyspler R, **Igreja Sa IC**, Nachtigal P, Stefela A, Pavek P, Micuda S. Int J Mol Sci. 2021 Jun 16;22(12):6468. DOI: 10.3390/ijms22126468. PMID: 34208774; PMCID: PMC8235314. [IF=6.208] (Q1); [AIS=1.064] (Q2)

The present study aimed to investigate the effect of atorvastatin in bile acid homeostasis in mice with diet-induced nonalcoholic steatohepatitis. The intent of our contribution to this study was to explore whether pharmacologic treatment of liver damage modulates sEng levels in circulation.

In this study, 2-months-old wild-type mice were fed with a standard chow diet (control group) and tap water *ad libitum* or FFC diet with added glucose and fructose to drinking water for 21 weeks (NASH-induced group). On week 21, the mice were assigned to treatment groups, atorvastatin (20 mg/kg), or vehicle were administered once daily by oral gavage. During the three weeks of pharmacologic treatment, the mice continued being fed with the respective diets.

Induction of NASH in mice resulted in increased circulating levels of sEng in comparison with the healthy mice control group, which was in accordance with our previous publication (172). However, when it comes to atorvastatin modulation of sEng levels, there was no statistically significant difference in the circulating levels of sEng when mice were treated with atorvastatin or vehicle for 3 weeks.

Although the therapeutic effect of atorvastatin in NASH was demonstrated by the significant reduction in plasma levels of ALT and improvement of histologic features of NASH, such as hepatic steatosis and inflammation, atorvastatin treatment did not improve fibrosis in this study. By comparison with studies previously published where atorvastatin treatment was administered for 2 months, the authors anticipated that the short length of the atorvastatin treatment in this study (3 weeks) might be the reason why the fibrosis effect was not visible.

These results lead us to question whether sEng levels were also not significantly altered due to the length of the treatment and whether they would reflect greater improvement in liver function by the long-term pharmacologic treatment of NASH.

## 4.4 MEMBRANE AND SOLUBLE ENDOGLIN ROLE IN CARDIOVASCULAR AND METABOLIC DISORDERS RELATED TO METABOLIC SYNDROME

Vicen M, **Igreja Sa IC**, Tripska K, Vitverova B, Najmanova I, Eissazadeh S, Micuda S, Nachtigal P. Cell Mol Life Sci. 2021 Mar;78(6):2405-2418. doi: 10.1007/s00018-020-03701-w. PMID: 33185696. [IF=9.207] (Q1); [AIS=2.069] (Q1)

In this review, we describe the status of knowledge available in the literature regarding the role of membrane-bound and soluble endoglin in cardiovascular and metabolic disorders related to metabolic syndrome reported by *in vivo* and *in vitro* studies published in original articles. We compiled the information regarding Eng and sEng structure and signaling pathway, as well as information screening through conditions such as hypercholesterolemia, endothelial dysfunction, inflammation, hyperglycemia, arterial hypertension, as well as liver alteration, and obesity-related to metabolic syndrome.

Eng expression in endothelium may function as an adhesion molecule and has been reported to be associated with the transmigration of leukocytes as a defense mechanism in response to acute inflammation. Additionally, currently available literature also reports that downregulation of Eng expression promotes endothelial permeability and impairment of the barrier function, which are common features present during the development of endothelial dysfunction in chronic conditions related to metabolic syndrome. However, outside of the endothelial scope, upregulation of Eng expression was linked to hepatic stellate cell activation and liver fibrosis. These seemingly contradictory results suggest the possibility of an equilibrium of Eng expression in the different organs and cell types to assure the proper function of the organism.

Regarding sEng, increased levels have been reported in patients with preeclampsia and in patients with metabolic syndrome-related conditions such as hypercholesterolemia, arterial hypertension, hyperglycemia, and type II diabetes mellitus. Additionally, sEng has also been linked to induce arterial hypertension, to participate in the development of endothelial dysfunction, and to play a role in hepatic cholesterol and bile acids metabolism. This suggests that high levels of sEng are not only present as they can support metabolic syndrome symptoms and complications.

Thus, in conclusion, we may claim that there is a potential association between Eng expression and sEng levels in conditions related to metabolic syndrome and liver impairment, and therefore we should take into account sEng levels in the circulation of patients prone to develop those conditions.

# 5 DISCUSSION

Increased circulating levels of sEng have been reported in patients with pathologies linked to metabolic syndrome accompanied by hypercholesterolemia (162, 163), as well as in patients with varying severity degrees and etiology of liver diseases (151, 153, 156, 164-166). The liver is the major metabolic organ in the organism and the responsible one for cholesterol and bile acid *de novo* synthesis (20, 21, 24, 25). However, it was unknown whether sEng plays a role in the modulation of cholesterol and bile acid metabolism in healthy and pathologic conditions and if pharmacologic treatment of the pathology results in the reduction of sEng levels in circulation.

In the first aim of this dissertation, we elucidated for the first time that high sEng levels in plasma results in a mild reduction of cholesterol in circulation by overexpression of hepatic influx transporters of cholesterol (i.e., Ldlr and Sr-b1) but increased hepatic deposition of cholesterol (173). Additionally, mice overexpressing human sEng presented increased conversion of cholesterol into BA in association with increased choleretic effect when compared to wild-type controls (173). Moreover, mice with high levels of sEng in their plasma also presented increased bile acid levels in circulation due to increased enteric reabsorption of BA into the circulation (173). These data suggested the potential role of sEng in bile acid and cholesterol liver metabolism.

Thus, we asked the question of whether sEng levels would modulate the cholesterol and BA metabolism under liver impairment. For that, we used a diet-induced NASH mouse model that closely phenocopies human NASH and metabolic syndrome (174-176). In this study, we reported for the first time that the NASH-induced group of mice presented increased levels of sEng in the circulation when compared to healthy controls. Increased levels of sEng were associated with increased hepatic expression of membrane-bound Eng and overexpression of MMP14, the cleavage enzyme responsible for cleavage of the extracellular domain of Eng and release sEng. Overexpression of Eng was in agreement with the results published from immunohistochemistry analysis of NASH-diagnosed human biopsies (159). To evaluate the sEng modulatory effect on the cholesterol, BA, and TAG metabolism, we induced NASH in transgenic mice overexpressing human sEng and their wild-type littermates. Mice with increased levels of sEng presented increased hepatic concentration of cholesterol due to impaired conversion of cholesterol into BA when compared to wild-type mice. In addition, we also observed increased hepatic concentration of TAG in sEng mice when compared to their wild-type littermates. This was due to reduced fatty acid oxidation and reduced assembly of VLDL-TAG via reduced expression of ApoB and Mttp. These results suggest that sEng may be not only considered as a biomarker of NASH, as increased sEng levels in plasma promote

aggravation of liver impairment. These data are in line with the potential importance of sEng in being a biomarker and inducer of preeclampsia and endothelial dysfunction (141, 144, 177, 178).

Given the increased sEng levels in the NASH group and the possibility of sEng being a biomarker of liver impairment, we hypothesized that pharmacologic treatment of liver damage would reduce sEng levels in circulation. For that, we administrated atorvastatin (daily p.o. for 3 weeks) to a NASH mouse model. Despite the fact that we observed, once more, increased sEng levels in the NASH group when compared to healthy controls, we did not find a statistically significant difference in the sEng levels in plasma atorvastatin-treated when compared to untreated mice (179). Interestingly, a study using an atherosclerosis mouse model treated with atorvastatin (50 mg/kg/day) was added to the diet for 2 months, resulting in a significant reduction in sEng levels in plasma (180). In agreement with this study, our pilot data have shown a reduction of sEng levels with a 2-month atorvastatin treatment of a mouse model of NASH (data not published). While the elevation of sEng levels is associated with liver damage, the slight improvement in liver impairment may not be substantial to result in a significant reduction of circulating sEng levels. Thus, we may not exclude the possibility of sEng levels being reduced among a greater improvement of the hepatic function.

Analysis of current literature on the role of Eng and sEng in conditions related to metabolic syndrome and liver impairment highlighted the lack of specific knowledge in the field and the need for further studies in the matter. Regarding the liver environment, increased Eng expression has been reported in patients with liver disease associated with fibrosis (151-156, 159). Indeed, Eng is known to play an important role in fibrosis development in the liver (148, 149). For example, upregulation of Eng is observed during hepatic stellate cells activation (146, 150). Although these studies suggest a profibrotic effect of endoglin, Alsamman et al. suggested Eng as protective against fibrotic injury when found in two different murine models where deficiency of Eng significantly aggravates liver fibrosis (157). Interestingly, Eng has been found downregulated in liver biopsy of patients with alcoholic cirrhosis (181), as well as during cholestatic liver disease in two distinct mouse models (unpublished data).

With this, we may speculate that different stimuli may be involved in Eng up/downregulation, and that the balanced Eng expression may be still involved in the anti/pro-fibrotic changes in the liver, or that Eng up- or down-regulated expression by different cells type within the liver may be involved in the different pathologic conditions. Even though Eng is not expressed by hepatocytes, Meurer et al. demonstrated that all liver cells have the capacity to direct Eng to exosomes independent of if they express endogenous Eng (including

hepatocytes) (147). For example, exosomes isolated from primary hepatic cells contain full-length Eng, and these particles may participate in the intercellular communication contributing to hepatic angiogenesis and the progression of liver fibrosis (147). Thus, to establish whether this reasoning/hypothesis is true or not, further studies would need to be performed where differential isolation of the different cell types present in the hepatic tissue would be required for accurate analysis of Eng expression as well as cell type-specific dysfunction.

Increased levels of sEng in circulation have been reported in patients with pathologies related to metabolic syndrome (i.e., hypercholesterolemia, type II diabetes mellitus, and arterial hypertension) (161-163). Increased levels of sEng were also found in patients with liver diseases (153), namely chronic hepatitis C (151), biliary atresia (164), cystic fibrosis associated with liver disease (165), liver fibrosis, and hepatic carcinoma (156, 166). Moreover, in this dissertation, we have shown, for the first time, the increase in sEng levels in the NASH mouse model (172). Additionally, sEng is also increased in a mouse model of cholestatic liver disease and in pregnant patients diagnosed with intrahepatic cholestasis (manuscript under revision). Given the knowledge that increased sEng levels have the capacity to modulate cholesterol and BA metabolism, as well as sabotage the essential defensive mechanism protecting against liver impairment. We may suggest that elevated levels of sEng especially combined with other risk factors of metabolic syndrome, may represent a marker of vascular injury or the progressive increase of hepatic decompensation. Even though increased sEng levels may correspond to the shedding of hepatic membrane-anchored Eng, and that is a known fact that endothelial cells can shed sEng via cleavage by MMP14, we cannot say that this alone is the sole source of sEng in circulation, once sEng shedding by other Eng expressing cell types or in other tissues has not been assessed.

# 6 CONCLUSION AND FUTURE DIRECTION IN RESEARCH

In conclusion, the collection of papers of this thesis contributed to the understanding of Eng and sEng involvement in cholesterol and bile acids metabolism, as well as their possible contribution to liver impairment.

Despite contradictory results, while evaluating the specific role of Eng in liver pathologies, we may claim that in all cases of liver impairment that have been studied so far, the presence of elevated sEng levels in circulation has been a constant (in both human patients and respective mouse models of disease) and that sEng have not been related to a favorable outcome of pathology in any of the reports. Thus, with this, we may suggest that sEng can be a possible marker of liver damage that also promotes liver impairment.

The research topic of this dissertation, as well as the outcomes published, may have generated more questions than answers, which may have opened the door for further analysis of Eng and sEng involvement in liver damage.

Keeping in mind that hepatocytes do not express endoglin and that they are the major key players in cholesterol and bile acids biosynthesis and elimination metabolism, we may hypothesize that sEng role in these modulatory effects may be due to its involvement in cell-to-cell communication or through extrahepatic factors. Thus, a future direction of this research topic would rely on the individual evaluation of the Eng signaling pathway in isolated cell types present in the hepatic environment and establish how the soluble form of Eng may be an influence in cell-to-cell communication.

# 7 CONTRIBUTION OF THE CANDIDATE TO THE PUBLISHED WORKS INCLUDED IN THE IN THE DISSERTATION

**I.** Dolezelova E*, **Igreja Sa IC***, Prasnicka A, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Cermanova J, Pericacho M, Visek J, Lasticova M, Micuda S, Nachtigal P. **High soluble endoglin levels regulate cholesterol homeostasis and bile acids turnover in the liver of transgenic mice.** Life Sci. 2019 Sep 1; 232:116643. [IF=3.647] (Q2); [AIS=0.674] (Q2)
* designates shared co-first authorship

- Equally contributed as a co-first author.
- Performed research and sample analysis: mRNA and protein expression of enzymes and transporters involved in cholesterol metabolism, and BA metabolism and turnover via qRT-PCR and western blotting, as well as Eng and sEng pathway.
- The main contribution to data analysis and to the text of the publication.

**II. Igreja Sa IC**, Tripska K, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Schreiberova J, Dolezelova E, Eissazadeh S, Vitverova B, Najmanova I, Vasinova M, Pericacho M, Micuda S, Nachtigal P. **Soluble endoglin as a potential biomarker of nonalcoholic steatohepatitis (NASH) development, participating in aggravation of NASH-related changes in mouse liver**. Int J Mol Sci. 2020 Nov 27;21(23):9021. [IF=5.924] (Q1); [AIS=1.123] (Q2)

- The first author, contributed to conceptualization, methodology, validation, formal analysis, data curation, and writing – original draft preparation.

**III.** Lastuvkova H, Faradonbeh FA, Schreiberova J, Hroch M, Mokry J, Faistova H, Nova Z, Hyspler R**, Igreja Sa IC**, Nachtigal P, Stefela A, Pavek P, Micuda S. **Atorvastatin Modulates Bile Acid Homeostasis in Mice with Diet-Induced Nonalcoholic Steatohepatitis**. Int J Mol Sci. 2021 Jun 16;22(12):6468. [IF=6.208] (Q1); [AIS=1.064] (Q2)

- Contributed to mouse experiments, sample analyses (mostly related to Eng and sEng pathway), data interpretation, and review and editing of the manuscript.

**IV**. Vicen M, **Igreja Sa IC**, Tripska K, Vitverova B, Najmanova I, Eissazadeh S, Micuda S, Nachtigal P. **Membrane and soluble endoglin role in cardiovascular and metabolic disorders related to metabolic syndrome.** Cell Mol Life Sci. 2021 Mar;78(6):2405-2418. [IF=9.207] (Q1); [AIS=2.069] (Q1)

- A significant contribution to writing, review, and editing of the manuscript.

# 8 OVERVIEW OF PUBLICATION ACTIVITY OF THE CANDIDATE

Dolezelova E*, **Igreja Sa IC***, Prasnicka A, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Cermanova J, Pericacho M, Visek J, Lasticova M, Micuda S, Nachtigal P. High soluble endoglin levels regulate cholesterol homeostasis and bile acids turnover in the liver of transgenic mice. Life Sci. **2019** Sep 1; 232:116643. DOI: 10.1016/j.lfs.2019.116643.PMID: 31299237. [IF=3.647] (Q2); [AIS=0.674] (Q2) * designates shared co-first authorship

Vitverova B, Najmanova I, Vicen M, Tripska K, **Igreja Sa IC**, Hyspler R, Pericacho M, Nachtigal P. Long term effects of soluble endoglin and mild hypercholesterolemia in mice hearts. PLoS One. **2020** May 29;15(5):e0233725. doi: 10.1371/journal.pone.0233725. PMID: 32470058; PMCID: PMC7259503. [IF=3.240] (Q2); [AIS=1.011] (Q2)

**Igreja Sa IC**, Tripska K, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Schreiberova J, Dolezelova E, Eissazadeh S, Vitverova B, Najmanova I, Vasinova M, Pericacho M, Micuda S, Nachtigal P. Soluble endoglin as a potential biomarker of nonalcoholic steatohepatitis (NASH) development, participating in aggravation of NASH-related changes in mouse liver. Int J Mol Sci. **2020** Nov 27;21(23):9021. DOI: 10.3390/ijms21239021. PMID: 33261044. [IF=5.924] (Q1); [AIS=1.123] (Q2)

Vicen M, **Igreja Sa IC**, Tripska K, Vitverova B, Najmanova I, Eissazadeh S, Micuda S, Nachtigal P. Membrane and soluble endoglin role in cardiovascular and metabolic disorders related to metabolic syndrome. Cell Mol Life Sci. **2021** Mar;78(6):2405-2418. doi: 10.1007/s00018-020-03701-w. PMID: 33185696. [IF=9.207] (Q1); [AIS=2.069] (Q1)

Visek J, Blaha M, Blaha V, Lasticova M, Lanska M, Andrys C, Tebbens JD, **Igreja Sa IC,** Tripska K, Vicen M, Najmanova I, Nachtigal P**.** Monitoring of up to 15 years effects of lipoprotein apheresis on lipids, biomarkers of inflammation, and soluble endoglin in familial hypercholesterolemia patients. Orphanet J Rare Dis. **2021** Feb 27;16(1):110. DOI: 10.1186/s13023-021-01749-w. [IF=4.303] (Q2) ; [AIS=1.349] (Q2)

Lastuvkova H, Faradonbeh FA, Schreiberova J, Hroch M, Mokry J, Faistova H, Nova Z, Hyspler R, **Igreja Sa IC**, Nachtigal P, Stefela A, Pavek P, Micuda S. Atorvastatin modulates bile acid homeostasis in mice with diet-induced nonalcoholic steatohepatitis**.** Int J Mol Sci. **2021** Jun 16;22(12):6468. DOI: 10.3390/ijms22126468. PMID: 34208774. [IF=6.208] (Q1); [AIS=1.064] (Q2)

Faradonbeh FA, **Igreja Sa IC**, Lastuvkova H, Cermanova J, Hroch M, Faistova H, Mokry J, Nova Z, Uher M, Nachtigal P, Pavek P, Micuda S. Metformin impairs bile acid homeostasis in ethinylestradiol-induced cholestasis in mice. Chem Biol Interact. **2021** Aug 25; 345:109525. DOI: 10.1016/j.cbi.2021.109525. Epub 2021 May 28. PMID: 34058177. [IF=5.168] (Q1); [AIS=0.669] (Q2)

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## 9 AWARDS AND FELLOWSHIPS

2019 **Best Poster (2nd Place)** – 23rd Congress on Atherosclerosis (CSAT) – Pilsen, Czech Republic.

2020 **Best Poster (1st Place)** – 24th Congress on Atherosclerosis (CSAT) – Brno, Czech Republic.

2021 **Young Investigator Fellowship** for the 89th EAS Virtual Congress, Finland.
**Best Publication (2nd Place)** – 25th Congress on Atherosclerosis (CSAT) –Prague, Czech Republic.

2022 **Fellowship for foreign internship** from the Czech Society for Atherosclerosis (CSAT) (6 months).
**Fellowship for foreign internship** from the Czech Hepatological Society (CHS – CLS JEP) (6 months).

# 10 PRESENTATIONS AT NATIONAL AND INTERNATIONAL CONFERENCES

2019 **Lecture** – "Effect of soluble endoglin on cholesterol and bile acids metabolism in NASH mouse model – A pilot study".
XIX. Interdisciplinary Meeting – Milovy, Czech Republic.
**Poster** – "High soluble endoglin levels affect bile acids and cholesterol metabolism in mice liver".
23rd Congress on Atherosclerosis (CSAT) – Pilsen, Czech Republic.

2020 **Lecture** – "Soluble endoglin does not affect cholesterol and bile acids metabolism in NASH mouse model".
10th Postgraduate and Postdoc Conference – Hradec Králové, Czech Republic.
**Poster** – "High soluble endoglin levels, do not modulate cholesterol and bile acids metabolism in NASH mouse model".
88th EAS Congress – Geneva, Switzerland.
**Poster** – "Labetalol treatment in estrogen-induced cholestasis – A pilot study".
88th EAS Congress – Geneva, Switzerland.
**Poster** – "Soluble endoglin aggravates NASH progression in mouse liver".
24th Congress on Atherosclerosis (CSAT) – Brno, Czech Republic.

2021 **Lecture** – "Soluble endoglin as a potential biomarker of NASH development, promoting NASH progression in mouse liver".
11th Postgraduate and Postdoc Conference – Hradec Králové, Czech Republic.
**Mini-lecture** – "Soluble endoglin as a potential biomarker of NASH development, participating in aggravation of NASH-related changes in mouse liver".
89th EAS Virtual Congress.
**Poster** – "Labetalol treatment aggravates estrogen-induced cholestasis".
89th EAS Virtual Congress.
**Lecture** – "Labetalol treatment exacerbates estrogen-induced liver damage".
25th Congress on Atherosclerosis (CSAT) – Prague, Czech Republic.

2022 **Lecture** – "Soluble endoglin and labetalol aggravates estrogen-induced intrahepatic cholestasis".
12th Postgraduate and Postdoc Conference – Hradec Králové, Czech Republic.

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# 12 LIST OF ANNEXES

**I.** Dolezelova E*, **Igreja Sa IC***, Prasnicka A, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Cermanova J, Pericacho M, Visek J, Lasticova M, Micuda S, Nachtigal P. **High soluble endoglin levels regulate cholesterol homeostasis and bile acids turnover in the liver of transgenic mice.** Life Sci. 2019 Sep 1; 232:116643. [IF=3.647] (Q2); [AIS=0.674] (Q2) * designates shared co-first authorship

**II. Igreja Sa IC**, Tripska K, Hroch M, Hyspler R, Ticha A, Lastuvkova H, Schreiberova J, Dolezelova E, Eissazadeh S, Vitverova B, Najmanova I, Vasinova M, Pericacho M, Micuda S, Nachtigal P. **Soluble endoglin as a potential biomarker of nonalcoholic steatohepatitis (NASH) development, participating in aggravation of NASH-related changes in mouse liver**. Int J Mol Sci. 2020 Nov 27;21(23):9021. [IF=5.924] (Q1); [AIS=1.123] (Q2)

**III.** Lastuvkova H, Faradonbeh FA, Schreiberova J, Hroch M, Mokry J, Faistova H, Nova Z, Hyspler R**, Igreja Sa IC**, Nachtigal P, Stefela A, Pavek P, Micuda S. **Atorvastatin Modulates Bile Acid Homeostasis in Mice with Diet-Induced Nonalcoholic Steatohepatitis**. Int J Mol Sci. 2021 Jun 16;22(12):6468. [IF=6.208] (Q1); [AIS=1.064] (Q2)

**IV**. Vicen M, **Igreja Sa IC**, Tripska K, Vitverova B, Najmanova I, Eissazadeh S, Micuda S, Nachtigal P. **Membrane and soluble endoglin role in cardiovascular and metabolic disorders related to metabolic syndrome.** Cell Mol Life Sci. 2021 Mar;78(6):2405-2418. [IF=9.207] (Q1); [AIS=2.2069] (Q1)