CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Biological and Medical science

Carotuximab effect on endoglin expression and signaling in liver fibrosis

Diploma thesis

Seyedehniloufar Mohammadi

Hradec Králové 2022

Supervisor: Prof. PharmDr. Petr Nachtigal, Ph.D.

Consultant: M.Sc. Samira Eissazadeh

I hereby confirm that this thesis is my original copyrighted work. I compiled and elaborated it independently in my own words and in accordance with copyright. All resources and literature I used are indexed in the bibliography and properly cited. The thesis was not written and intended as misuse for obtaining the same or different academic degree.

In Hradec Králové **Seyedehniloufar Mohammadi**

Acknowledgments

I would like to thank Professor Nachtigal for accepting me as a small part of his incredible team, guiding me through all stages, and being patient with all the challenges in this thesis. I can't think of a more foresighted supervisor. Afterward, I'd like to thank my thesis consultant, M.Sc. Samira Eissazadeh, for her invaluable advice and guidance. I couldn't have finished this thesis without her assistance.

I'd also like to thank my amazing study partners and friends, Dina and Kimia, for being by my side throughout the entire five-year study process. I can't imagine completing my studies without your help.

Finally, I'd like to thank my parents for always believing in me and supporting my ambitions. Thank you for always showing me a way in difficult situations. I will always do everything in my power to make you proud.

I'll be forever grateful for the opportunity to be a part of Charles University. This wonderful university assisted me in achieving my goal of becoming a pharmacist while also maturing considerably over a five-year period.

Abstrakt

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Katedra biologických a lékařských věd

Školitel: prof. PharmDr. Petr Nachtigal, Ph. D

Student: Seyedehniloufar Mohammadi

Název diplomové práce: Účinky karotuximabu na expresi a signalizaci endoglinu u jaterní fibrózy

Cíl práce

Endoglin je 180 kDa transmembránový glykoprotein, který působí jako koreceptor pro vazbu na superrodinu TGFβ a nachází se ve dvou formách v krvi: jako membránový endoglin (Eng) a rozpustný endoglin (sEng) cirkulující v krvi. Bylo prokázáno, že Eng může hrát důležitou roli v procesu jaterní fibrózy, zánětu a endotrlové dysfunkce. Podle různých studií může mít Eng profibrotickou nebo antifibrotickou aktivitu. Přesný dopad exprese Eng a změn signalizace a hladin sEng během jaterních poruch stále není znám. Carotuximab, TRC105 (chimerický IgG1), je monoklonální protilátka, která se váže na Eng a ovlivňuje jeho expresi, signalizaci a hladiny sEng. Hypotézou této práce je, že carotuximab zabraňuje jaterním změnám modulací Eng a sEng během rozvoje experimentální jaterní fibrózy a zánětu předpokládáme.

Metody

Poškození jater bylo indukováno u tříměsíčních samců myší C57BL/6 dietou 3,5-diethoxykarbonyl-1,4-dihydrokolidin (DDC), zatímco kontrolní zvířata dostávala standardní potravu. Skupině TRC105 byl podáván carotuximab (15 mg/kg) dvakrát týdně, zatímco kontrolním zvířatům byl podáván fyziologický roztok, následovalo usmrcení myší po čtyřech týdnech s následným odběrem krve a molekulární analýzou vzorků jater. Biochemickou analýzou byly stanoveny hladiny alanintransaminázy ALT, celkového bilirubinu TBIL a Vzorky jater byly odebrány pro Western blot analýzu. Biochemickou analýzou byly stanoveny hladiny alanintransaminázy ALT, celkového bilirubinu TBIL

Výsledek

Poškození jater a fibróza u DDC myší byly potvrzeny signifikantním zvýšením hladiny ALT, TBIL a zvýšeným poměrem jater k tělesné hmotnosti. Ačkoli dieta DDC významně zvýšila

hladiny sEng a expresi MMP-14, došlo také k významnému snížení exprese Eng v játrech. Ve všech analýzách však nebyl žádný statisticky významný rozdíl mezi skupinou, které byl podáván carotuximab, a skupinou, která dostávala pouze DDC dietu. Táto práce ukazuje na to že DDC dieta pravděpodobně vede ke štěpení Eng pomocí MMP-14, a tudíž by mohl být eEng považován za cirkulující biomarker poškození jater po léčbě DDC.

Závěr

Táto práce ukazuje na to, že DDC dieta pravděpodobně vede ke štěpení Eng pomocí MMP-14, a tudíž by mohl být eEng považován za cirkulující biomarker poškození jater po léčbě DDC. Samotná léčba TRC105 však nemá významný vliv na expresi Eng u jaterní fibrózy při dietě DDC.

Abstract

Charles University, Faculty of Pharmacy in Hradec Králové

Department of Biological and Medical Sciences

Supervisor: Prof. PharmDr. Petr Nachtigal, Ph.D

Student: Seyedehniloufar Mohammadi

Title of the thesis: Carotuximab effects on endoglin expression and signaling in liver fibrosis

Objective of the thesis

Endoglin is a 180 kDa transmembrane glycoprotein that acts as a coreceptor for binding to the Transforming growth factor β (TGFβ) superfamily and is found in two forms in the blood: membrane endoglin (Eng) and soluble endoglin (sEng) circulating in the blood. It has been demonstrated that Eng might play an important role in the process of liver fibrosis, inflammation, and endothelial dysfunction. According to different studies, Eng can have prefibrotic or antifibrotic activity. The precise impact of Eng expression and signalling changes, and sEng levels during liver disorders are still unknown. Carotuximab, TRC105 (chimeric IgG1), is a therapeutic monoclonal antibody that binds to Eng and influences its expression, signaling, and sEng levels. In experimental liver fibrosis and inflammation, we hypothesize that **c**arotuximab prevents liver alterations by modulating Eng and sEng.

Methods

The liver damage was induced in three-month-old C57BL/6 male mice by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet while control animals were received a standard chow diet. The TRC105 group was administrated with **c**arotuximab (15 mg/kg) twice a week while control animals were administrated with physiological solution, followed by sacrificing the mice after four weeks with subsequent blood collection and molecular analysis of liver samples. Alanine transaminase (ALT)**,** total bilirubin (TBIL) levels were determined by biochemical analysis and the liver samples were collected for the western blot.

Results

The liver impairment and fibrosis in DDC mice were confirmed by the significant increase in the level of ALT**,** TBIL and increased ratio of liver to the body weight. Although the DDC diet significantly increased sEng levels and MMP-14 expression, there was a significant

reduction in the expression of Eng in the liver. However, in all analyses, there was no statistically significant difference between the TRC105-administered group and the DDC diet-only group.

Conclusion

DDC diet results in cleavage of Eng by MMP-14 leading to high levels of sEng. Thus, sEng might be considered a circulating biomarker of liver damage after DDC treatment. However, TRC105 treatment itself does not have significant effect on the Eng expression in liver fibrosis in the DDC diet.

Table of contents

List of figures

List of abbreviations

ADCC	"Antibody-dependent cell-mediated cytotoxicity"
ALK1	" Activin receptor-like kinase 1"
ALP	"Alkaline Phosphatase "
ALT	"Alanine transaminase "
APAP	"Acetaminophen"
APS	"Ammonium persulfate "
AMD	"Age-related macular degeneration"
ATP	"Adenosine triphosphate"
BCA	" Bicinchoninic acid assay"
BMP	"Bone morphogenic proteins"
CFLD	"Cystic fibrosis-associated liver disease"
CYP2E1	"Cytochrome P450 2E1"
DEN	" Diethylnitrosamine"
DMN	" Dimethylnitrosamine"
DMT2	" Diabetes mellitus type 2"
ECM	"Extracellular matrix"
Eng	" Endoglin"
eNOS	"Endogenous nitric oxide synthase"
ERK1/2	" Extracellular signal-regulated kinase 1/2"
FFA	"Free fatty acid"
HCC	"Hepatocellular carcinoma"

HDL	“High density lipoproteins”
HRP	“Horse Radish Peroxidase”
HSCs	“ Hepatic stellate cell”
HUVECs	“Human umbilical vein endothelial cells”
LAP	“Latency-associated peptide”
LSECs	“ liver sinusoidal endothelial cells”
LTBP	“Latent TGF- β binding protein”
mAb	“ Monoclonal antibody”
NAFL	“Nonalcoholic fatty liver”
NAFLD	“Non-alcoholic fatty liver disease”
NAPQI	“N-acetyl-p-phenylquinone imine”
NASH	“Nonalcoholic steatohepatitis”
NGAL	“Neutrophil gelatinase-associated lipocalin”
NO	“ Nitric oxide”
PVDF	“Polyvinylidene fluoride”
TGF-β	“Transforming growth factor β”
TGF-βRI	“TGF-β receptor I”
TGF-βRII	“TGF-β receptor II”
sEng	“ Soluble endoglin”
SDS	“Sodium dodecyl sulfate”
Smad	“Small mothers against decapentaplegic”
TAA	“Thioacetamide”
TBIL	“Total bilirubin”
Temed	“Tetramethylethylenediamine”

TIMPs	“Inhibitors of Metalloproteinases”
TLR4	“Toll-like receptor 4”
VEGF	“Vascular endothelial growth factor”
VLDL	“Very low density lipoproteins”
ZP	“ Zona pellucida”

1. Introduction

Endoglin (CD105) is a 180KDa glycoprotein that acts as a coreceptor for binding to the Transforming growth factor β (TGF-β) superfamily of proteins (López-Novoa & Bernabeu, 2010; S. K. Meurer et al., 2011; St-Jacques, 1994). Eng expression occurs in various cells including endothelial cells, macrophages, hepatic stellate cells, fibroblasts, monocytes, macrophages and vascular smooth muscle cells (Bot et al., 2009; Lastres et al., 1992; S. Meurer et al., 2019; Rathouska et al., 2015; St-Jacques, 1994). There are two forms of Eng presently discovered, particularly, membrane form (Eng) and soluble endoglin (sEng) which is circulating in the blood stream (Rathouska et al., 2015). sEng is the extracellular domain of membrane Eng that is cleaved by metalloprotease-14 (MMP-14) enzyme. sEng is released into the circulatory system during pathological conditions such as preeclampsia, hypertension, and diabetes mellitus type 2 (DMT2), playing an important role in them (Venkatesha et al., 2006, Malhotra et al., 2013, Doghish et al., 2019). sEng levels were also elevated in liver diseases, implying that it could be a potential biomarker of liver injury, such as fibrosis and/or nonalcoholic steatohepatitis (NASH) (Igreja Sá et al., 2020).

Liver fibrosis is the process of healing the liver as a result of a chronic liver injury caused by a variety of factors such as hepatitis (viral and autoimmune), NASH, chronic alcohol consumption, nonalcoholic fatty liver disease, and cholestatic disease (Iwaisako et al., 2012). These pathological conditions cause hepatocyte damage and hepatic stellate cell (HSCs) activation in the liver. HSCs are the starting point of hepatic myofibroblasts by contributing to collagen-producing cells via fibrogenic factors that facilitate collagen secretion from portal fibrocytes, fibroblasts, and bone marrow-derived myofibroblasts (Rygiel et al., 2008).

TGF- β is one of the most potent profibrotic cytokines, and its role in fibrotic conditions has been established (Biernacka et al., 2011). The role of Eng in liver fibrosis is linked to the same mechanism of action, in which Eng regulates TGF- β as a coreceptor. TGF- β regulates the phenotype and function of fibroblasts. Activated fibroblasts differentiate into myofibroblasts. TGF- β isoforms are key regulators of cell responses such as differentiation, migration, proliferation, and gene expression, and they are all involved in fibrotic responses (Frangogiannis, 2020).

Carotuximab (Tracon Pharmaceuticals Inc), also known as TRC105 (chimeric IgG1), is a monoclonal antibody (mAb) that affects Eng expression, signaling, and sEng level by binding to Eng. Carotuximab was first developed as an anti-cancer drug (Rosen et al., 2014). This thesis focuses on the activity of this new mAb on Eng expression and sEng as a circulating biomarker of liver damage following a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet.

2. Theoretical part

1. The Liver

The liver is the largest internal organ in the body, and it performs numerous important functions such as bile production and excretion, carbohydrate, fat, and protein metabolism, detoxification, synthesis, and secretion of plasma proteins such as albumin and clotting factor, and phagocytosis with the help of Kupffer cells.

2.1.1. Liver Histology

The liver is in the upper part of the abdominal cavity and is separated into two lobes by a thin fibrous structure known as the falciform ligament which integrates the liver to the front body wall. The larger right lobe is divided into two parts: the inferior (Quadrate) lobe and the posterior lobe (Caudate).

The liver is made up of many hexagonal shaped microscopic units which is called hepatic lobules, and they work together to maintain liver function. These polygonal structures are built up of radially arranged Hepatocytes around the central vein. Sinusoids, permeable capillaries that receive blood from the hepatic artery and portal vein and deliver it to the centrilobular venule, separate these hepatocytes.

Six portal triads are located at the angles of each hexagon corner. Portal triads are formed by three vessels: an artery that supplies oxygen, a venule that receives low-oxygen blood, and bile ducts that receive bile produced by hepatocytes from the bile canaliculi.

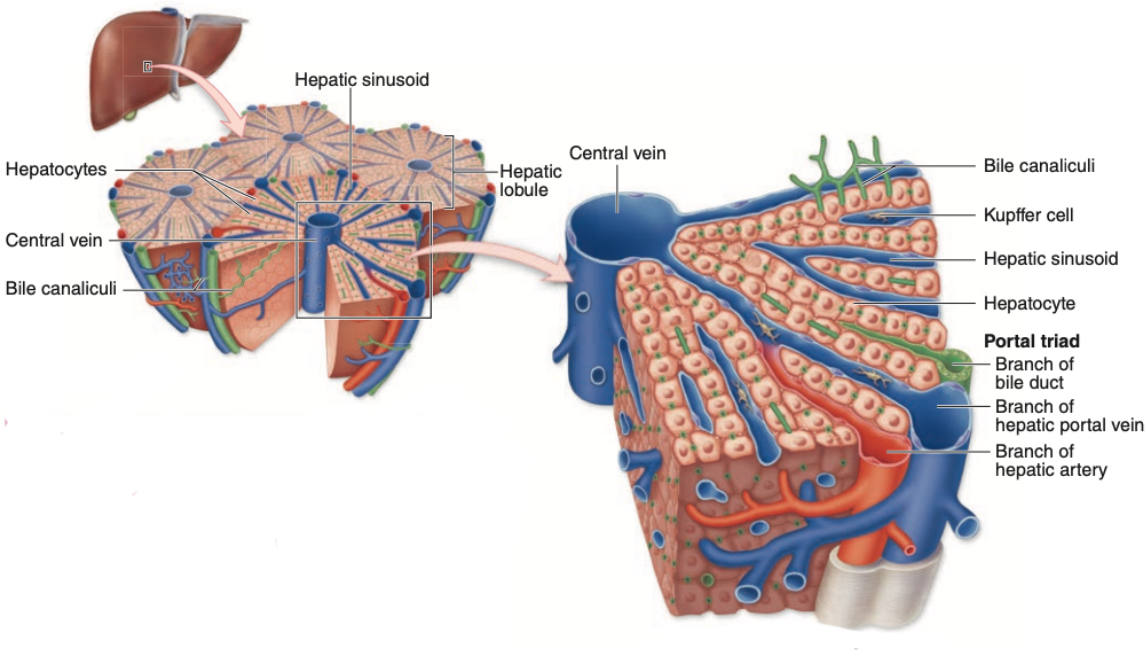

Figure 1 *Shows the hepatic lobules, composed of hepatocytes, portal triad, hepatic sinusoid and the central vein in the center. "Adapted from Junqueiras Basic Histology 2018-15th edition, chapter 16, page 338" Figure Credits. (Mescher, 2017)* *https://accessmedicine.mhmedical.com/content.aspx?bookid=2430&sectionid=190219970*

The liver contains a variety of cells including hepatocytes, liver sinusoidal endothelial cells (LSECs), Kupffer cells and HSCs. Hepatocytes are large polygonal epithelial cells with large nuclei and eosinophilic cytoplasm that is heavily populated with mitochondria. Hepatocytes have a polyhedral structure of six sides most of the time. They can have one or two nuclei, and these nuclei can contain up to twice as many chromosomes as a normal cell. Aside from having a lot of mitochondria, hepatocytes have quite a lot of Golgi membrane stacks, peroxisomes, and endoplasmic reticulum (Schulze et al., 2019). Tight junctions divide the plasma membrane of hepatocytes into basolateral (sinusoidal) and apical (canalicular) domains, which contain various channels, receptors, and surface proteins (Hung et al., 2020).

Endothelial cells give rise to LSECs. Since they lack the basement membrane, these cells are the most permeable endothelial cells in our bodies. They fill the gap between blood cells and other liver cells including HSCs and hepatocytes (Hung et al., 2020).

Cholangiocytes are epithelial cells of the bile duct. The bile ducts are lined with cholangiocytes, both small and large. Small cholangiocytes are cuboidal and line the small bile ducts, whereas large cholangiocytes are columnar and line the larger bile ducts. They also have

cilia, which allows mechano-, chemo-, and osmo-sensors to respond to bile flow changes (Maroni et al., 2015).

Kupffer cells are a type of macrophage found in the liver. They have an amoeboid structure attached to sinusoidal endothelial cells. Kupffer cells have a bean shaped nucleus with star shaped cytoplasm extensions. They contain an oval nuclei and black carbon particles in their cytoplasm (Basit et al., 2020).

HSCs are spindly cells with oval nuclei that reside in the perisinusoidal space. They have a cytoplasm that is filled with lipid droplets which store vitamin A and other fat-soluble vitamins. In response to the cell injury, these cells become activated and differentiate into myofibroblasts (Senoo et al., 2010).

2.1.2. Liver Function

The liver is an amazing organ that performs a variety of vital functions. The production of bile is one of the most important functions of the liver. Bile is a green-yellowish fluid that aids digestion as well as the absorption of fat and fat-soluble vitamins. Bile is produced by hepatocytes and flows through the common bile duct before being released into the duodenum or stored in the gallbladder. Later, the liver recycles used bile by receiving it from the portal blood after digestion, removing salts and extraneous substances, and returning it to the gallbladder.

The storage or metabolism of fat-soluble vitamins is another important function of the liver. Most fat-soluble vitamins enter the liver via intestinal absorption as chylomicrons or VLDL (Kalra & Tuma, 2018).

The metabolism of bilirubin is one of the most crucial functions of the liver. Kupffer cells are responsible for trapping and destroying old red and white blood cells, bacteria, and foreign substances that enter the sinusoids. Hemoglobin is separated into hem and globin within these cells. Its constituents, globin is later degraded to amino acids that are used to make new proteins, and the heme is converted to biliverdin via iron cleavage. Later, iron is stored in the liver or used by bone marrow cells to produce new red blood cells, and biliverdin is transformed to bilirubin and released into the bloodstream in either conjugated or free form (Ozougwu, 2017).

The liver is also involved in the metabolism of carbohydrates, fats, and proteins. Most liver cells, hepatocytes, play a variety of important roles in metabolism. Glycogenolysis is the

process of converting glycogen to glucose. After a meal, they do the opposite and regenerate glycogen by glucose using a process known as glycogenesis. They also play a key function in the production of new proteins such as coagulation factors, albumin, and lipoproteins such as very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL). VLDL carries fatty acids and cholesterol to adipocytes, whereas HLD carries cholesterol from the periphery to the liver. During the deamination process, these astounding cells also remove the amine group from amino acids while producing Adenosine triphosphate (ATP).

The liver is responsible for converting toxic lipophilic metabolites into more hydrophilic metabolites and excreting them from the body. The two-phase pathway, phase I (oxidation, reduction, and hydrolysis) and II (conjugation), begins with molecules entering hepatocytes and ending in bile (Almazroo et al., 2017) . Many factors, including genetics, alcohol, and nutrition, can have an impact on this process.

Since liver has an extensive vascular network, it plays an important role in vascular and hematologic Functions. Hematopoiesis occurs in a variety of organs during fetal development, including the liver. Hematopoietic stem cells are in the portal tract of the liver (Lewis et al., 2021). The liver plays an essential part in the hemostatic system by synthesizing the large percentage of coagulation factors and proteins that are involved in fibrinolysis. The liver is responsible for the production of thrombopoietin, a key hormone that sensitizes platelets, as well as most plasma proteins, including Albumin, fibrinogen and globulins (except gamma globulin) (Lisman et al., 2002).

2.2. Non-alcoholic fatty liver disease (NAFLD)

NAFLD is the most common cause of chronic liver disease. NAFLD is caused by fat accumulation in the liver when there is no or little alcohol consumption, which is linked to inflammation. NAFLD, which is essentially the hepatic manifestation of the metabolic syndrome, is mostly linked to insulin resistance, which includes DMT2, obesity, and hyperlipidemia. nonalcoholic fatty liver (NAFL) and NASH are two types of NAFLD. NAFL is characterized by steatosis without inflammation, whereas NASH is characterized by hepatic inflammation, which is distinct from alcohol inflammation. NAFL has a lower potential to advance fibrosis and death from liver disease, whereas NASH is thought to be more progressive, with a higher risk of progression to advanced fibrosis and mortality (Singh et al., 2015, Byrne & Targher, 2015).

NAFLD is classified as primary or secondary based on the pathogenesis. Insulin resistance and metabolic syndrome are linked to primary NAFLD (obesity, DMT2, or dyslipidemia, and insulin resistance, hypertension). Secondary NAFLD occurs when the liver becomes the site of lipid storage for reasons other than a metabolic syndrome, such as drug use, abdominal surgery, acute fatty liver of pregnancy, and so on (Grundy, 2005). The metabolic syndrome is a collection of risk factors that increase the likelihood of atherosclerotic cardiovascular disease, DMT2, and their complications. The most common underlying risk factors are obesity and insulin resistance. Dyslipidemia, high blood pressure, high glucose, a prothrombotic state, and a proinflammatory state are the five risk factors for metabolic syndrome in general(Grundy, 2005)

2.2.1. NAFLD and type 2 diabetes

As previously stated, NAFLD regularly coexists with obesity, insulin resistance, hyperlipidemia, DMT2, and cardiovascular disease.

Insulin resistance occurs when cells simply stop to respond to the hormone insulin. As a result, the pancreas's Beta cells produce more insulin. Insulin and blood sugar levels rise over time, potentially leading to type 2 diabetes. One of the primary causes of insulin receptor and signaling damage is a high caloric intake, which results in defective suppression of free fatty acid (FFA) release from adipose cells as well as defective nitric oxide (NO) release. Insulin resistance and inflammation form a destructive cycle in the presence of lipotoxicity, with each

condition promoting the other and accelerating the development of NAFLD and other metabolic disorders (Hong & Choi, 2020).

The link between NAFLD and DMT2 was first discovered in a study that found that high alanine aminotransferase (ALT) predicts the development of DMT2 in Pima Indians (Vozarova et al., 2002). Afterwards, various studies demonstrated that elevated serum liver enzymes could increase the risk of T2D in populations of different nationalities, independent of other risk factors such as diet and lifestyle (Ballestri et al., 2016; Goessling et al., 2008; Kunutsor et al., 2013).

And at last, the presence of NAFLD raises the risk of DMT2 and facilitates the development of complications. Contrarily, the presence of DMT2 increases the likelihood of NAFLD progressing to more severe forms of liver disorders.

2.2.2. Liver fibrosis in NAFLD

NAFLD has four main stages:

Simple fatty liver, also known as steatosis, is the stage at which the liver cells begin to accumulate fat. There is no inflammation, and it is asymptomatic for most people because it does not progress.

NASH happens when fat accumulation in liver cells begins to be accompanied by inflammation. When the liver begins to repair the inflamed tissue, a scar forms, which can eventually lead to fibrosis.

Fibrosis is the third stage in which scar tissue is formed around the liver and its surrounding blood vessels after inflammation. However, the liver can still function normally, and removing the inflammation may help to prevent further damage.

If scar tissue begins to replace a large portion of the normal liver tissue over time, the liver's function is compromised. This can progress to cirrhosis, the most severe stage, which occurs after years of inflammation and causes the liver to shrink and symptoms such as skin yellowing to appear.

All the previously discussed cause-and-effect relationships accelerate this progression and make it more difficult for the liver to rebuild itself.

2.3. Endoglin (Eng)

Eng (CD105) is a 180KDa glycoprotein that acts as a coreceptor for binding to the TGF-β superfamily of proteins. (López-Novoa & Bernabeu, 2010; S. K. Meurer et al., 2011; St-Jacques, 1994). Eng expression occurs in various cells including endothelial cells, macrophages, hepatic stellate cells, fibroblasts, monocytes, macrophages and vascular smooth muscle cells (Bot et al., 2009; Lastres et al., 1992; S. Meurer et al., 2019; Rathouska et al., 2015; St-Jacques, 1994). There are two forms of Eng presently discovered, particularly, membrane Eng and sEng which is circulating in the blood stream (Rathouska et al., 2015).

2.3.1. Structure and Function

Eng, made of two 95kDa disulphide-linked subunits, has three different domains: 561 amino acids extracellular domain, a single hydrophobic domain, and a 47-residue cytoplasmic tail. The extracellular domain contains an Arg-Gly-Asp tripeptide, four N-linked glycosylation sites, and an O-linked glycosylation part. Eng's extracellular part has two main domains: the orphan domain and the zona pellucida (ZP) domain. The ZP domain makes Eng a member of the ZP domain protein family which has the 260 amino acid residues in the extracellular region. The orphan domain is basically the NH2-terminal that has no structural similarity to the any other proteins in the family. This terminal contains five N-linked glycosylation sites and an O-glycan domain. Typically, this terminal is glycosylated, and it is involved in binding of BMP9, a Bone morphogenic proteins of the TGF-B subfamily member. The intracellular domain contains serine and threonine residues that act as the main site of the phosphorylation, resulting in the formation of a functional receptor complex (Gougos & Letarte, 1990, Koleva et al., 2006; López-Novoa & Bernabeu, 2010; Nassiri et al., 2011, Jovine et al., 2005). Since Eng has a short cytoplasmic domain, it cannot initiate the entire signaling cascade and therefore functions as a coreceptor (Gougos & Letarte, 1990).

There are two isoforms of Eng; Short and Long. Short-Eng has 14 amino acids in its cytoplasmic domain, whereas Long-Eng has 47 amino acids (Bellón et al., 1993). Both of these isoforms can interact with their ligands but the difference between is that long-Eng has a high level of phosphorylation and is predominantly expressed in endothelial cells, whereas Short-Eng has a low level of phosphorylation. Additionally, their affinity for the receptor and ability to regulate TGF-β-dependent responses differ Long-form endoglin promotes angiogenesis by inducing endogenous nitric oxide synthase (eNOS) expression whereas short-form endoglin

appears to contribute to aging-related cardiovascular pathology(Jang & Choi, 2014).Generally, long-endoglin appears to promote signaling via the activin receptor-like kinase 1 (ALK1) pathway, whereas short-endoglin appears to promote signaling via the activin receptor-like kinase 5 (ALK5) pathway (Velasco et al., 2008). To summarize, short endoglin inhibits endothelial cell migration, proliferation, and angiogenesis, whereas long endoglin stimulates them (Vicen et al., 2021).

sEng is the extracellular domain of membrane Eng that is cleaved by MMP-14 enzyme. sEng is released into the circulatory system during pathological conditions such as preeclampsia, hypertension and DMT2, playing an important role in them (Venkatesha et al., 2006, Malhotra et al., 2013, Doghish et al., 2019).

TGF- β family has three isoforms known as of TGF- β1, TGF- β2 and TGF- β3. TGF- β binds to two types of receptors: TGF-β receptors I and II (TGF-βRI and TGF-βRII). Eng binds to TGF-β1 and TGF-β 3, but not TGF-β2. Binding to TGF-β activates the type II receptor, which then transphosphorylates and activates the type I receptor. Type 1 then phosphorylates and transmits signals to Smads (Barbara et al., 1999; Cheifetz et al., 1992).

Small mothers against decapentaplegic (Smad) proteins are the primary signal transducers for receptors of the TGF-B superfamily. When these proteins are activated, they move from the cytoplasm to the nucleus, where they, along with other transcription factors, can activate or suppress transcription and thus regulate target gene expression (Attisano & Lee-Hoeflich, 2001). Upon phosphorylation of TGF β-RI substrate proteins, ALK1 phosphorylates Smad1/5/8 along with Smad4 and ALK5 phosphorylates Smad2/3 and translocate to the nucleus with Smad4. As a result, ALK1 activation stimulates cell proliferation and migration, whereas ALK5 activation inhibits these responses (Goumans et al., 2002; Lebrin et al., 2005). Eng appears to be an important modulator of the balance between ALK1 and ALK5 signaling because it promotes TGF- B1/ALK1 and BMP-9/ALK1 signaling in endothelial cells and enhances BMP-7 signaling via the Smad1/Smad5 pathway in myoblasts (Gore et al., 2014; Lebrin et al., 2004).

To summarize, Eng modulates both complexes in opposing ways; it promotes signaling via ALK-1 while inhibiting signaling via ALK-5. Following that, ALK-1 and ALK-5 activate distinct R-Smad pathways, causing opposing endothelial cell responses in proliferation, migration, and pro- or anti-angiogenic gene expression. Mutations in Eng or ALK1 causes a

vascular disorder with autosomal dominant inheritance called “Hereditary Hemorrhagic Telangiectasia” or HHT (Albiñana et al., 2017).

Eng expression is low in resting endothelial cells, but it increases during almost all inflammatory processes, including angiogenesis, inflammation in tissues and wounds, tumor vessels, embryogenesis, after vascular injury, ischemia in the heart and kidney, and so on(Adam et al., 1998). Eng expression is normally low in normal smooth muscle cells, but it is upregulated in atherosclerotic plaques. Eng expression is also increased during the monocyte-macrophage transition and in tissues that are undergoing fibrosis such as liver and kidney (Lastres et al., 1992, Clemente et al., 2006; Rodríguez-Peña et al., 2002).

2.3.2. Endoglin role in liver fibrosis

TGF- β is one of the most potent profibrotic cytokines, and its role in fibrotic conditions has been established (Biernacka et al., 2011). The role of Eng in liver fibrosis is linked to the same mechanism of action, in which Eng regulates TGF- β as a coreceptor. TGF- β regulates the phenotype and function of fibroblasts. Activated fibroblasts differentiate into myofibroblasts. TGF- β isoforms are key regulators of cell responses such as differentiation, migration, proliferation, and gene expression, and they are all involved in fibrotic responses (Frangogiannis, 2020).

TGF- β can be synthesized by endothelial cells, macrophages, fibroblasts, epithelial cells, lymphocytes, and platelets in the injured tissues. In the early stages, hepatic macrophages initiate fibrosis by recruiting proinflammatory immune cells and secreting proinflammatory cytokines and chemokines. Macrophages produce a large amount of latent TGF- β and cadherin 11, which cause macrophages and myofibroblasts to adhere. This is required to activate TGF-β stores in the nearby of the fibroblasts (Walton et al., 2017).

TGF–β activates fibrosis through Smad and non-Smad pathways. TGF- β binding results in phosphorylation of Smad2/3 transcription factors, which then travel to the nucleus with the assistance of Smad4. Following that, they promote the expression of genes that are responsible for the fibrotic response such as fibronectin, collagen, plasminogen activator inhibitor-1, proteoglycans, connective tissue growth factor, integrins, and matrix metalloproteases (MMPs) (Walton KL et al, 2017). Many studies have shown that the SMAD-1/5 pathway, another Smad-

dependent TGF-dependent pathway, is anti-fibrotic, particularly BMP-7 (Taipale et al., 1996). However, the precise mechanism of action might be still unknown.

TGF- β activates fibrosis through non-Smad pathway via a complex process involving Latency-associated peptide (LAP) and integrins. LAP is a protein derived from the TGF- β gene product's N-terminus, binds TGF- β1, forming a latent complex that aids in secretion. There are two types of the latent complexes, small and large (Taipale et al., 1996). A disulfide bond connects a small latent complex to a protein called latent TGF- β binding protein (LTBP) (Murphy-Ullrich & Poczatek, 2000). Latent complexes are activated by heterodimeric transmembrane receptors called integrins. Integrins serve as cell anchoring molecules as well as signaling molecules. As a result, they cause cellular cytoskeleton adhesion to the extracellular matrix (ECM) and regulate a variety of vital cell functions such as adhesion, polarity, differentiation, migration, and cell division. Integrin αvβ6 is exclusively expressed in epithelial cells (G & Erkki, 1999). In general, two of these integrins (αvβ6 and αvβ8) can activate latent TGF- β1 and TGF- β3 in the extracellular space. Integrin αvβ v6 mediates TGF-β activation by covalently linking small latent complexes to the latent TGF- β binding protein and ECM proteins, whereas αvβ8 can bind LAP-1 and activate TGF- β1. This causes MMP-14 to cleave LAP and release TGF- β1 (Nishimura, 2009). MMPs are matrix metallopeptidases that play critical roles in wound healing and tissue repair in response to injury. Their regulation is controlled by tissue inhibitors of metalloproteinases (TIMPs). Both matrix and non-matrix proteins can be degraded by this enzyme in the extracellular environment (Nagase et al., 2006).

2.3.3. Soluble endoglin role in liver disorders

As previously stated, the proteolytic MMP14 cleavage of membrane endoglin produces sEng, which enters the circulation. MMP-14 cleaves the extracellular portion of L-endoglin at position 586 at the NH-terminus. The location of membrane Eng cleavage can have a significant impact on the development of a variety of pathologies. The 80 kDa sEng molecule is normally cleaved at position 586 from the surface of Human umbilical vein endothelial cells (HUVECs). However, sEng is not cleaved at position 586 in pre-eclampsia and possibly other diseases (Rathouska et al., 2015). The elevation of sEng in pre-eclamptic women increased the severity of the disease and accelerated its progression (R. J. Levine et al., 2006). sEng was initially involved in a variety of disease groups. sEng has been found in tumor patients' plasma (Pérez-Gómez et al., 2010). sEng levels in the plasma of patients with cystic fibrosis-associated liver

disease (CFLD) and hepatocellular carcinoma (HCC) combined with cirrhosis have been found to be higher (Yagmur et al., 2007). It has previously been demonstrated that an increase in plasma sEng may be a circulating biomarker of NASH. In addition, sEng levels were increased in liver diseases suggesting sEng might represent a potential biomarker of liver injury, including fibrosis and NASH. This is most likely due to increased Eng expression in the liver and elevated levels of MMP14, which causes sEng cleavage (Igreja Sá et al., 2020). However, the precise impact of Eng expression and signaling changes, as well as sEng levels, during cholestasis/fibrosis is unknown.

2.4. Liver fibrosis animal models

The use of animal models is important not only for determining the mechanisms underlying fibrosis initiation and progression, but also for developing new strategies. Animal models can help us study specific signaling pathways by collecting many samples for subsequent studies. It can also aid in the study of specific pathways in genetically modified animals. (Delire et al., 2015).

Liver fibrosis is usually classified according to the modeling method used. This section provides a summary of the various fibrosis treatment methods and their characteristics.

Chemical fibrosis is a type of fibrosis that is caused by chemical agents. Scientists cause chemical fibrosis with the help of different agents including thioacetamide, carbon tetrachloride, acetaminophen, and nitrosamines (Bao et al., 2021). Thioacetamide (TAA) is an organic colorless or white crystal compound that is commonly used to induce experimental liver fibrosis. TAA's active metabolite causes fibrosis by binding to lipids and proteins (Bao et al., 2021). The mechanism of action is complex, but oxidative bioactivation of its toxic metabolites causes bile duct proliferation and portal fibrosis, altering the pattern and increasing liver total collagen content (Müller et al., 1988).

Another method that can be used in chemical fibrosis is CCl4. This agent works in the liver by inducing similar mechanisms to inflammation and fibrosis. CCl4 is a non-polar organic compound that is colorless. This substance is extremely toxic and can dissolve a wide range of substances, including fat and proteins. CCl4 can cause direct damage to liver cells by altering the permeability of lysosomes and mitochondrial membranes. The first step is to cause damage through oxidative stress and lipid peroxidation (Bao et al., 2021; Weber et al., 2003).

Nitrosamines are extremely hepatotoxic, causing fibrosis through cytochrome P450 2E1 (CYP2E1)-dependent metabolism. By forming NA complexes, this can result in a variety of toxic metabolites and hepatotoxicity. As a result, individual rats develop fibrotic changes and collagen deposition (Gao et al., 2017). Two nitrosamines implicated in chemical fibrosis are dimethylnitrosamine (DMN) and diethylnitrosamine (DEN). The percentage of collagen fibrosis in DMN-induced rat liver fibrosis has been linked to plasma levels of hyaluronic acid, laminin, and collagen (C.-H. Li et al., 2005).

Acetaminophen (APAP) is another agent that can be used to induce liver fibrosis. Acetaminophen is well-known for its hepatotoxicity. Acetaminophen chemical fibrosis is

caused by APAP-induced liver injury, which causes mitochondrial oxidative stress. APAP metabolites such as N-acetyl-p-phenylquinone imine (NAPQI) are strongly hepatotoxic. NAPQI increases mRNA expression of various factors and leads to phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) which controls a widespread range of stimulated cellular processes, mainly proliferation, differentiation, and stress response (Bai et al., 2017).

Alcohol-induced fibrosis is another method for inducing fibrosis in animals, a research model that causes liver fibrosis by combining alcohol and a high-fat diet. Toll-like receptor 4 (TLR4) signaling is activated with chronic alcohol administration and the NASH-induced high-fat diet, resulting in fibrosis (Gäbele et al., 2011).

The other methods for inducing fibrosis in animals are: The Immune Damage-Induced Liver Fibrosis Model, which is caused by autoimmune hepatitis or virus infection, the Surgery-Induced Liver Fibrosis Model, which is based on cholestasis obstruction of both bile flow formation and excretion resulting in excessive ECM deposition, and Organoid Liver Fibrosis Modes, which are caused by 3D organ-like cells (T. Li & Apte, 2015).

2.4.1. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) model of liver fibrosis

DDC diet has been an interesting topic for the scientists over the past years. In our study, we fed DDC diet for 4 weeks in 6 three-month-old C57BL male mice.

Chronic feeding of DDC to mice was first established to study Mallory bodies, which are cytoplasmic body inclusions of hepatocytes associated with NASH as well as other metabolic liver diseases (Denk et al., 2000; Fickert et al., 2003).

DDC diet resulted in increased biliary porphyrin secretion and induction of vascular cell adhesion molecule, osteopontin, and tumor necrosis factor-expression in bile duct epithelial cells in an animal model (mice). Glutathione and phospholipid excretion have also been shown to decrease significantly over time (Fickert et al., 2007). In the same study, the DDC diet induced a new xenobiotic model in mice for sclerosing cholangitis, which is caused by inflammation, and biliary fibrosis leading to a scar formation in the bile duct (Ishida et al., 2016).

By examining plasma creatinine, another study discovered that the level of creatinine in the plasma was significantly higher after the DDC diet. The DDC diet also resulted in elevated levels of urinary Neutrophil gelatinase-associated lipocalin (NGAL), a marker of tubular damage. Urinary examinations revealed a higher urinary albumin/creatinine ratio, as well as a high level of bicarbonate concentration. Overall, they found that DDC causes renal impairment through tubular cell injury, resulting in chronic kidney disease (Ishida et al., 2016). In a separate study, it was discovered that DDC diet activates the constitutive androstane receptor in mice, resulting in oval cell proliferation in the mice's liver. Oval cells, which have an oval nucleus, appear in the injured liver after toxins inhibit hepatocyte proliferation (Yamazaki et al., 2011).

Moreover, DDC is a porphyrinogenic agent and it induces aminolaevulinate synthetase, an important enzyme in the heme-biosynthetic pathway, in mouse liver. DDC inhibits mitochondrial and microsomal haem synthesis *in vivo* by inhibiting ferrochelatase activity (J. Y. Kim et al., 2017). As a conclusion, the DDC diet is useful for testing various hepatic and renal pathologies *in vivo*.

2.5. Carotuximab

Carotuximab, (Tracon Pharmaceuticals Inc), also known as TRC105 (chimeric IgG1), is a human/murine mAb that binds human and murine Eng and has an impact on Eng expression, signaling, and sEng levels. It binds to the orphan domain of Eng and affecs the Eng signaling pathway via SMAD expression and phosphorylation (Liu et al., 2014; Seon et al., 2011). As previously stated, the Orphan domain is the NH2-terminal domain that has no significant structural similarity to any other protein family and is involved in the binding to BMP9 (Nolan-Stevaux et al., 2012). Carotuximab was initially developed as an anti-cancer medication. It was initially developed for use in oncology, with the goal of targeting proliferating endothelial cells in the vasculature of solid tumors (Liu et al., 2021; Rosen et al., 2014). It is currently being tested in clinical trials for age-related macular degeneration (AMD) (Liu et al., 2021).

2.5.1. Structure, function, pharmacodynamic

This human/mouse chimeric IgG1 antibody binds CD105 and result in a complicated mechanism of action. TRC105 binds to Eng and causes antibody-dependent cell-mediated cytotoxicity (ADCC), which is mediated by a group of cells including NK cells, neutrophils, and monocytes and results in cell death. It also induces MMP-14-mediated ENG cleavage, resulting in an increase in sEng, which can aid in the reduction of ENG. As previously stated, sEng regulates endoglin signaling via MMP-14 cleavage of its receptor domain. TRC105 also prevents ENG from binding to BMP9, thereby inhibiting the signaling pathway described below (Liu et al., 2021).

BMPs are composed of an N-terminal signalling pathways peptide that directs secretion, a C-terminal mature peptide, and a prodomain that effectively supports folding. BMPs are typically synthesized as large proproteins containing 400–500 amino acids that form dimers via a disulphide bridge in the C-terminus (Bragdon et al., 2011). The amino acid domain in the C terminal is proteolytically cleaved by an enzyme, such as serine endoproteases or furin for BMP4. The prodomain, which oversees the mature ligand's proper folding, dimerization, and secretion, remains non-covalently attached to the mature active BMP dimer (except BMP2). Except for BMP9 and BMP7, the majority of the prodomain complexes are silent After cleavage, the ligand dimer activates the type II transmembrane serine/threonine kinase receptors, which then activate the type I receptor. Finally, Smads are activated by the Type I-receptor (Anderson & Wharton, 2017; Bidart et al., 2012).

BMP9 is the main ligand for ALK1 which is primarily expressed by non-parenchymal cells in the liver. Several studies have suggested that BMP9 may play a role in liver fibrosis, glucose metabolism, hemopoietic factor, osteogenic factor, and cholinergic neuron differentiation(Chen et al., 2003; J. Z. Li et al., 2003; P. Li et al., 2018; López-Coviella et al., 2000; Ploemacher et al., 1999). BMP9 may play an important role in angiogenesis by inhibiting endothelial cell growth and migration and inducing genes related to ALK1 activation (David et al., 2007). As a result, when TRC105 binds to the orphan domain of Eng, it reduces BMP9 cell signaling and inhibits these mechanisms.

Furthermore, TRC105 urges extracellular domain shedding by directly coupling Eng and MMP-14 to sEng at the cell surface. Not only does sEng have an antagonist effect on Eng, but it also binds to BMP-9 directly, inhibiting angiogenesis (Kumar et al., 2014). This was discovered in a study which showed that sEng specifically binds to BMP9 and BMP10, leading to suppress of the growth of the tumour and formation of blood vessels (Castonguay et al., 2011).

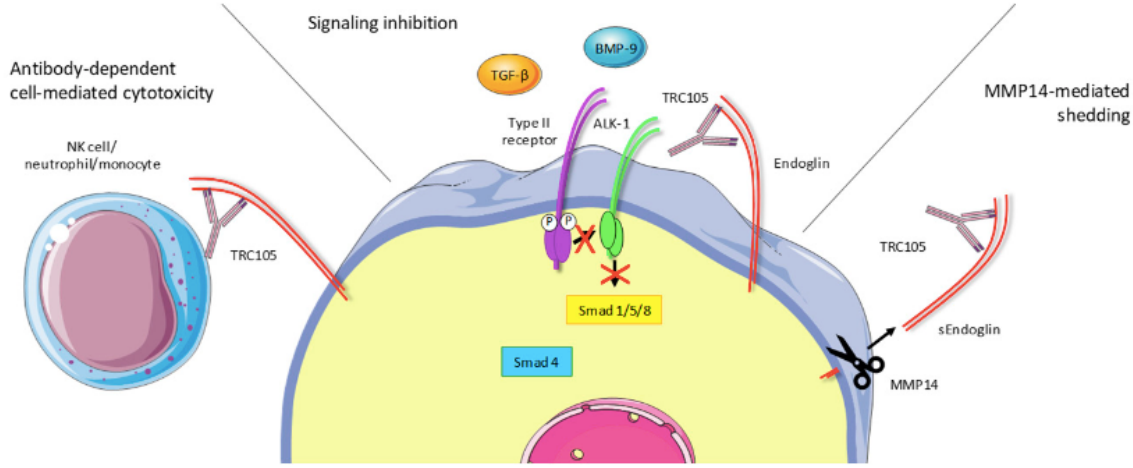

Figure 2 *shows three possible mechanism of actions of carotuximab "Adapted from Endoglin Targeting: Lessons Learned and Questions That Remains, (Liu et al., 2021) "*

It was also discovered that sEng can bind circulating TGF- β1, reducing its ability to interact with its membrane receptors and thus disrupting the TGF- β1 signaling pathway. As a result, sEng inhibits TGF- β1 receptor binding and downstream signaling, affecting eNOS activation and vasodilation. In conclusion, increased levels of sEng inhibit TGF- β1 binding to its membrane receptors in endothelial cells, preventing Smad2/3 activation, inhibiting eNOS activation and eNOS dependent vasodilation (Karzai et al., 2015; Venkatesha et al., 2006).

Many studies have already been conducted to demonstrate the adverse effects. In a study of 20 patients who received 20 mg/kg every two weeks, the most common adverse effects were problems related to the infusion, headache, fever, epistaxis, and anemia. TRC105 was discovered to be well tolerated in general, with manageable side effects. Serious side effects such as hypertension, proteinuria, thromboembolic events, and severe bleeding were uncommon (Karzai et al., 2015). In a recent open-label, dose-escalating, sequential cohort study, which was a phase I study, patients with AMD were given carotuximab injections in different groups, and no serious adverse effects were reported when carotuximab was given as a single intravitreal injection to patients with persistent exudative AMD(Gonzalez et al., 2021). TRC105 was tolerated at 10 mg/kg every week and 15 mg/kg every 2 weeks in another phase I, open-label study about antitumor activity in patients with advanced refractory tumor cells, with a safety profile different from that of VEGF inhibitors (Rosen et al., 2012). As a final thought, there have been numerous studies conducted on the safety of this monoclonal antibody.

2.5.2. Carotoximab treatment

Carotuximab was initially developed as an anti-cancer medication. It was initially developed for use in oncology, with the goal of targeting proliferating endothelial cells in the vasculature of solid tumors (Liu et al., 2014; Rosen et al., 2014). The overall result of the TRC105 treatment was angiogenesis suppression with an increase in sEng. The treatment was said to be safe, with clinical activity indications, and it served as the foundation for further clinical development (Liu et al., 2021).

Following that, the combination of carotuximab and the vascular endothelial growth factor (VEGF) antibody bevacizumab was discovered to have long-term activity in a VEGF inhibitor refractory patient population with a reduction in tumor density, as confirmed by CT scan (Gordon et al., 2014).

Afterward, the effect of carotuximab in combination with VEGF inhibitors was then studied in other cancers such as sarcoma(Ravi et al., 2018) , breast cancer (E. Levine et al., 2013), prostate cancer (Madhav et al., 2018), renal cell carcinoma (C. Shen & Kaelin, 2013) , glioblastoma (Ahluwalia et al., 2016) , and HCC (Duffy et al., 2015, 2017).

Furthermore, in a 2018 study, they worked on a combination therapy of anti VEGF-A and anti-Eng treatment of subretinal fibrosis, which leads to irreversible vision loss in neovascular

AMD. Combination therapy was shown to be more effective than monotherapy in inhibiting vascular leak (W. Shen et al., 2018).

2.6. Western blot analysis

2.6.1. The principle of the method

Western blot is an analytical technique using for proteins detection of a specific sample. The Western blot method was introduced to the world of scientific in 1979, allowing detection of particular proteins via autoradiography, UV light, or the peroxidase reaction product (Towbin et al., 1979). The western blot process is summarized as follows: protein sample extraction, gel electrophoresis of the proteins sample, transfer of the proteins from the gel to a membrane support, the interested proteins are labelled via specific antibodies, and immunodetection of a target antigen (Hnasko & Hnasko, 2015).

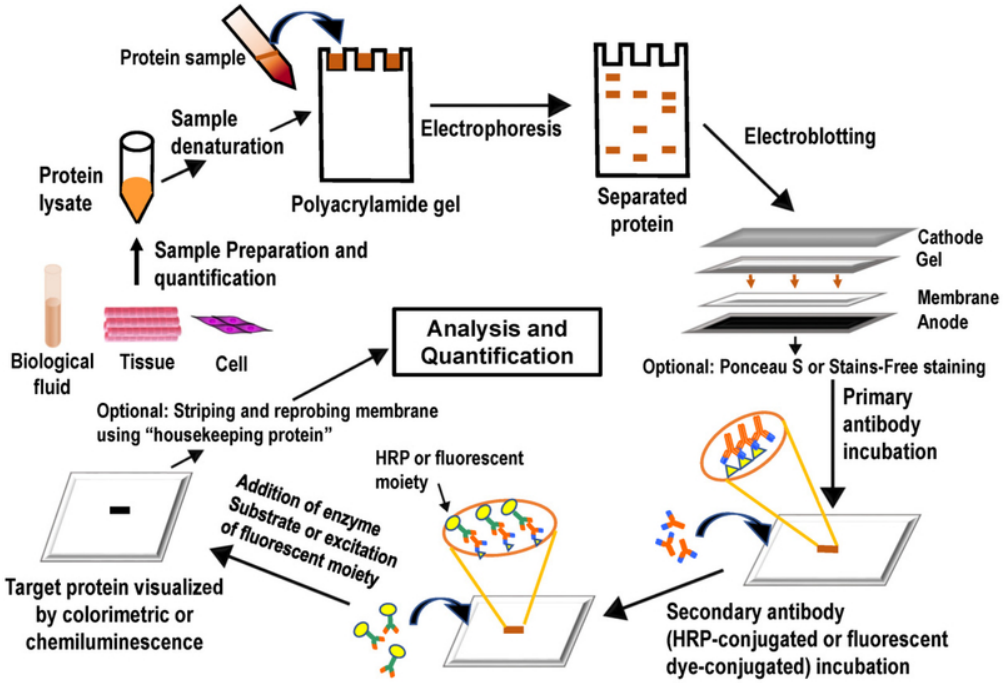

Figure 3 *shows the principle of the western blot method, Adapted from Protein purification and analysis: next generation Western blotting techniques, (Mishra et al., 2017)"*

2.6.2. Sample preparation

The preparation of the sample is the first step in this method. This step is critical because protein extraction and purification have a significant impact on the results. The cells or tissues are frozen with liquid nitrogen and kept in -80°C, following by lysing the samples. The most common way for lysing the cells and tissues is a mechanical homogenisation to be sure about lysis of the cells and disruption of the cell membranes. Homogenization should be done at 4°C

and in a specific buffer containing protease inhibitor which can avoid protein denaturation. The proteins we're looking for can be found in different part of the cells such as cell membrane, mitochondria, or the nucleus. The protein concentration of the samples should be determined after protein extraction and this step is highly critical for calculating the exact amount of protein mass to apply on the each well in the gel. The Bicinchoninic acid assay (BCA) assay is the biochemical assay used to measure the overall quantity of protein in a solution. A purple colour is formed by bicinchoninic acid after the reduction of Cu+2 to Cu+1 in the alkaline solution. The protein's four amino acids, cysteine, cystine, tyrosine, and tryptophan, cause this reduction. This is an important step in reducing differences in the protein composition (He, 2011).

After determination of protein concentration, samples are diluted to desire protein concentration by Mili-Q water, following by adding sample buffer for visualization during the electrophoresis process. In the final step of samples preparation, stained samples are heated at 95°C in order to induce protein denaturation.

2.6.3. Gel electrophoresis

The samples would then be placed on the polyacrylamide gel. The principle of gel electrophoresis is based on the difference of two gels: upper gel naming stacking and lower gel identifying separating. Both gels are made of Mili Q, which is ultrapure water extracted directly from potable water at a flow rate of 8 L/hr, gel buffers, Acrylamide Bis solution, sodium dodecyl sulfate (SDS), which is required for the electrophoresis part, Ammonium persulfate (APS), an oxidizing agent, and finally tetramethylethylenediamine, also known as Temed. The only differences between these two gels are the pH of the separation and stacking buffers and also the concentration of the acrylamide; the pH of the stacking gel is 6.8 and also has a lower concentration of the acrylamide, therefore it is extremely porous while the pH of the separating gel is 8.8 with a higher concentration of acrylamide which cause protein distribution on the gel according to their molecular weight. Consequently, the Low molecular weight proteins move through the gel more quickly than high molecular weight proteins, and vice versa.

The marker and prepared samples with sample buffer from previous step should be loaded into the separated wells in the gel. The applied voltage forces the negatively charged proteins to move towards the gel, from anode side to the cathode side and as a result, the proteins separate into individual lines so-called protein bands. Moreover, the marker represents a

mixture of known molecular weight proteins allowing identification of the protein band of interest according to its molecular weight.

2.6.4. Blotting

Following by electrophoresis, the electricity current drives negatively charged proteins from the gel to the membrane. There are two types of membranes: nitrocellulose and polyvinylidene fluoride (PVDF). The pore size, buffer used, and detection are slightly different. The transfer will take place in a complex known as the " Sandwich." The cassette, sponge, gel, filter papers and membrane make the sandwich. The membrane is placed between the gel and the positive electrode, and consequently, the traveling proteins can bind to the membrane. There are two methods for transferring namely, Semi-dry or more reliable Wet way.

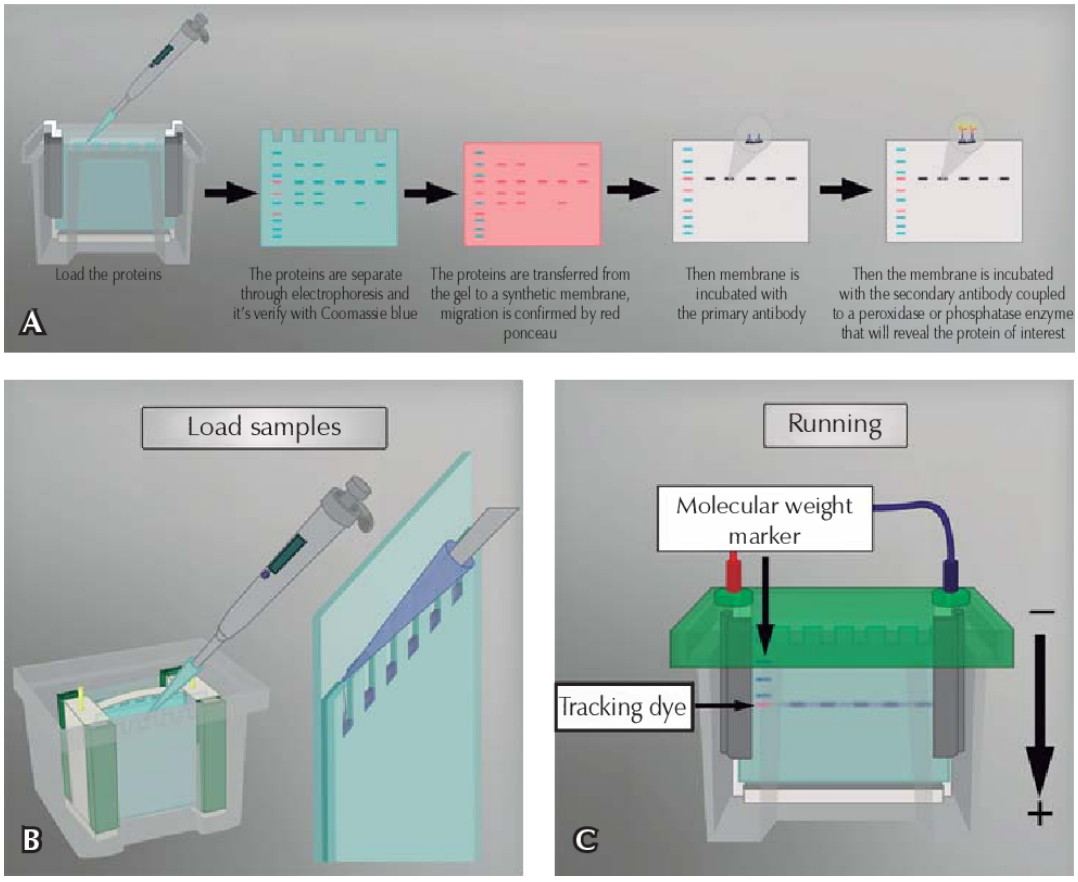

Figure 4 *shows the sample loading and running gels in the electrophoresis Adapted from "Western blot: a tool in the biomedical field" (Martínez et al., 2017)*

2.6.5. Blocking and antibodies

It is extremely essential to avoid unwanted interaction between the antibodies and the membrane before applying the antibodies on the membrane. By concealing the non – specific binding of the antibodies in the membrane, the blocking agent reduces the possibility of a false result. Blocking is a protein diluted solution, and a non-fat milk is the most common blocking solution.

After the blocking step, the membrane is incubated in a determined concentration of the primary antibody to bind to the protein of interest. This procedure requires an overnight incubation at +4°C. The membranes would then be washed with TBST buffer to remove the unbounded antibodies. Following by washing, the membrane is incubated in secondary antibody and this antibody binds the primary antibody, which is usually Horse Radish Peroxidase (HRP) and Alkaline Phosphatase (ALP). Lastly, the membranes are washed again to remove any remaining unbound secondary antibody.

2.6.6. Chemiluminescence detection

The detection with Chemiluminescence would be the final step. This occurs in a machine known as ChemiDoc. ChemiDoc is an imaging machine that uses a simple and high-resolution system to image and quantify gels and blots. Once the chemiluminescent reagent is added on the membrane, HRP cleaves such a substrate to produce a signal of color that can be detected by the machine. The obtained data do not provide the absolute amount of protein, but relatively comparison between the samples is possible. Therefore, Western blot is a common method to compare specific proteins expression in different cells and tissues.

3. Aim

In this diploma thesis, we hypothesize that carotuximab affects liver alterations by modulating Eng and sEng in experimental liver fibrosis and inflammation caused by the DDC diet. We tried to clarify the role of endoglin in mouse model of liver cholestasis and fibrosis. Finally, based on the results, we would like to conclude whether carotuximab can prevent liver alteration and function in proposed pathological experimental conditions by modulating Eng expression, its signaling and sEng level.

4. Experimental part

4.1. Method

4.1.1. Animals

Experiment was performed with eighteen 3-month-old C57BL/6 male mice weighting 21-28 g, housed with a 12-hour light/dark cycle, and permitted ad libitum consumption of water. The mice were divided into three groups: the control group (n=6), the DDC group, and the TRC105 group. Whereas the control group was fed by standard chow diet, the DDC group (n=6) and TRC105 group (n=6) were fed by 0.1% DDC-supplemented diet. During the experimental time, the control group and DDC group were administrated by physiological solution (15 mg/kg) while the TRC105 group was administrated by TRC105 (15 mg/kg) twice a week i. p. following by sacrificing the mice after four weeks with subsequent blood collection and molecular analysis of liver samples. All animals received care according to the guidelines set by the Ethical Committee of the Charles University, Faculty of Pharmacy in Hradec Králové for ensuring laboratory animal's welfare. All planned experiments were in accordance with the Czech Law No. 246/1992 Sb. (Date: October 27, 2020)

4.1.2. Western blot

4.1.2.1. Sample preparation

Liver tissues were collected and frozen in liquid nitrogen quickly, following by keeping in -80°C until homogenization. The liver tissues were homogenized, and total fraction and crude plasma membrane fraction were prepared. The BCA method was then used to determine the concentration of the protein sample. As it is very important to have the same amount of protein per well in the gel, the samples were diluted by Mili-Q to have 25 ug of protein in each well.

The samples were centrifuged for 45 seconds, then boiled them for 5 minutes before centrifuging them for 30 seconds. The samples were then added to the polyacrylamide gel that we had previously prepared.

It is important to assemble a Mini-PROTEAN Tetra Cell kit (*Bio-Rad laboratories, Inc., CA, USA*) properly before preparation of the gels for the electrophoresis, including of a transparent stand and two smaller green stands. For each gel, two different sized glass plates,

longer and shorter is needed. The longer has raised bands on its sides which the shorter is placed on them and a space between the slides can be created for poring the gel. The glass plates then carefully were inserted into the green stand and then fixed by closing the side green door. Subsequently, the ready slides were placed on the transparent stand. Appropriate seating of the slides is extremely important to prevent exit of the poured gel.

4.1.2.2. Gel preparation

4.1.2.2.1. Separating gel

Based on the molecular weight of the desired protein, different percentages of separating gel should be created. For detection of Eng and MMP14 protein expression, 7.5% and 10% gel were prepared respectively (Table 1). Firstly, all required component according to the table 1 were mixed in a beaker properly. Speed was important as the addition of TEMED initiates the polymerization of the gel. Secondly, the prepared gel solution was pipetted between the glass slides. The pipetting should be done in a manner to minimize air access, following by coating the gel with isobutanol along its entire length. The polymerization time varied 30-60 minutes and a small residual gel in the beaker served as a control for the progress of gel polymerization. Once the gel was polymerized, the isobutanol was poured out and the slides was washed with Mili-Q. The excess water between the slides was then dried via filter paper.

***Table 1**: Composition of separating gel*

Component	**7.5% gel**	**10% gel**
Milli-Q water	5,500 ml	4,900 ml
Separating gel buffer	2,500 ml	2,500 ml
Acrylamide-Bis Solution	1,900 ml	2,500 ml
10% (w/v) SDS	0,100 ml	0,100 ml
10% APS	0,030 ml	0,030 ml
TEMED	0,015 ml	0,015 ml

4.1.2.2.2. Stacking gel

All required component for stacking gel were mixed in a beaker appropriately according to the table 2. Following the addition of TEMED, the stacking gel was immediately placed on

the top of the polymerized separating gel, all the way to the edge of the shorter slides. Subsequently, a plastic comb was pressed into the unpolymerized stacking gel quickly and carefully. As a result of pouring out of the gel after inserting the comb, it is necessary to add gel solution into both right and left corners of the comb. It takes 30-60 minutes for gel polymerization and again as a control of the polymerization phase, the rest of the gel in the beaker was checked.

Table 2: *Composition of stacking gel*

Component	**5% gel**
Milli-Q water	6,150 ml
Stacking gel buffer	2,50 ml
Acrylamide-Bis Solution	1,250 ml
10% (w/v) SDS	0,100 ml
10% APS	0,030 ml
TEMED	0,015 ml

4.1.2.3. Applications of samples on gels

The samples were diluted by pipetting the determined volume of sample and Mili-Q water into new Eppendorf tubes. The tubes were then vortexed, and an equivalent volume of sample buffer was added to each sample (6 ul sample+ 6 ul sample buffer).

Then, they were centrifuged at 5000 rpm for 45 second and placed in a water bath preheated to 95 °C for 5 min. Centrifugation was repeated for another 30 second before applying the samples on the gel.

This was followed by the preparation of running buffer for electroporation. 100 ml of buffer (10x Tris/Glycine/SDS, *Bio-Rad Laboratories, Inc., CA, USA*) was measured into a measuring cylinder and reached it to 1000 ml with MilliQ water. The green slides were removed from the transparent rack and the slides with gels were freed from the racks by carefully rotating the green doors. These were then positioned into the Mini-PROTEAN® Tetra Cell attachment and their shorter slides facing inwards. The electrophoretic chamber system was assembled carefully and properly and checked for leaking also. The created space between the slides was filled with running buffer. While the wells were under running buffer, the comb was pulled out carefully in order to not destroy the wells. Then, the marker and samples were applied into the

wells. A 10 ul of the marker was applied in the first well as a known molecular weight (Precision Plus Protein ™Dual Color Standards, *Bio-Rad Laboratories, Inc., CA, USA*) for being use as a reference, following by pipetting a 10 ul of samples from left to right by inserting the pipette tip between the glass slides under the running buffer level and injecting the samples slowly and carefully. At the end, the remaining space of the tank was filled up to the line marking the 2 gels with running buffer.

4.1.2.4. Gel electrophoresis

The tank was closed with a lid containing electrodes and it connected to the power source according to the color code (red to red, black to black). The instrument was set according to table 3. As the instrument was heating up during the gel electrophoresis, two ice pads were placed on both sides of the electrophoresis tank during the electrophoresis process.

Table 3*: Values of gel electrophoresisconditions*

Voltage	200 V
Electric current	120 mA
Time	35 minutes

4.1.2.5. Blotting

Preparation of membranes

Before transferring of the proteins, it is essential to activate the membranes. A PVDF membrane (Immobilon®-P PVDF Membrane, *Sigma-Aldrich Inc., MO, USA) was* cut according to a template (9.5 cm*6.5 cm). The membrane was covered on both sides with a blue paper to avoid direct contact with the membranes. The membrane was carefully removed by using tweezer and placed in a bath of methanol for 30 seconds with shaking since the membrane is not directly wettable following by transferring the membrane to a Transfer buffer and keep it on the shaker for 25 minutes. The activation was repeated with the second membrane.

Preparation of transfer (blotting) buffer

Transfer buffer must be prepared before proteins can be transferred from the gel to the membrane. This preparation starts with 100 ml of transfer buffer (10x Tris/Glycine/Buffer for

Western Blots and Native Gels, Bio-Rad Laboratories, Inc., CA, USA) in a graduated cylinder, followed by 700 ml Mili-Q water and 200 ml methanol. Since 10x transfer buffer reacts with methanol strongly and make the container so hot, it is very important to add Mili-Q between them.

Wet way

For this thesis, the wet way method with Mini Trans-Blot® Cell kit (*Bio-Rad Laboratories, Inc., CA, USA)* was used for transferring proteins from the gel to the membrane. Two soaked black sponges and a soaked white think filter paper were placed on the black side of the cassette, to assemble the so-called sandwich. The sponges and filters were soaked in pre-prepared transfer buffer previously. The black side of the cassette represented the negative pole and the transparent side the positive pole.

The slides glasses containing the gel were carefully opened, the gel was removed and transferred on the center of the thick filter paper following by placing the activated membrane on top of it. Once the membrane was placed on the gel, it was no longer allowed to be moved. Then, another thick filter paper was placed on top of it and the air bubbles were carefully removed by using a roller. Lastly, two black sponges were placed on it and the sandwich was closed. The prepared cassette containing the sandwich was placed in the rack according to color, so that the black side of the cassette was oriented towards the black side and the transparent side of the cassette towards the red side of the rack. The rack was placed in the tank that was filled with transfer buffer up to the blotting mark. After closing the tank and connecting the electrodes to the source, the voltage, current and time values were set on the instrument (see Table 4).

Table 4*: Values of electrophoretic transfer conditions*

Voltage	140 V
Current	300 mA
Time	1.5 hr

After the transferring time, the membrane removed carefully from the sandwich and placed in a tray containing transfer buffer.

4.1.2.6. Blocking antibody application

Wash buffer TBS-T

For the subsequent preparation of the so-called blocking buffer and membrane washing, it was necessary to prepare the washing buffer (TBS-T) in advance. 100 ml of 10× concentrated TBS (Tris buffer saline) buffer was mixed with 900 ml of ultrapure water, 1 ml of Tween® 20 (*SERVA Electrophoresis GmbH, Germany*) was added and the solution was allowed to mix on a magnet stirrer.

Blocking of membranes

The membranes were removed from the transfer buffer tray, oriented in a way that the marker was located on the left side. According to the expected molecular weight of the protein of interest the membranes were cut into strips and marked with pencil. Table 5 presents the size of studied protein in this research.

Table 5*: Molecular weights of the proteins*

Protein	Molecular weight (kDa)
Endoglin	130
MMP14	60-66
GAPDH*	37

**GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) represents a control for the applied samples.*

The membranes were placed in a blocking buffer (TBS-T-5% milk) to avoid any non-specific reactions between the membrane and the antibody. Blocking buffer was prepared by dissolving 5 g of low-fat milk powder (Blotting-Grade Blocker, *Bio-Rad Laboratories, Inc., CA, USA*) in 95 ml of washing buffer (TBS-T), and the membranes were blocked in it for 1 h on a shaker at room temperature.

Incubation of membranes with primary and secondary antibodies

Primary antibody precisely binds to the protein of interest on the membrane. The used primary antibodies for this thesis diluted in blocking buffer according to table 6. The membranes were placed in the pre-prepared parafilm boxes with facing upwards and the primary antibody was immediately pipetted onto the membrane, following by keeping them on

the shaker at room temperature for one hour. After that, they were refrigerated overnight. The membranes were washed with TBS-T for 10 minutes the next day, and the process was repeated five times. After that, the primary antibody was incubated with a secondary antibody that had HRP bound to it. The secondary antibodies were diluted to the specified concentration in blocking buffer (see Table 7) and applied to the washed membranes and placed prepared parafilm trays. This time the incubation lasted 1 hour, already at laboratory temperature, and was followed by washing in the same manner as after incubation with the primary antibody.

***Table 6**: Primary antibodies dilution*

Primary antibody	Producer	Catalog number	Host	
GAPDH	Cell Signaling Technology (*MA, USA*)	2118	Rabbit	1:8000
Endoglin	Santa Cruz (*Inc., TX, USA*)	sc19793	Goat	1:1000
MMP14	Abcam (Cambridge, United Kingdom)	ab51074	Rabbit	1:2000

***Table 7**: Secondary antibodies and dilution*

Protein	Secondary antibody**	Producer	Catalog number	Host	Dilution
GAPDH	Anti-rabbit	Cell Signaling	7074S	Goat	1:10000
Endoglin	Anti-Goat	*(Sigma-Aldrich Inc., MO, USA)*	sc19793	Rabbit	1:2000
MMP14	Anti-Rabbit	(*Abcam, Cambridge, UK*)	Ab6112	Goat	1:4000

***Secondary antibodies were labeled with the enzyme HRP (horseradish peroxidase), which ensures cleavage of the detection reagent and color reaction*

4.1.2.7. Chemiluminescence detection

The chemiluminescent substrates should be prepared firstly. The reagent number 1 and 2 were mixed 1:1 ratio (500 ul reagent 1+ 500 ul reagent 2 per each membrane), following by placing membranes on a strong plastic sheet in a way that proteins facing upwards. The chemiluminescent reagent (*Thermo Fisher Scientific Inc., IL, USA*), Pico (Super Signal West Pico PLUS Chemiluminescent Substrate) was applied to the membranes. The exposure time of Pico substrate cleaved by horseradish peroxidase bound to the secondary antibody, was determined for each protein of interest. The reaction time could be adjusted if necessary (see Table 8). After that, the reagent was allowed to drain from the membrane and the membrane was immediately placed in the prepared plastic cassette. Excess bubbles were squeezed out using a tissue and placed the membrane in the ChemiDoc machine. The quantification of the wetern blot images were done by ImageLab imaging software version 6.0.1 (Bio–Rad). The immunodetection of GAPDH confirmed the equal loading of proteins onto the gel. Afterward, the obtained results were statistically evaluated using GraphPad Prism 8 software.

Table 8*: Reaction time of reagent (chemiluminescent substrate)*

Protein	Reagent/ time	Exposure time
GAPDH	Pico/ 3 min	30 sec
Endoglin	Pico/ 4 min	150 sec
MMP14	Pico/ 4 min	50 sec

4.1.3. ELISA

The concentration of sEng in the plasma of both control and DDC mice was determined using an Enzyme-Linked ImmunoSorbent Assay (ELISA). In duplicates, plasma was tested using the Mouse Endoglin/CD105 Quantikine ELISA kit (DNDG00, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The results obtained in unit of ng/ml were compared between the control and DDC group.

4.1.4. Biochemical analysis

Blood sample was collected from the inferior vena cava, following by using 100 ul of each blood sample for measuring liver enzymes in the blood using commercial Preventive Care Profile Plus test and VetScan2 device (Abaxis Germany).

4.1.5. Statistical analysis

All analyses were carried out using the GraphPad Prism 8.0 software (San Diego, CA, USA). Statistical evaluation was performed by Non-parametric ANOVA (Kruskal – Wallis test), using GraphPad Prism 8.0 software (San Diego, CA). A p value < 0.05 was considered statistically significant.

5. Results

5.1. DDC diet induces liver impairment in mice

5.1.1. The effect of DDC on the ratio of liver weight to the body

When compared to mice fed the chow diet, mice fed the DDC diet had a significant increase (30%) in the ratio of liver weight to body weight, suggesting liver damage induced by DDC diet. Although the DDC diet successfully induced liver damage, carotuximab did not significantly influence the ratio liver weight to body weight (Figure 5).

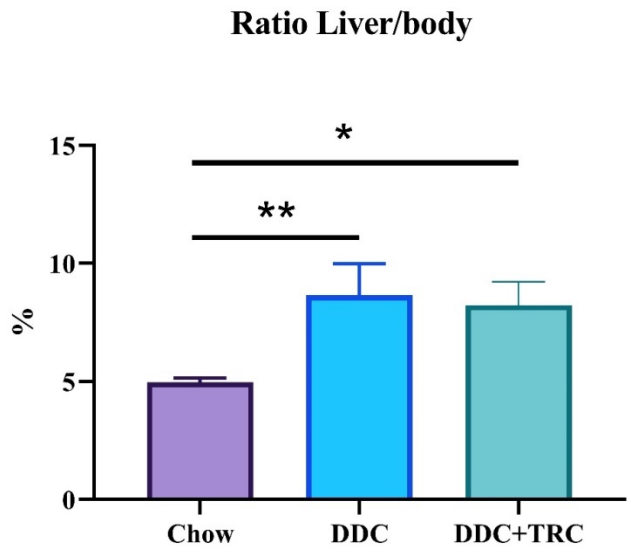

Figure 5 *DDC diet effects on the ratio liver weight to body weight. The data are presented as mean ± S.E.M, (n = 6). * p < 0.05 ** p < 0.01, *** p < 0.001, using the Kruskal – Wallis test.*

5.1.2. The effect of DDC on liver enzyme

To confirm the liver injury in mice fed a DDC diet, the activity of ALT in plasma was measured. When compared to the control group, there was a significant increase in the level of ALT in the plasma of mice fed a DDC diet, implying that the DDC diet causes liver impairment (Figure 6).

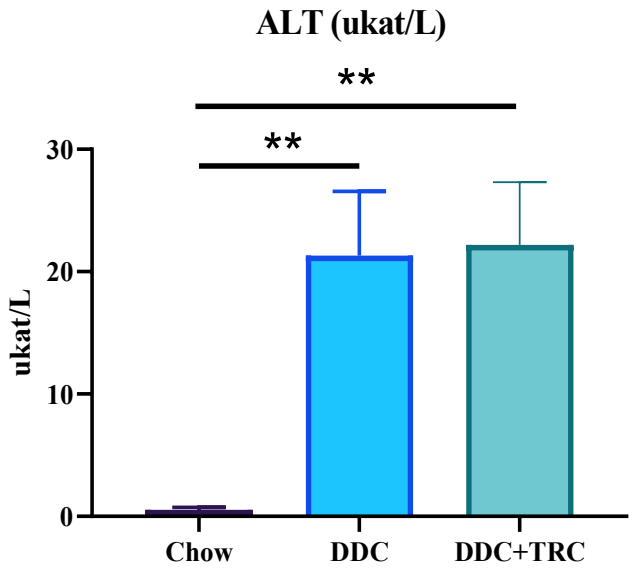

Figure 6 *Impact of the DDC diet on ALT activity in plasma The data are presented as mean ± S.E.M. * p <0.05 ** p < 0.01, *** p < 0.001, using the Kruskal – Wallis test. n= 6 mice per group*

5.1.3. The impact of DDC on total bilirubin

DDC-fed mice had significantly higher levels of TBIL than chow-fed mice, indicating liver injury in DDC-fed mice. Although the DDC diet successfully induced liver impairment, carotuximab did not significantly influence the ratio liver weight to body weight, ALT activity and total bilirubin in plasma (figure 7).

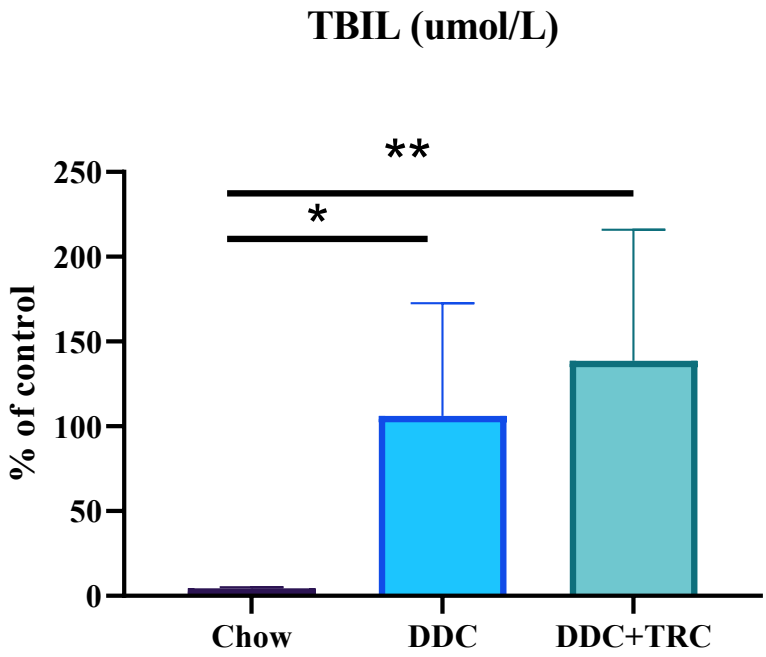

***Figure** 7 Impact of the DDC diet on the level of Total bilirubin in plasma. The data are presented as mean ± S.E.M, (n = 6). * p < 0.05 ** p < 0.01, *** p < 0.001, using the Kruskal – Wallis test. n= 6 mice per group.*

5.2. The effect of DDC and TRC105 on Eng expression, signalling and sEng

5.2.1. DDC modulates Eng expression in liver

Eng protein expression was measured in liver samples to detect the effect of the DDC diet and Carotuximab impact on mice liver. As it is shown in Figure 8, The western blot analysis revealed that the level of Eng protein expression in DDC fed mice was significantly lower than in the control group. Additionally, carotuximab did not show any impact on the damaged liver concerning protein expression of Eng (Figure 8).

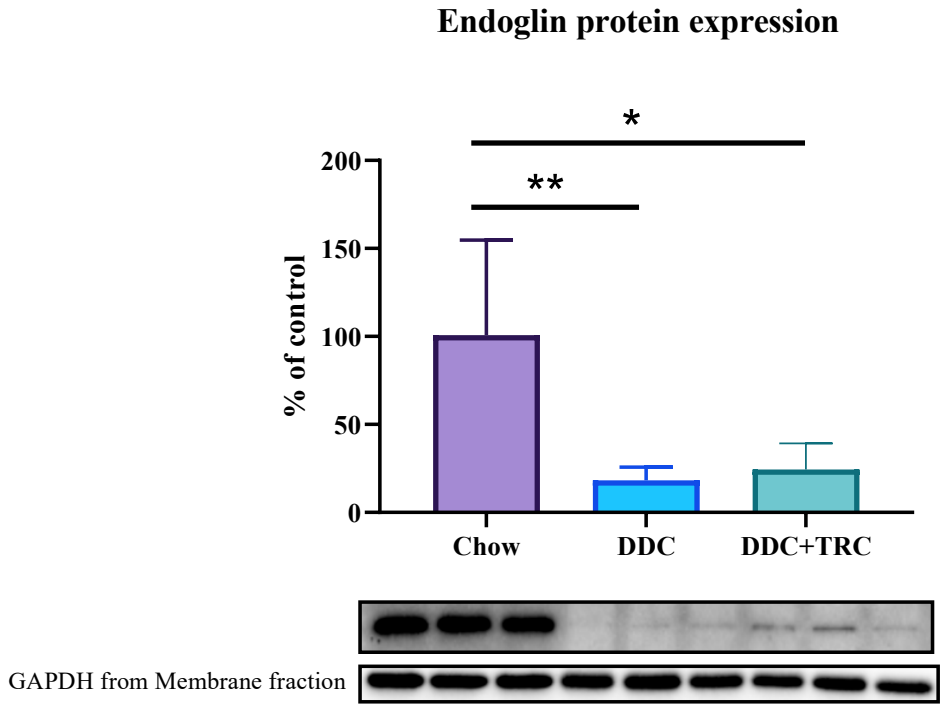

Figure 8 *Endoglin protein expression in mice liver. Densitometric quantification of immunoreactive bands (top panel: densitometric analysis, control = 100%) was recalculated to the GAPDH signal (bottom panel: representative immunoblots). The data are presented as mean ± S.E.M. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$, using the Kruskal – Wallis test. n= 6 mice per group.*

5.2.1.1. The impact of DDC and carotuximab on MMP14 expression

Western blot analysis reported a significant increase in MMP14 protein expression in DDC fed mice compared to the control group, implying liver fibrosis and Eng cleavage in DDC fed mice. On the other hand, MMP14 protein expression did not reach statistically significant between DDC and TRC treated groups concerning protein expression of MMP14 (Figure 9).

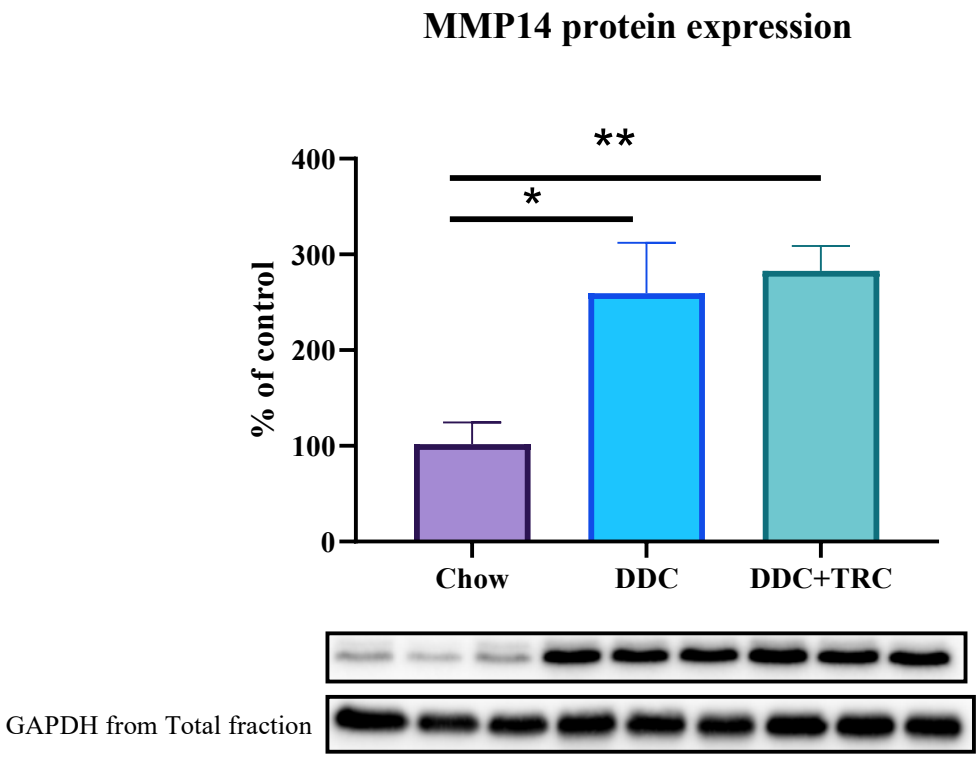

Figure 9 *MMP14 protein expression in mice liver. Densitometric quantification of immunoreactive bands (top panel: densitometric analysis, control = 100%) was recalculated to the GAPDH signal (bottom panel: representative immunoblots). The data are presented as mean ± S.E.M. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$, using the Kruskal – Wallis test. n= 6 mice per group.*

5.2.1.2. The impact of DDC and caotuximab on the level of sEng

The ELISA analysis was performed to measure the level of sEng in the plasma. As expected, the significantly higher level of sEng was detected in the plasma of the DDC group mice compared to the control group suggesting cleavage of liver tissue Eng via MMP14 protein and releasing of sEng into the blood stream (Figure 10).

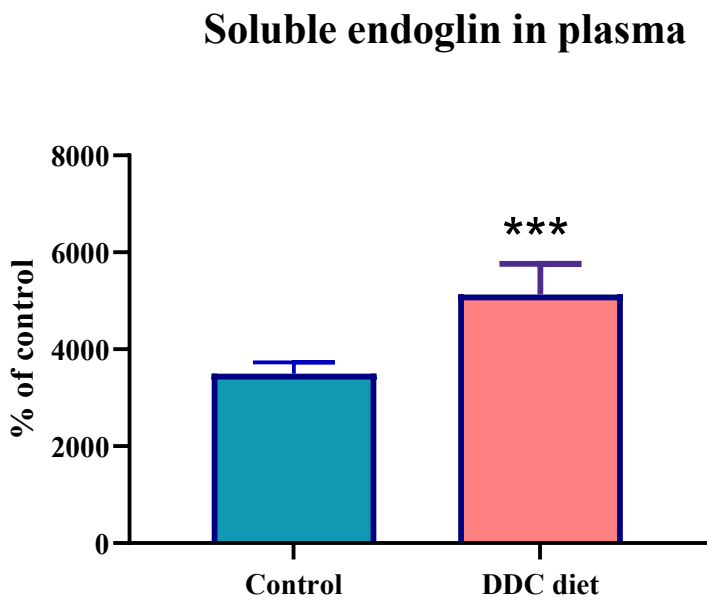

Figure 10 *Changes in the level of sEng in the plasma of the control and DDC group. The data are presented as mean ± S.E.M. * p < 0.05 ** p < 0.01, *** p < 0.001. n= 6 mice per group.*

6. Discussion

Eng (CD105) is a 180KDa glycoprotein that functions as a coreceptor for binding to the TGF-β superfamily. Endothelial cells, fibroblasts, vascular smooth muscle cells, HSCs, activated monocytes, and macrophages are the primary cells that express Eng (Bot et al., 2009; Lastres et al., 1992; López-Novoa & Bernabeu, 2010; S. Meurer et al., 2019; S. K. Meurer et al., 2011; Rathouska et al., 2015). Eng has been a popular topic among scientists in recent years. Some studies claim that Eng has profibrotic activity, while others claim that it has antifibrotic activity. The cleavage of membrane endoglin by the proteolytic MMP14 results in the formation of sEng, which enters the circulation. sEng was previously involved in several disease groups. sEng was initially involved in a variety of disease groups. sEng has been found in tumor patients' plasma (Pérez-Gómez et al., 2010). sEng levels in the plasma of patients with CFLD and HCC combined with cirrhosis have been found to be higher (Yagmur et al., 2007). It has previously been demonstrated that an increase in plasma sEng may be a circulating biomarker of NASH. In addition, sEng levels were increased in liver diseases suggesting sEng might represent a potential biomarker of liver injury, including fibrosis and/or nonalcoholic steatohepatitis. This is most likely due to increased Eng expression in the liver and elevated levels of MMP14, which causes sEng cleavage (Igreja Sá et al., 2020). However, the precise impact of Eng expression and signaling changes, as well as sEng levels, during liver cholestasis and fibrosis is not conclusive.

Liver fibrosis is the unusual buildup of ECM proteins such as collagen that takes place in many chronic liver diseases. The most common cause of chronic liver disease is NAFLD(Byrne & Targher, 2015). NAFLD is caused by fat accumulation in the liver when there is no or little alcohol consumption, which is linked to inflammation. NAFLD, which is essentially the hepatic manifestation of the metabolic syndrome, is mostly linked to insulin resistance, which includes diabetes, obesity, and hyperlipidemia.

Carotuximab is a monoclonal antibody that binds to Eng, affecting not only its expression but also its signaling and sEng levels. Carotuximab was first developed as an anti-cancer drug. It was initially developed for use in oncology, with the goal of targeting proliferating endothelial cells in solid tumor vasculature (Liu et al., 2021; Rosen et al., 2014). It is currently being tested in clinical trials for AMD (Liu et al., 2021). A wide range of research have been carried over the years to explain carotuximab's efficacy in anti-Eng therapy. However, there are

no studies showing whether Carotuximab effects on Eng expression in liver could affect liver pathology.

In our study, the levels of sEng in the bloodstream and Eng expression were discussed in relation to liver fibrosis (in animal model of liver cholestasis and fibrosis). We induced liver fibrosis with the DDC diet. Chronic DDC-feeding in mice is a well-established model of cholestatic liver injury originally, which is specifically associated with metabolic liver injury, as observed in alcoholic and nonalcoholic steatohepatitis, based on DDC ability to induce chronic oxidative cell stress. Conversely, cholestatic effects exerted by DDC depends upon the ability to stimulate biliary porphyrin secretion that after a 4-week treatment leads to the generation of intraductal pigment plugs. Due to these properties, chronic DDC feeding results in periductal fibrosis, which slowly progressed over time (Mariotti et al., 2018).

Afterward, we measured Eng expression and sEng levels after the fibrosis. Later, we discussed about the effect of carotuximab on Eng expression and sEng.

In the experiment, three-month-old male mice were divided into three groups based on diet intake and carotuximab administration. The control group was our first group. We fed them the chow diet and administered them with the physiological solution (15 mg/kg) twice a week. The DDC group was the second. We fed them DDC diet and administered physiological solution (15 mg/kg) twice per week. The third group was given a DDC diet, but instead of a physiological solution, they were given TRC105 (15 mg/kg) twice a week. The liver tissues were collected after the mice were sacrificed.

Elevated levels of ALT and TBIL were discovered in biochemical analysis. ALT and TBIL have been shown to be biomarkers of liver fibrosis. A high ALT level is linked to exacerbating liver inflammation and fibrosis. TBILI has been also linked to hepatocellular disease and biliary obstruction (Guerra Ruiz et al., 2021; W. R. Kim et al., 2008; Schultze, 2007). The increased enzyme level was accompanied by an increase in the liver-to-body weight ratio. This confirmed the liver damage observed in mice. However, carotuximab had no effect on the ratio of liver weight to body weight, ALT activity, or total bilirubin in plasma.

Surprisingly, the western blot analysis for the three groups' samples revealed a significant decrease in Eng protein expression in DDC fed mice compared to the control group. Additionally, carotuximab did not show any impact on the damaged liver concerning protein expression of Eng. These data are contradictory to our previous paper where we showed increase endoglin expression in liver in NASH animal model compared to the control mice

(Igreja Sá et al., 2020). The explanation could be related to the different pathological stimulus here by DDC diet (cholestasis model) and NASH diet. In any case, we propose that endoglin is not participating in fibrosis in DDC fibrosis animal model. Indeed, Carotuximab treatment did not affect endoglin expression because it was already reduced by DDC feeding which was never demonstrated before. Moreover, we may propose that cholestatic induced fibrosis is not related to endoglin expression.

Following that, a significant increase in MMP14 protein expression in DDC fed mice compared to the control group was confirmed by the western blot, implying liver fibrosis and Eng cleavage in DDC fed mice in which did not differ significantly between the DDC and TRC105 treated groups.

Finally, ELISA was used to find the concentration of sEng in the circulation. As expected, significantly higher levels of sEng were detected in the plasma of DDC group mice compared to the control group, implying Eng cleavage via MMP14 protein and sEng release into the bloodstream. These data are in line with our previously published paper where liver injury caused by NASH resulted in increase of MMP-14 expression and increased sEng levels.

7. Conclusion

In our study, we wanted to investigate the role of endoglin in a mouse model of liver fibrosis and evaluate the effect of TRC105 on liver alteration by modulating both Eng and sEng levels in a mouse model of liver injury.

Biochemical analysis confirmed that we successfully caused liver impairment with the DDC diet and confirmed the liver impairment with increased levels of ALT and total Bilirubin, followed by an increased liver-to-body ratio. After confirming the liver impairment, we measured the levels of Eng, sEng, and MMP14.

Surprisingly, the western blot method showed that the presence of DDC diet significantly reduced the expression of Eng. The project was continued by measuring the expression of MMP14 enzyme, which was found to be higher in DDC-fed mice. Finally, it was revealed that the level of sEng in the plasma was considerably higher in mice fed DDC. However, in all analyses, there was no statistically significant difference between the DDC diet-only group and the TRC105-administered group.

We can conclude that the level of MMP14 enzyme was increased in the presence of DDC diet, resulting in more cleavage of membrane Eng and more sEng in the plasma. This suggest that soluble endoglin might be biomarker of liver fibrosis/cholestasis development. However, carotuximab treatment had no significant effect on the Eng and sEng levels in the DDC diet.

8. References

Adam, P. J., Clesham, G. J., & Weissberg, P. L. (1998). Expression of endoglin mRNA and protein in human vascular smooth muscle cells. Biochemical and Biophysical Research Communications, 247(1). https://doi.org/10.1006/bbrc.1998.8734

Ahluwalia, M. S., Rogers, L. R., Chaudhary, R. T., Newton, H. B., Seon, B. K., Jivani, M. A., Adams, B. J., Shazer, R. L., & Theuer, C. P. (2016). A phase 2 trial of TRC105 with bevacizumab for bevacizumab refractory glioblastoma. Journal of Clinical Oncology, 34(15_suppl), 2035. https://doi.org/10.1200/JCO.2016.34.15_suppl.2035

Albiñana, V., Zafra, M. P., Colau, J., Zarrabeitia, R., Recio-Poveda, L., Olavarrieta, L., Pérez-Pérez, J., & Botella, L. M. (2017). Mutation affecting the proximal promoter of Endoglin as the origin of hereditary hemorrhagic telangiectasia type 1. BMC Medical Genetics, 18(1). https://doi.org/10.1186/s12881-017-0380-0

Almazroo, O. A., Miah, M. K., & Venkataramanan, R. (2017). Drug Metabolism in the Liver. Clinics in Liver Disease, 21(1), 1–20. https://doi.org/https://doi.org/10.1016/j.cld.2016.08.001

Anderson, E. N., & Wharton, K. A. (2017). Alternative cleavage of the bone morphogenetic protein (BMP), Gbb, produces ligands with distinct developmental functions and receptor preferences. Journal of Biological Chemistry, 292(47). https://doi.org/10.1074/jbc.M117.793513

Attisano, L., & Lee-Hoeflich, S. T. (2001). The Smads. Genome Biology, 2(8), REVIEWS3010–REVIEWS3010. https://doi.org/10.1186/gb-2001-2-8-reviews3010

Bai, Q., Yan, H., Sheng, Y., Jin, Y., Shi, L., Ji, L., & Wang, Z. (2017). Long-term acetaminophen treatment induced liver fibrosis in mice and the involvement of Egr-1. Toxicology, 382, 47–58. https://doi.org/https://doi.org/10.1016/j.tox.2017.03.008

Ballestri, S., Zona, S., Targher, G., Romagnoli, D., Baldelli, E., Nascimbeni, F., Roverato, A., Guaraldi, G., & Lonardo, A. (2016). Nonalcoholic fatty liver disease is associated with an almost twofold increased risk of incident type 2 diabetes and metabolic syndrome. Evidence from a systematic review and meta-analysis. Journal of Gastroenterology and Hepatology (Australia), 31(5). https://doi.org/10.1111/jgh.13264

Bao, Y.-L., Wang, L., Pan, H.-T., Zhang, T.-R., Chen, Y.-H., Xu, S.-J., Mao, X.-L., & Li, S.-W. (2021). Animal and Organoid Models of Liver Fibrosis. Frontiers in Physiology, 12, 666138. https://doi.org/10.3389/fphys.2021.666138

Barbara, N. P., Wrana, J. L., & Letarte, M. (1999). Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-β superfamily. Journal of Biological Chemistry, 274(2). https://doi.org/10.1074/jbc.274.2.584

Basit, H., Tan, M. L., & Webster, D. R. (2020). Histology, Kupffer Cell. In StatPearls.

Bellón, T., Corbi, A., Lastres, P., Calés, C., Cebrián, M., Vera, S., Cheifetz, S., Massague, J., Letarte, M., & Bernabéu, C. (1993). Identification and expression of two forms of the human transforming growth factor-β-binding protein endoglin with distinct cytoplasmic regions. European Journal of Immunology, 23(9). https://doi.org/10.1002/eji.1830230943

Bidart, M., Ricard, N., Levet, S., Samson, M., Mallet, C., David, L., Subileau, M., Tillet, E., Feige, J. J., & Bailly, S. (2012). BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain. Cellular and Molecular Life Sciences, 69(2). https://doi.org/10.1007/s00018-011-0751-1

Biernacka, A., Dobaczewski, M., & Frangogiannis, N. G. (2011). TGF-β signaling in fibrosis. Growth Factors (Chur, Switzerland), 29(5), 196–202. https://doi.org/10.3109/08977194.2011.595714

Bot, P. T. G., Hoefer, I. E., Sluijter, J. P. G., van Vliet, P., Smits, A. M., Lebrin, F., Moll, F., de Vries, J.-P., Doevendans, P., Piek, J. J., Pasterkamp, G., & Goumans, M.-J. (2009). Increased expression of the transforming growth factor-beta signaling pathway, endoglin, and early growth response-1 in stable plaques. Stroke, 40(2), 439–447. https://doi.org/10.1161/STROKEAHA.108.522284

Bragdon, B., Moseychuk, O., Saldanha, S., King, D., Julian, J., & Nohe, A. (2011). Bone Morphogenetic Proteins: A critical review. In Cellular Signalling (Vol. 23, Issue 4). https://doi.org/10.1016/j.cellsig.2010.10.003

Byrne, C. D., & Targher, G. (2015). NAFLD: A multisystem disease. In Journal of Hepatology (Vol. 62, Issue S1). https://doi.org/10.1016/j.jhep.2014.12.012

Castonguay, R., Werner, E. D., Matthews, R. G., Presman, E., Mulivor, A. W., Solban, N., Sako, D., Pearsall, R. S., Underwood, K. W., Seehra, J., Kumar, R., & Grinberg, A. v. (2011). Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. Journal of Biological Chemistry, 286(34). https://doi.org/10.1074/jbc.M111.260133

Cheifetz, S., Bellón, T., Calés, C., Vera, S., Bernabeu, C., Massagué, J., & Letarte, M. (1992). Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. Journal of Biological Chemistry, 267(27), 19027–19030. https://doi.org/https://doi.org/10.1016/S0021-9258(18)41732-2

Chen, C., Grzegorzewski, K. J., Barash, S., Zhao, Q., Schneider, H., Wang, Q., Singh, M., Pukac, L., Bell, A. C., Duan, R., Coleman, T., Duttaroy, A., Cheng, S., Hirsch, J., Zhang, L., Lazard, Y., Fischer, C., Barber, M. C., Ma, Z. D., … Birse, C. E. (2003). An integrated functional genomics screening program reveals a role for BMP-9 in glucose homeostasis. Nature Biotechnology, 21(3). https://doi.org/10.1038/nbt795

Clemente, M., Núñez, O., Lorente, R., Rincón, D., Matilla, A., Salcedo, M., Catalina, M. v., Ripoll, C., Iacono, O. L., Bañares, R., Clemente, G., & García-Monzón, C. (2006). Increased intrahepatic and circulating levels of endoglin, a TGF-β1 co-receptor, in patients with chronic hepatitis C virus infection: Relationship to histological and serum markers of hepatic fibrosis. Journal of Viral Hepatitis, 13(9), 625–632. https://doi.org/10.1111/j.1365-2893.2006.00733.x

David, L., Mallet, C., Mazerbourg, S., Feige, J. J., & Bailly, S. (2007). Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood, 109(5). https://doi.org/10.1182/blood-2006-07-034124

Delire, B., Stärkel, P., & Leclercq, I. (2015). Animal Models for Fibrotic Liver Diseases: What We Have, What We Need, and What Is under Development. Journal of Clinical and Translational Hepatology, 3(1), 53–66. https://doi.org/10.14218/JCTH.2014.00035

Denk, H., Stumptner, C., & Zatloukal, K. (2000). Mallory bodies revisited. In Journal of Hepatology (Vol. 32, Issue 4). https://doi.org/10.1016/S0168-8278(00)80233-0

Doghish, A. S., Bassyouni, A. A., Mahfouz, M. H., Abd El-Aziz, H. G., & Zakaria, R. Y. (2019). Plasma endoglin in Type2 diabetic patients with nephropathy. Diabetes and

Metabolic Syndrome: Clinical Research and Reviews, 13(1). https://doi.org/10.1016/j.dsx.2018.11.058

Duffy, A. G., Ma, C., Ulahannan, S. v., Rahma, O. E., Makarova-Rusher, O., Cao, L., Yu, Y., Kleiner, D. E., Trepel, J., Lee, M. J., Tomita, Y., Steinberg, S. M., Heller, T., Turkbey, B., Choyke, P. L., Peer, C. J., Figg, W. D., Wood, B. J., & Greten, T. F. (2017). Phase I and preliminary phase II study of TRC105 in combination with sorafenib in hepatocellular carcinoma. Clinical Cancer Research, 23(16). https://doi.org/10.1158/1078-0432.CCR-16-3171

Duffy, A. G., Ulahannan, S. v., Cao, L., Rahma, O. E., Makarova-Rusher, O. v., Kleiner, D. E., Fioravanti, S., Walker, M., Carey, S., Yu, Y., Venkatesan, A. M., Turkbey, B., Choyke, P., Trepel, J., Bollen, K. C., Steinberg, S. M., Figg, W. D., & Greten, T. F. (2015). A phase II study of TRC105 in patients with hepatocellular carcinoma who have progressed on sorafenib. United European Gastroenterology Journal, 3(5). https://doi.org/10.1177/2050640615583587

Fickert, P., Stöger, U., Fuchsbichler, A., Moustafa, T., Marschall, H. U., Weiglein, A. H., Tsybrovskyy, O., Jaeschke, H., Zatloukal, K., Denk, H., & Trauner, M. (2007). A new xenobiotic-induced mouse model of sclerosing cholangitis and biliary fibrosis. American Journal of Pathology, 171(2). https://doi.org/10.2353/ajpath.2007.061133

Fickert, P., Trauner, M., Fuchsbichler, A., Stumptner, C., Zatloukal, K., & Denk, H. (2003). Mallory body formation in primary biliary cirrhosis is associated with increased amounts and abnormal phosphorylation and ubiquitination of cytokeratins. Journal of Hepatology, 38(4). https://doi.org/10.1016/S0168-8278(02)00439-7

Frangogiannis, N. (2020). Transforming growth factor-β in tissue fibrosis. The Journal of Experimental Medicine, 217(3), e20190103–e20190103. https://doi.org/10.1084/jem.20190103

G, G. F., & Erkki, R. (1999). Integrin Signaling. Science, 285(5430), 1028–1033. https://doi.org/10.1126/science.285.5430.1028

Gäbele, E., Dostert, K., Dorn, C., Patsenker, E., Stickel, F., & Hellerbrand, C. (2011). A New Model of Interactive Effects of Alcohol and High-Fat Diet on Hepatic Fibrosis. Alcoholism: Clinical and Experimental Research, 35(7). https://doi.org/10.1111/j.1530-0277.2011.01472.x

Gao, J., Wang, G. J., Wang, Z., Gao, N., Li, J., Zhang, Y. F., Zhou, J., Zhang, H. X., Wen, Q., Jin, H., & Qiao, H. L. (2017). High CYP2E1 activity correlates with hepatofibrogenesis induced by nitrosamines. Oncotarget, 8(68). https://doi.org/10.18632/oncotarget.22937

Goessling, W., Massaro, J. M., Vasan, R. S., D'Agostino, R. B., Ellison, R. C., & Fox, C. S. (2008). Aminotransferase Levels and 20-Year Risk of Metabolic Syndrome, Diabetes, and Cardiovascular Disease. Gastroenterology, 135(6). https://doi.org/10.1053/j.gastro.2008.09.018

Gonzalez, V. H., Berger, B., Goldberg, R., Gordon, C. M., Khurana, R. N., Angeles, R., & Shams, N. (2021). Safety and Tolerability of Intravitreal Carotuximab (DE-122) in Patients With Persistent Exudative Age-Related Macular Degeneration: A Phase I Study. Translational Vision Science & Technology, 10(14), 27. https://doi.org/10.1167/tvst.10.14.27

Gordon, M. S., Robert, F., Matei, D., Mendelson, D. S., Goldman, J. W., Chiorean, E. G., Strother, R. M., Seon, B. K., Figg, W. D., Peer, C. J., Alvarez, D., Adams, B. J., Theuer, C. P., & Rosen, L. S. (2014). An open-label phase Ib dose-escalation study of TRC105 (anti-endoglin antibody) with bevacizumab in patients with advanced cancer. Clinical Cancer Research, 20(23). https://doi.org/10.1158/1078-0432.CCR-14-1143

Gore, B., Izikki, M., Mercier, O., Dewachter, L., Fadel, E., Humbert, M., Dartevelle, P., Simonneau, G., Naeije, R., Lebrin, F., & Eddahibi, S. (2014). Key Role of the Endothelial TGF-β/ALK1/Endoglin Signaling Pathway in Humans and Rodents Pulmonary Hypertension. PLoS ONE, 9(6), e100310. https://doi.org/10.1371/journal.pone.0100310

Gougos, A., & Letarte, M. (1990). Primary structure of endoglin, and RGD-containing glycoprotein of human endothelial cells. Journal of Biological Chemistry, 265(15). https://doi.org/10.1016/s0021-9258(19)38892-1

Goumans, M.-J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., & ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. The EMBO Journal, 21(7), 1743–1753. https://doi.org/10.1093/emboj/21.7.1743

Grundy, S. M. (2005). Metabolic syndrome scientific statement by the American Heart Association and the National Heart, Lung, and Blood Institute. In Arteriosclerosis,

Thrombosis, and Vascular Biology (Vol. 25, Issue 11). https://doi.org/10.1161/01.ATV.0000189155.75833.c7

Guerra Ruiz, A. R., Crespo, J., López Martínez, R. M., Iruzubieta, P., Casals Mercadal, G., Lalana Garcés, M., Lavin, B., & Morales Ruiz, M. (2021). Measurement and clinical usefulness of bilirubin in liver disease. Advances in Laboratory Medicine / Avances En Medicina de Laboratorio, 2(3). https://doi.org/10.1515/almed-2021-0047

He, F. (2011). BCA (Bicinchoninic Acid) Protein Assay. BIO-PROTOCOL, 1(5). https://doi.org/10.21769/bioprotoc.44

Hnasko, T. S., & Hnasko, R. M. (2015). The Western Blot. In R. Hnasko (Ed.), ELISA: Methods and Protocols (pp. 87–96). Springer New York. https://doi.org/10.1007/978-1-4939-2742-5_9

Hong, S. H., & Choi, K. M. (2020). Sarcopenic obesity, insulin resistance, and their implications in cardiovascular and metabolic consequences. In International Journal of Molecular Sciences (Vol. 21, Issue 2). https://doi.org/10.3390/ijms21020494

Hung, T.-M., Hsiao, C.-C., Lin, C.-W., & Lee, P.-H. (2020). Complex Cell Type-Specific Roles of Autophagy in Liver Fibrosis and Cirrhosis. Pathogens (Basel, Switzerland), 9(3), 225. https://doi.org/10.3390/pathogens9030225

Igreja Sá, I. C., 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. (2020a). Soluble Endoglin as a Potential Biomarker of Nonalcoholic Steatohepatitis (NASH) Development, Participating in Aggravation of NASH-Related Changes in Mouse Liver. International Journal of Molecular Sciences, 21(23), 9021. https://doi.org/10.3390/ijms21239021

Ishida, T., Kotani, H., Miyao, M., Kawai, C., Jemail, L., Abiru, H., & Tamaki, K. (2016). Renal impairment with sublethal tubular cell injury in a chronic liver disease mouse model. PLoS ONE, 11(1). https://doi.org/10.1371/journal.pone.0146871

Iwaisako, K., Brenner, D. A., & Kisseleva, T. (2012). What's new in liver fibrosis? The origin of myofibroblasts in liver fibrosis. Journal of Gastroenterology and Hepatology (Australia), 27(SUPPL.2). https://doi.org/10.1111/j.1440-1746.2011.07002.x

Jang, Y.-S., & Choi, I.-H. (2014). Contrasting Roles of Different Endoglin Forms in Atherosclerosis. Immune Network, 14(5). https://doi.org/10.4110/in.2014.14.5.237

Kalra, A., & Tuma, F. (2018). Physiology, Liver. In StatPearls.

Karzai, F. H., Apolo, A. B., Cao, L., Madan, R. A., Adelberg, D. E., Parnes, H., McLeod, D. G., Harold, N., Peer, C., Yu, Y., Tomita, Y., Lee, M. J., Lee, S., Trepel, J. B., Gulley, J. L., Figg, W. D., & Dahut, W. L. (2015). A phase i study of TRC105 anti-endoglin (CD105) antibody in metastatic castration-resistant prostate cancer. BJU International, 116(4). https://doi.org/10.1111/bju.12986

Kim, J. Y., An, H. J., Kim, W. H., Park, Y. Y., Park, K. D., & Park, K. K. (2017). Apamin suppresses biliary fibrosis and activation of hepatic stellate cells. International Journal of Molecular Medicine, 39(5). https://doi.org/10.3892/ijmm.2017.2922

Kim, W. R., Flamm, S. L., di Bisceglie, A. M., & Bodenheimer, H. C. (2008). Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. In Hepatology (Vol. 47, Issue 4). https://doi.org/10.1002/hep.22109

Kumar, S., Pan, C. C., Bloodworth, J. C., Nixon, A. B., Theuer, C., Hoyt, D. G., & Lee, N. Y. (2014). Antibody-directed coupling of endoglin and MMP-14 is a key mechanism for endoglin shedding and deregulation of TGF-β signaling. Oncogene, 33(30). https://doi.org/10.1038/onc.2013.386

Kunutsor, S. K., Apekey, T. A., & Walley, J. (2013). Liver Aminotransferases and Risk of Incident Type 2 Diabetes: A Systematic Review and Meta-Analysis. American Journal of Epidemiology, 178(2), 159–171. https://doi.org/10.1093/aje/kws469

Lastres, P., Bellon, T., Cabañas, C., Sanchez-Madrid, F., Acevedo, A., Gougos, A., Letarte, M., & Bernabeu, C. (1992). Regulated expression on human macrophages of endoglin, an Arg-Gly-Asp-containing surface antigen. European Journal of Immunology, 22(2). https://doi.org/10.1002/eji.1830220216

Lebrin, F., Deckers, M., Bertolino, P., & ten Dijke, P. (2005). TGF-β receptor function in the endothelium. Cardiovascular Research, 65(3), 599–608. https://doi.org/10.1016/j.cardiores.2004.10.036

Lebrin, F., Goumans, M.-J., Jonker, L., Carvalho, R. L. C., Valdimarsdottir, G., Thorikay, M., Mummery, C., Arthur, H. M., & ten Dijke, P. (2004). Endoglin promotes endothelial cell

proliferation and TGF-beta/ALK1 signal transduction. The EMBO Journal, 23(20), 4018–4028. https://doi.org/10.1038/sj.emboj.7600386

Levine, E., Forero, A., O'Connor, T., Seon, B., Jivani, M., Adams, B., & Theuer, C. (2013). A phase Ib dose-escalation study of TRC105 (anti-endoglin antibody) in combination with capecitabine for advanced solid tumors (including patients with progressive or recurrent HER2-negative metastatic breast cancer). Journal of Clinical Oncology, 31, 3057. https://doi.org/10.1200/jco.2013.31.15_suppl.3057

Levine, R. J., Lam, C., Qian, C., Yu, K. F., Maynard, S. E., Sachs, B. P., Sibai, B. M., Epstein, F. H., Romero, R., Thadhani, R., & Karumanchi, S. A. (2006). Soluble Endoglin and Other Circulating Antiangiogenic Factors in Preeclampsia. New England Journal of Medicine, 355(10). https://doi.org/10.1056/nejmoa055352

Lewis, K., Yoshimoto, M., & Takebe, T. (2021). Fetal liver hematopoiesis: from development to delivery. In Stem Cell Research and Therapy (Vol. 12, Issue 1). https://doi.org/10.1186/s13287-021-02189-w

Li, C.-H., Piao, D.-M., Xu, W.-X., Yin, Z.-R., Jin, J.-S., & Shen, Z.-S. (2005). Morphological and serum hyaluronic acid, laminin and type IV collagen changes in dimethylnitrosamine-induced hepatic fibrosis of rats. World Journal of Gastroenterology, 11(48), 7620–7624. https://doi.org/10.3748/wjg.v11.i48.7620

Li, J. Z., Li, H., Sasaki, T., Holman, D., Beres, B., Dumont, R. J., Pittman, D. D., Hankins, G. R., & Helm, G. A. (2003). Osteogenic potential of five different recombinant human bone morphogenetic protein adenoviral vectors in the rat. In Gene Therapy (Vol. 10, Issue 20). https://doi.org/10.1038/sj.gt.3302075

Li, P., Li, Y., Zhu, L., Yang, Z., He, J., Wang, L., Shang, Q., Pan, H., Wang, H., Ma, X., Li, B., Fan, X., Ge, S., Jia, R., & Zhang, H. (2018). Targeting secreted cytokine BMP9 gates the attenuation of hepatic fibrosis. Biochimica et Biophysica Acta - Molecular Basis of Disease, 1864(3). https://doi.org/10.1016/j.bbadis.2017.12.008

Li, T., & Apte, U. (2015). Bile Acid Metabolism and Signaling in Cholestasis, Inflammation, and Cancer. In Advances in Pharmacology (Vol. 74). https://doi.org/10.1016/bs.apha.2015.04.003

Lisman, T., Leebeek, F. W. G., & de Groot, P. G. (2002). Haemostatic abnormalities in patients with liver disease. In Journal of Hepatology (Vol. 37, Issue 2). https://doi.org/10.1016/S0168-8278(02)00199-X

Liu, Y., Paauwe, M., Nixon, A. B., & Hawinkels, L. J. A. C. (2021). Endoglin targeting: Lessons learned and questions that remain. In International Journal of Molecular Sciences (Vol. 22, Issue 1). https://doi.org/10.3390/ijms22010147

Liu, Y., Tian, H., Blobe, G. C., Theuer, C. P., Hurwitz, H. I., & Nixon, A. B. (2014). Effects of the combination of TRC105 and bevacizumab on endothelial cell biology. Investigational New Drugs, 32(5). https://doi.org/10.1007/s10637-014-0129-y

López-Coviella, I., Berse, B., Krauss, R., Thies, R. S., & Blusztajn, J. K. (2000). Induction and maintenance of the neuronal cholinergic phenotype in the central nervous system by BMP-9. Science, 289(5477). https://doi.org/10.1126/science.289.5477.313

López-Novoa, J. M., & Bernabeu, C. (2010). The physiological role of endoglin in the cardiovascular system. In American Journal of Physiology - Heart and Circulatory Physiology (Vol. 299, Issue 4). https://doi.org/10.1152/ajpheart.01251.2009

Madhav, A., Andres, A., Duong, F., Mishra, R., Haldar, S., Liu, Z., Angara, B., Gottlieb, R., Zumsteg, Z. S., & Bhowmick, N. A. (2018). Antagonizing CD105 enhances radiation sensitivity in prostate cancer. Oncogene, 37(32). https://doi.org/10.1038/s41388-018-0278-0

Malhotra, R., Paskin-Flerlage, S., Zamanian, R. T., Zimmerman, P., Schmidt, J. W., Deng, D. Y., Southwood, M., Spencer, R., Lai, C. S., Parker, W., Channick, R. N., Morrell, N. W., Elliott, C. G., & Yu, P. B. (2013). Circulating angiogenic modulatory factors predict survival and functional class in pulmonary arterial hypertension. Pulmonary Circulation, 3(2). https://doi.org/10.4103/2045-8932.110445

Mariotti, V., Strazzabosco, M., Fabris, L., & Calvisi, D. F. (2018). Animal models of biliary injury and altered bile acid metabolism. In Biochimica et Biophysica Acta - Molecular Basis of Disease (Vol. 1864, Issue 4). https://doi.org/10.1016/j.bbadis.2017.06.027

Maroni, L., Haibo, B., Ray, D., Zhou, T., Wan, Y., Meng, F., Marzioni, M., & Alpini, G. (2015). Functional and Structural Features of Cholangiocytes in Health and Disease. In CMGH (Vol. 1, Issue 4). https://doi.org/10.1016/j.jcmgh.2015.05.005

Martínez, K., Salazar, Á., Fernández, J., Pineda, C., Aguilar, C., & López, A. (2017). Western blot: a tool in the biomedical field. Investigacion En Discapacidad, 6(3).

Mescher, A. L. (2017). Junqueira ' s Basic Histology Text & Atlas. In Mc Graw Hill (Issue January).

Meurer, S. K., Tihaa, L., Borkham-Kamphorst, E., & Weiskirchen, R. (2011). Expression and functional analysis of endoglin in isolated liver cells and its involvement in fibrogenic Smad signalling. Cellular Signalling, 23(4). https://doi.org/10.1016/j.cellsig.2010.12.002

Meurer, S., Wimmer, A. E., Leur, E. van de, & Weiskirchen, R. (2019). Endoglin Trafficking/Exosomal Targeting in Liver Cells Depends on N-Glycosylation. Cells, 8(9). https://doi.org/10.3390/cells8090997

Mishra, M., Tiwari, S., & Gomes, A. v. (2017). Protein purification and analysis: Next generation western blotting techniques. In Expert Review of Proteomics (Vol. 14, Issue 11). https://doi.org/10.1080/14789450.2017.1388167

Müller, A., Machnik, F., Zimmermann, T., & Schubert, H. (1988). Thioacetamide-induced cirrhosis-like liver lesions in rats usefulness and reliability of this animal model. Experimental Pathology, 34(4). https://doi.org/10.1016/S0232-1513(88)80155-5

Murphy-Ullrich, J. E., & Poczatek, M. (2000). Activation of latent TGF-β by thrombospondin-1: mechanisms and physiology. Cytokine & Growth Factor Reviews, 11(1), 59–69. https://doi.org/https://doi.org/10.1016/S1359-6101(99)00029-5

Nagase, H., Visse, R., & Murphy, G. (2006). Structure and function of matrix metalloproteinases and TIMPs. In Cardiovascular Research (Vol. 69, Issue 3). https://doi.org/10.1016/j.cardiores.2005.12.002

Nishimura, S. L. (2009). Integrin-mediated transforming growth factor-beta activation, a potential therapeutic target in fibrogenic disorders. The American Journal of Pathology, 175(4), 1362–1370. https://doi.org/10.2353/ajpath.2009.090393

Nolan-Stevaux, O., Zhong, W., Culp, S., Shaffer, K., Hoover, J., Wickramasinghe, D., & Ruefli-Brasse, A. (2012). Endoglin Requirement for BMP9 Signaling in Endothelial Cells Reveals New Mechanism of Action for Selective Anti-Endoglin Antibodies. PLoS ONE, 7(12). https://doi.org/10.1371/journal.pone.0050920

Ozougwu, J. (2017). Physiology of the liver. 4, 13–24.

Pérez-Gómez, E., del Castillo, G., Santibáñez, J. F., López-Novoa, J. M., Bernabéu, C., & Quintanilla, M. (2010). The role of the TGF-β coreceptor endoglin in cancer. In TheScientificWorldJournal (Vol. 10). https://doi.org/10.1100/tsw.2010.230

Ploemacher, R. E., Engels, L. J. A., Mayer, A. E. M., Thies, S., & Neben, S. (1999). Bone morphogenetic protein 9 is a potent synergistic factor for murine hemopoietic progenitor cell generation and colony formation in serum-free cultures. Leukemia, 13(3). https://doi.org/10.1038/sj.leu.2401363

Rathouska, J., Jezkova, K., Nemeckova, I., & Nachtigal, P. (2015). Soluble endoglin, hypercholesterolemia and endothelial dysfunction. In Atherosclerosis (Vol. 243, Issue 2). https://doi.org/10.1016/j.atherosclerosis.2015.10.003

Ravi, V., Brohl, A. S., Chawla, S. P., Attia, S., Riedel, R. F., Liebner, D. A., Thornton, K. A., Basu Mallick, A., Mehta, C. R., Liu, L., Alvarez, D., Theuer, C. P., Robinson, S. I., Penel, N., Stacchiotti, S., Tap, W. D., Jones, R. L., & Maki, R. G. (2018). TAPPAS: An adaptive enrichment phase 3 trial of TRC105 and pazopanib versus pazopanib alone in patients with advanced angiosarcoma. Journal of Clinical Oncology, 36(15_suppl), TPS11590–TPS11590. https://doi.org/10.1200/JCO.2018.36.15_suppl.TPS11590

Rodríguez-Peña, A., Eleno, N., Düwell, A., Arévalo, M., Pérez-Barriocanal, F., Flores, O., Docherty, N., Bernabeu, C., Letarte, M., & López-Novoa, J. M. (2002). Endoglin upregulation during experimental renal interstitial fibrosis in mice. Hypertension, 40(5). https://doi.org/10.1161/01.HYP.0000037429.73954.27

Rosen, L. S., Gordon, M. S., Robert, F., & Matei, D. E. (2014). Endoglin for Targeted Cancer Treatment. Current Oncology Reports, 16(2). https://doi.org/10.1007/s11912-013-0365-x

Rosen, L. S., Hurwitz, H. I., Wong, M. K., Goldman, J., Mendelson, D. S., Figg, W. D., Spencer, S., Adams, B. J., Alvarez, D., Seon, B. K., Theuer, C. P., Leigh, B. R., & Gordon, M. S. (2012). A phase I first-in-human study of TRC105 (anti-endoglin antibody) in patients with advanced cancer. Clinical Cancer Research, 18(17). https://doi.org/10.1158/1078-0432.CCR-12-0098

Rygiel, K. A., Robertson, H., Marshall, H. L., Pekalski, M., Zhao, L., Booth, T. A., Jones, D. E. J., Burt, A. D., & Kirby, J. A. (2008). Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease. Laboratory Investigation, 88(2). https://doi.org/10.1038/labinvest.3700704

Schultze, A. E. (2007). Book Review: Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. Veterinary Pathology, 44(1). https://doi.org/10.1354/vp.44-1-129-a

Schulze, R. J., Schott, M. B., Casey, C. A., Tuma, P. L., & McNiven, M. A. (2019). The cell biology of the hepatocyte: A membrane trafficking machine. In Journal of Cell Biology (Vol. 218, Issue 7). https://doi.org/10.1083/jcb.201903090

Senoo, H., Yoshikawa, K., Morii, M., Miura, M., Imai, K., & Mezaki, Y. (2010). Hepatic stellate cell (vitamin A-storing cell) and its relative – past, present and future. Cell Biology International, 34(12). https://doi.org/10.1042/cbi20100321

Seon, B. K., Haba, A., Matsuno, F., Takahashi, N., Tsujie, M., She, X., Harada, N., Uneda, S., Tsujie, T., Toi, H., Tsai, H., & Haruta, Y. (2011). Endoglin-targeted cancer therapy. Current Drug Delivery, 8(1), 135–143. https://doi.org/10.2174/156720111793663570

Shen, C., & Kaelin, W. G. (2013). The VHL/HIF axis in clear cell renal carcinoma. Seminars in Cancer Biology, 23(1), 18–25. https://doi.org/https://doi.org/10.1016/j.semcancer.2012.06.001

Shen, W., Lee, S. R., Yam, M., Zhu, L., Zhang, T., Pye, V., Mathai, A. E., Shibagaki, K., Zhang, J. Z., Matsugi, T., & Gillies, M. C. (2018). A combination therapy targeting endoglin and VEGF-A prevents subretinal fibro-neovascularization caused by induced müller cell disruption. Investigative Ophthalmology and Visual Science, 59(15). https://doi.org/10.1167/iovs.18-25628

Singh, S., Allen, A. M., Wang, Z., Prokop, L. J., Murad, M. H., & Loomba, R. (2015). Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. Clinical Gastroenterology and Hepatology : The Official Clinical Practice Journal of the American Gastroenterological Association, 13(4), 643-e40. https://doi.org/10.1016/j.cgh.2014.04.014

St-Jacques, S. (1994). Molecular characterization and in situ localization of murine endoglin reveal that it is a transforming growth factor-beta binding protein of endothelial and stromal cells. Endocrinology, 134(6). https://doi.org/10.1210/en.134.6.2645

Taipale, J., Saharinen, J., Hedman, K., & Keski-Oja, J. (1996). Latent transforming growth factor-beta 1 and its binding protein are components of extracellular matrix microfibrils. Journal of Histochemistry & Cytochemistry, 44(8), 875–889. https://doi.org/10.1177/44.8.8756760

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America, 76(9). https://doi.org/10.1073/pnas.76.9.4350

Velasco, S., Alvarez-Muñoz, P., Pericacho, M., ten Dijke, P., Bernabéu, C., López-Novoa, J. M., & Rodríguez-Barbero, A. (2008). L- and S-endoglin differentially modulate TGFβ1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts. Journal of Cell Science, 121(6). https://doi.org/10.1242/jcs.023283

Venkatesha, S., Toporsian, M., Lam, C., Hanai, J. I., Mammoto, T., Kim, Y. M., Bdolah, Y., Lim, K. H., Yuan, H. T., Libermann, T. A., Stillman, I. E., Roberts, D., D'Amore, P. A., Epstein, F. H., Sellke, F. W., Romero, R., Sukhatme, V. P., Letarte, M., & Karumanchi, S. A. (2006). Soluble endoglin contributes to the pathogenesis of preeclampsia. Nature Medicine, 12(6). https://doi.org/10.1038/nm1429

Vicen, M., Igreja Sá, I. C., Tripská, K., Vitverová, B., Najmanová, I., Eissazadeh, S., Micuda, S., & Nachtigal, P. (2021). Membrane and soluble endoglin role in cardiovascular and metabolic disorders related to metabolic syndrome. In Cellular and Molecular Life Sciences (Vol. 78, Issue 6). https://doi.org/10.1007/s00018-020-03701-w

Vozarova, B., Stefan, N., Lindsay, R. S., Saremi, A., Pratley, R. E., Bogardus, C., & Antonio Tataranni, P. (2002). High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes. Diabetes, 51(6). https://doi.org/10.2337/diabetes.51.6.1889

Walton, K. L., Johnson, K. E., & Harrison, C. A. (2017). Targeting TGF-β Mediated SMAD Signaling for the Prevention of Fibrosis. Frontiers in Pharmacology, 8, 461. https://doi.org/10.3389/fphar.2017.00461

Weber, L. W. D., Boll, M., & Stampfl, A. (2003). Hepatotoxicity and Mechanism of Action of Haloalkanes: Carbon Tetrachloride as a Toxicological Model. Critical Reviews in Toxicology, 33(2), 105–136. https://doi.org/10.1080/713611034

Yagmur, E., Rizk, M., Stanzel, S., Hellerbrand, C., Lammert, F., Trautwein, C., Wasmuth, H. E., & Gressner, A. M. (2007). Elevation of endoglin (CD105) concentrations in serum of patients with liver cirrhosis and carcinoma. European Journal of Gastroenterology and Hepatology, 19(9). https://doi.org/10.1097/MEG.0b013e3282202bea

Yamazaki, Y., Moore, R., & Negishi, M. (2011). Nuclear receptor CAR (NR1I3) is essential for DDC-induced liver injury and oval cell proliferation in mouse liver. Laboratory Investigation, 91(11). https://doi.org/10.1038/labinvest.2011.115