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**Carotuximab effects on inflammation during liver fibrosis development**

(Diploma thesis)

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## **Author's Declaration**

I declare that this thesis is my original work. All literature and other resources which were used during the preparation of this thesis are listed in bibliography and properly cited.

Date:

Signature:

## **AKNOWLEDGEMENT**

I feel so privileged to be supervised by my mentor PharmDr. Jana Rathouska, Ph.D. she guided me in every step of this journey, and I am truly honoured to work with such a fantastic scientist.

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## Abstract

Chronic liver inflammation is a process that results in the distortion of the liver parenchyma. Constant damage caused by inflammation would lead to formation of fibrotic tissue which can impair the normal liver physiology. Liver fibrosis is one of the common causes of morbidity and mortality worldwide. Non-alcoholic fatty liver disease (NAFLD) is a pathological state that is characterised by excessive accumulation of lipid in the hepatocytes (hepatic steatosis) which can trigger the inflammatory response in the liver. Carotuximab (TRC 105) is a monoclonal antibody that has been used for the treatment of a number of cancers, including hepatocellular carcinoma (HCC).

The aim of this study was to investigate the role of carotuximab in liver inflammation in a mouse model of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced liver fibrosis. Eighteen 3-month-old C57BL/6 male mice were recruited in this study. They were divided into three groups: control group (n=6) – fed chow diet for the period of 4 weeks, DDC group (n=6) – fed DDC diet for the period of 4 weeks, DDC group treated with carotuximab (DDC+TRC, n=6) - fed DDC diet for the period of 4 weeks and to that TRC 105 (15mg/kg) was administered. The impact of the diet and TRC 105 on mouse weight, as well as the effect on liver damage (levels of alkaline phosphatase, ALP), were evaluated. Several markers of inflammation (NF- $\kappa$ B, ICAM-1, COX-2, HO-1) were evaluated by Western blot analysis.

The results of this study confirmed the presence of liver damage and upregulation of inflammatory markers after DDC diet. However, no considerable effect on inflammatory markers was revealed after TRC 105 treatment, in relation to the group treated with DDC diet only. Thus, further studies will be necessary to investigate a possible impact of carotuximab treatment on hepatic inflammation.

## Abstrakt

Chronický zánět jater je proces, který vede k poškození jaterního parenchymu. Chronické poškození způsobené zánětem vede k tvorbě fibrotické tkáně, která může narušit normální fyziologii jater. Fibróza jater je celosvětově jednou z nejčastějších příčin morbidity a mortality. Nealkoholické ztukovatění jater (NAFLD) je patologický stav, který je charakterizován nadměrným hromaděním lipidů v hepatocytech (jaterní steatóza), což může vyvolat zánětlivou reakci v játrech. Carotuximab (TRC 105) je monoklonální protilátka, která se používá k léčbě řady nádorových onemocnění, včetně hepatocelulárního karcinomu (HCC).

Cílem této studie bylo zjistit úlohu carotuximabu při zánětu jater na myším modelu jaterní fibrózy vyvolané 3,5-diethoxykarbonyl-1,4-dihydrocholidem (DDC). Do této studie bylo zařazeno osmnáct tříměsíčních myších samců C57BL/6. Byly rozděleny do tří skupin: kontrolní skupina (n=6) - krmená standardní dietou po dobu 4 týdnů, skupina DDC (n=6) - krmená DDC dietou po dobu 4 týdnů, skupina DDC léčená carotuximabem (DDC+TRC, n=6) - krmená DDC dietou po dobu 4 týdnů s podáním TRC 105 (15mg/kg). Byl hodnocen vliv diety a TRC 105 na hmotnost myši a také vliv na poškození jater (hladiny alkalické fosfatázy, ALP). Vybrané markery zánětu (NF- $\kappa$ B, ICAM-1, COX-2, HO-1) byly hodnoceny pomocí Western blot analýzy.

Výsledky této studie potvrdily přítomnost poškození jater a zvýšení tvorby zánětlivých markerů po DDC dietě. Po aplikaci TRC 105 však nebyl zjištěn žádný významný vliv na markery zánětu, ve vztahu ke skupině krmené samotnou DDC dietou. Bude tedy nutné provést další studie, aby se odhalil možný vliv podání carotuximabu na jaterní zánět.

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## List of abbreviations

◆ $\alpha$ -SMA	Alpha smooth muscle actin
◆ $\alpha$ -TTP	$\alpha$ -tocopherol transfer protein
◆ ADCC	Antibody-dependent cell-mediated cytotoxicity
◆ ALP	Alkaline phosphatase
◆ APS	Ammonium persulfate
◆ ATP	Adenosine triphosphate
◆ BBB	Blood brain barrier
◆ BCA	Bicinchoninic acid
◆ BMP	Bone morphogenetic protein
◆ CCL4	Carbon tetrachloride
◆ CO	Carbon monoxide
◆ COX	Cyclooxygenase
◆ CYP2E1	Cytochrome P450 2E1
◆ DDC	3,5-diethoxycarbonyl- 1,4-dihydrocollidine
◆ DEN	Diethyl nitrosamine
◆ DMN	Dimethyl nitrosamine
◆ DNA	Deoxyribonucleic acid
◆ EBP- $\alpha$	Enhancer binding protein- $\alpha$
◆ FA	Fatty acid
◆ FFA	Free fatty acid
◆ FGF	Fibroblast growth factor
◆ GAPDH	Glyceraldehyde -3-phosphate dehydrogenase
◆ HBV	Hepatitis B virus
◆ HCC	Hepatocellular carcinoma
◆ HDL	High-density lipoprotein
◆ HER-2	Herceptin receptor 2
◆ HO-1	Heme oxygenase-1
◆ HRP	Horseradish peroxidase



◆ HSC	Hepatic stellate cell
◆ ICAM-1	Intracellular adhesion molecule-1
◆ IFN	Interferon
◆ IgG1	Immunoglobulin G1
◆ IL-1	Interleukin 1
◆ IL-17	Interleukin 17
◆ IL-6	Interleukin 6
◆ LPS	Lipopolysaccharide
◆ MCD	Methionine/choline deficient diet
◆ Mdr2	Multidrug resistance-associated protein 2
◆ MMP-14	Matrix metalloproteinase 14
◆ NAFL	Non-alcoholic fatty liver
◆ NAFLD	Non-alcoholic fatty liver disease
◆ NASH	Non-alcoholic steatohepatitis
◆ NF-κB	Nuclear factor Kappa-light chain-enhancer of activated B cell
◆ NK	Natural killers
◆ NO	Nitric oxide
◆ PBC	Primary biliary cholangitis
◆ PNPLA-3	Patatin-like phospholipase domain-containing protein 3
◆ PPAR-γ	Peroxisome proliferator-activated receptor gamma
◆ PSC	Primary sclerosing cholangitis
◆ PVDF	Polyvinylidene fluoride
◆ RBC	Red blood cell
◆ ROS	Reactive oxygen species
◆ SDS	Sodium dodecyl sulphate
◆ SEC	Sinusoidal endothelial cell
◆ sICAM-1	Soluble intracellular adhesion molecule-1
◆ SOD2	Superoxide dismutase 2
◆ SREBP1c	Sterol regulatory element binding protein-1c
◆ T2DM	Type 2 diabetes mellitus
◆ TAG	Triacylglycerol
◆ TBS	Tris buffer saline

- ◆ TEMED      Tetramethyl ethylene diamine
- ◆ TGFβ1      Transforming growth factor beta 1
- ◆ TIMP      Tissue inhibitor metalloproteinase
- ◆ TLR4      Toll-like receptor 4
- ◆ TM6SF2      Transmembrane 6 superfamily 2
- ◆ TNF-α      Tumour necrosis factor-α
- ◆ UV      Ultraviolet
- ◆ VEGF      Vascular endothelial growth factor
- ◆ VLDL      Very-low density lipoprotein

# 1. Introduction

Long term untreated liver inflammation will lead to some irreversible changes which directly influence the parenchyma portion of the organ and consequently affects the physiological roles of the liver. High level of alcohol consumption, exposure to the particular viruses that can induce inflammation, metabolic syndrome, elevated level of the fat derivatives, cholestasis, are among the most common causes of the chronic liver inflammation (Zhou et al., 2014).

Inflammatory cells in the liver are responsible for the primary response to the stimuli. Macrophages are one of the important types of immune cells that participate in the inflammatory response in human body, and they are generally classified into M1 (proinflammatory) and M2 (anti-inflammatory) type. Kupffer cells are fixed macrophages in the organ that are mostly located in the liver sinusoids. The disbalance between two types of Kupffer cells known as M1 type and M2 type can cause the liver inflammation. M1 type circulating macrophages play a crucial role in the initiation of the cascade of proinflammatory response. Kupffer cells and circulating macrophages can be distinguished from each other by the surface markers which they are carrying. Neutrophils are another group of phagocytic cells that are quickly attracted to the site of inflammation in order to expand the immune response. Apart from the innate response, T-lymphocytes would also participate in the proinflammatory agents generation. Among the cytokines that are secreted from the T-helper cells, IL-17 would possess a strong profibrogenic effect (Koyama et al., 2017).

It has been shown that imbalances in the levels of some proteins have a significant role in hepatic inflammation. Nuclear factor kappa-light chain-enhancer of activated B cells (NF- $\kappa$ B) is a nuclear transcription factor which is present in almost all mammal cells. It is involved in the regulation of gene transcriptions, generation of cytokines, and apoptosis. Elevated level of NF- $\kappa$ B can be linked with several conditions, including inflammation, cancer, and autoimmune diseases (Zhang et al., 2021). Intracellular adhesion molecule-1 (ICAM-1) has an important role in adhesion of immune cells to the vascular wall during the inflammatory responses. Some studies have suggested that the increased level of ICAM-1 is associated with the inflammation. Production of ICAM-1 is upregulated in macrophages specifically during the inflammation (Wiesolek et al., 2019). Heme oxygenase-1 (HO-1) is an enzyme catalysing the degradation of heme. Some studies have shown that the overproduction of HO-1 during inflammation can reduce the extent of inflammatory process (Ryter et al., 2021). Cyclooxygenase (COX) is an

enzyme responsible for the metabolising of arachidonic acid. Prostaglandin is one of the important metabolites of arachidonic acid which is synthesized by COX. COX-2 plays a vital role in inflammation by supplying of required prostaglandin (Martinez O., 2003).

Endoglin (CD105), the auxiliary receptor for TGF- $\beta$  cytokine signalling, is a transmembrane glycoprotein composed of two domains. It has been demonstrated that endoglin can play an important role in vascular dysfunction that are mainly accompanied by the inflammatory responses. Some studies have suggested the significant links between the inflammation and the level of soluble endoglin (Meurer et al., 2020).

TRC 105, known as carotuximab, is a novel monoclonal antibody that was primarily developed to treat number of cancers. Carotuximab is a chimeric IgG1 CD105 monoclonal antibody that is able to bind endoglin in order to inhibit the signalling and endoglin function in some pathological states, particularly those with high level of endoglin expression (Duffy et al., 2017).

A large number of scientific groups are making efforts to reveal the possible consequences of liver damage development and contribute to effective treatment of liver fibrosis as one of the most common causes of morbidity and mortality worldwide.

## **2. The liver**

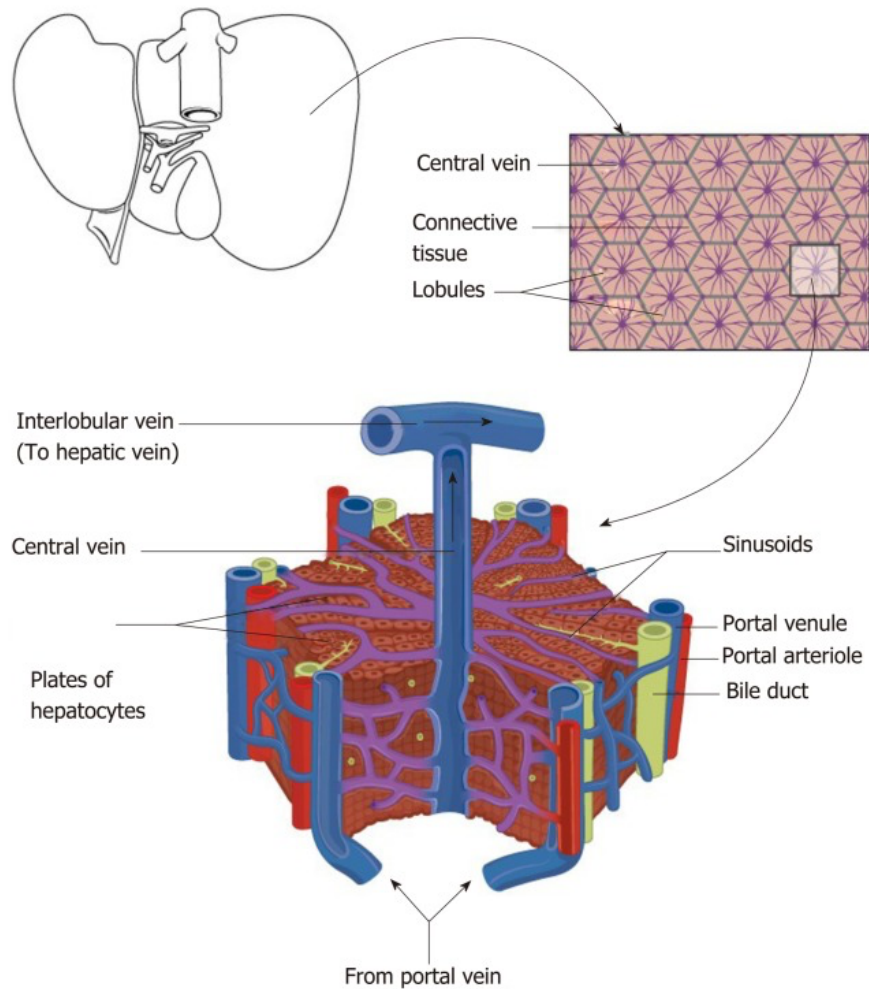
### **2.1. Histological structure of the liver**

Liver is known as the largest internal organ of the human body that is composed of two lobes. Two lobes of the liver are attached to each other through falciform ligament. Liver is located in the upper right quadrant of the abdominal cavity and is extended towards the epigastric area. The rib cage provides the mechanical protection to the liver (Abdel-Misih et al., 2010). Hepatic lobules are the major functional unit of the liver that are hexagonally shaped. Hepatocytes are organised in a disk-shaped structure forming the hepatic lobules. A huge network of capillaries is distributed among the hepatocytes to drain the blood into the central vein (Huppert et al., 2018).

#### **2.1.1. Hepatic lobule, hepatocytes, and sinusoids**

During the embryonic development, a definitive endoderm that contains the multipotent progenitor cells differentiate to give rise to the organs that are located in the abdominal cavity, including the thyroid gland, the lungs, the pancreas, and the liver. Later on, hepatoblasts arise from the hepatic endoderm which undergoes thickening to be converted from columnar epithelium to a pseudostratified epithelium. During the hepatic organogenesis, fibroblast growth factors (FGF) signalling as well as bone morphogenetic proteins (BMP) are required for the normal liver development.

Hepatoblasts undergo differentiation to form mature and fully functional hepatocytes. Hepatocytes are arranged in a disk-like structure to give rise to hepatic lobule which is the main functional unit of the liver. Hepatic lobule is a hexagonally shaped structure that comprises the central vein, bile canaliculi, portal vein, hepatic arteries, and hepatocyte zonation. At each angle of the hexagonal structure there is a portal triad consisting of portal vein, hepatic artery, and bile duct. The organisation of hepatic lobule contributes to the efficient blood flow through the parenchyma portion of the liver. Intrahepatic bile duct system is composed of cholangiocytes responsible for carrying the produced bile by hepatocytes. Sinusoids are a huge network of capillaries that are lined by fenestrated endothelium and are extended throughout the hepatic lobule in order to facilitate the blood flow from the portal vein and hepatic artery to the central vein (Huppert et al., 2018).



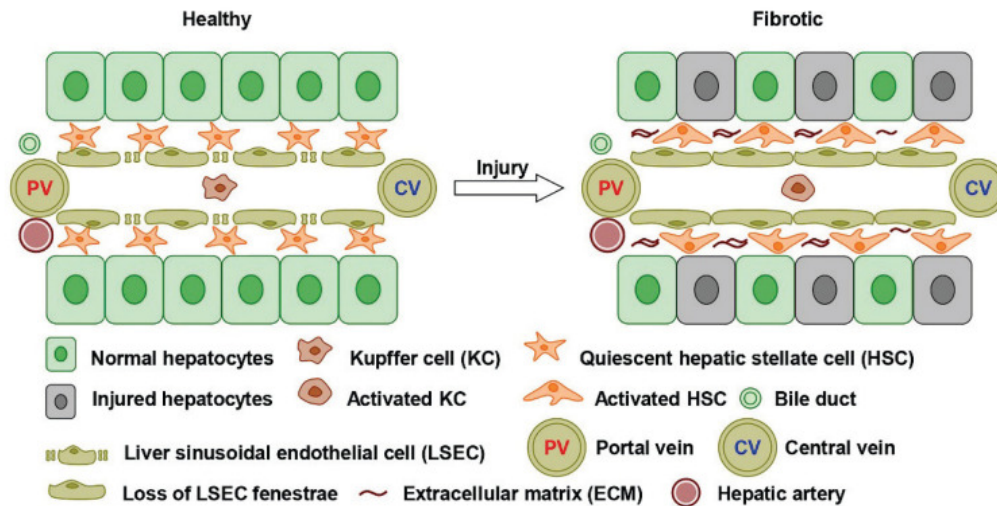
**Figure 1:** *The structure of hepatic lobule (adopted from Tanwar et al., 2020).*

### 2.1.2. Kupffer cells and hepatic stellate cells

Kupffer cells are the fixed macrophages of the liver responsible for elimination of the pathogens, initiation of the immunological response in the liver, and clearance of the endotoxins. Additionally, Kupffer cells are an integral part of innate immunity since they perform phagocytosis. Furthermore, they participate in production of proinflammatory cytokines, including interleukin 1 (IL-1), interleukin 6 (IL-6) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Tanwar et al., 2020).

Hepatic stellate cells (HSC) are distributed among the hepatocytes, and they take part in the

formation of perisinusoidal cells. HSCs are considered as the vitamin A storing cells. HSCs are the fixed mesenchymal cells exhibiting the properties of the resident fibroblasts. In the presence of stimuli HSCs undergo trans-differentiation to form proliferative myofibroblasts. Activated myofibroblasts would play a vital role in liver fibrosis (Higashi et al., 2017).



**Figure 2:** *Hepatic stellate cells and Kupffer cells in the context of liver fibrosis. (adopted from Luo et al., 2021).*

## 2.2. Physiological properties of the liver

The liver performs several physiological roles in the body. It takes part in the metabolism of carbohydrates, lipids, and proteins. It is also involved in the metabolism and detoxification of endogenous and exogenous molecules, including drugs and hormones. It participates in the formation of heme and excretion of bilirubin which is the final product of heme degradation. The liver plays an important role in the digestion of dietary cholesterol by synthesis of bile acid. Precursor of vitamin D requires two steps activation to be fully functional. The first step of hydroxylation of vitamin D precursor takes place in the liver. The liver is the storage site for many vitamins and minerals, including vitamin A, B12, D, E, K (Hen et al., 2016, Toleikis et al., 2020, Rendic et al., 2018, Haines et al., 2020, Chiang et al., 2018, Roth et al., 2018, Blaner et al., 2016).

### **2.2.1. Metabolism of carbohydrates and lipids**

The liver has a major role in the metabolism of the carbohydrates and maintenance of the physiological level of the essential monosaccharides, particularly glucose. It applies its role through some metabolic pathways, including the glycolysis, gluconeogenesis, glycogenolysis, and glycogenesis. Regulation of glucose homeostasis is crucial since glucose is considered as the primary fuel for ATP generation in various tissues. In the feeding state, an excess amount of glucose is accumulated in the liver in a form of glycogen with the aid of glycogen synthase. During the fasting state, glycogen phosphorylase is stimulated to begin the phosphorylation of the glucose units and release them. There are some endogenous agents that are also able to activate the glycogen phosphorylase, including glucagon, cortisol, and epinephrine. Glucose can be catabolised under aerobic or anaerobic conditions to form pyruvate and ATP. During the fasting state glucose can be synthesized from the lactate and certain amino acids by a metabolic pathway known as gluconeogenesis (Han et al., 2016).

The liver is involved in the metabolism of lipids. Fatty acids are considered as a vital source of energy, they constantly undergo beta oxidation pathway in the mitochondria of the hepatocytes to give rise to considerable amount of ATP (Toleikis et al., 2020). In the absence of glucose, fatty acids are mobilized to be converted to ketone bodies in a metabolic pathway known as ketogenesis which primarily takes place in the liver. Ketone bodies are able to cross blood brain barrier (BBB) to be consumed as a source of energy in case of glucose shortage (Chen et al., 2019). Formation of lipoproteins is highly crucial since fat derivatives have some lipophilic characteristics. Therefore, they are not able to be transported in a hydrophilic environment of the plasma. There is a wide range of lipoproteins including chylomicrons, very-low-, low-, and high-density lipoproteins. Very low-density lipoproteins (VLDL) are primarily synthesized in the hepatocytes and VLDL are considered as the major carrier of triacylglycerol (TAG) in the blood stream (Jayaraman et al., 2019).

### **2.2.2. Metabolism and detoxification of endogenous and exogenous compounds**

The liver constantly participates in the metabolism of endogenous and exogenous compounds. First-pass effect is a decisive route for the fate of the administered drug. The drug



molecule is transported to the liver via portal vein, and it is taken up by the hepatocytes for the metabolism. Drug metabolism is divided into two steps, the first step of biotransformation includes hydrolysis, oxidation, and reduction of the drug molecule, in order to increase its hydrophilicity. The second step of biotransformation that is mainly carried out by the superfamily of cytochrome p450 to prepare the drug molecule for excretion.

Not only drugs but also many endogenous compounds including steroids, steroid derivatives, eicosanoids, and hormones are metabolized in the liver (Rendic et al., 2018).

### **2.2.3. Heme synthesis, degradation, and excretion of bilirubin**

The liver is considered as one of the main sites of heme production. Heme is an important component of haemoglobins which binds oxygen with high affinity. The metalloproteins have a vital role in tissue oxygenation. A considerable amount of heme is deposited in the red blood cells (RBC) that circulate throughout the human body to supply sufficient amount of oxygen to tissues. Aged or damaged RBCs are eliminated from the circulation with the aid of splenic macrophages which results in release of the heme group. Heme oxygenase is an enzyme that is responsible for conversion of heme into biliverdin. Subsequently, biliverdin is degraded to bilirubin by the action of biliverdin reductase. Bilirubin is a lipophilic molecule. Thus, it requires albumin as a carrier to be transported to the liver for further actions. Bilirubin-albumin complex is dissociated, and bilirubin is taken up by the hepatocytes. Bilirubin is conjugated with two molecules of glucuronate and the resulted complex has enough hydrophilicity to be excreted via bile (Haines et al., 2020).

### **2.2.4. Synthesis of bile**

One of the major functions of the liver is the bile acid production. The liver is the only organ which contains all the necessary enzymes for the bile synthesis. Presence of bile is highly crucial for the catabolism of the dietary cholesterol. Accumulation of high amount of bile acids can be toxic to the organ. Thus, the production and secretion of the bile is tightly regulated by the liver. Bile acid is required for the absorption of steroids and fat-soluble vitamins in the intestine. Additionally, bile acids have an antibacterial effect to suppress the overgrowth of pathogens in the intestine. Cholesterol molecules are the main precursors for the bile synthesis. Bile acids are stored in the gall bladder, and they are secreted into the duodenum after food

intake by the action of cholecystokinin. Enterohepatic circulation contributes to efficient reabsorption of the major secreted portion of the bile (Chiang et al., 2018).

### **2.2.5. Activation of vitamin D**

Vitamin D has many vital physiological roles in the human body, such as bone mineralization, enhancement of calcium absorption in the intestine, regulation of immune system, multiplication and differentiation of various cell types. Endogenous production of vitamin D is considered as the major source of this essential compound. Vitamin D becomes fully activated after subsequent hydroxylation of its precursor. Exposure of skin to ultraviolet radiation will give rise to conversion of 7-dehydrocholesterol into cholecalciferol. The liver is responsible for the first step of hydroxylation of cholecalciferol with the aid of 25-hydroxylase. The kidneys would play the final role in the activation of vitamin D by carrying out the final step of hydroxylation and generation of calcitriol (Roth et al., 2018. Pappa et al., 2008).

### **2.2.6. Storage site of minerals and vitamins**

The liver is the main site of storage for various vitamins and minerals. Vitamin A is taken up by the hepatocytes and subsequently stored in the HSCs (Blaner et al., 2016). Presence of bile salts is vital for the sufficient absorption of fat-soluble vitamins, including vitamin A, D, E, and K. Excess amount of vitamin D is stored in the liver (Pappa et al., 2008). Vitamin E plays an important role in preventing oxidative stress-induced cell damage. There are eight isoforms of vitamin E.  $\alpha$ -tocopherol is the main isoform of vitamin E that plays a cytoprotective role in the human body. Hepatic  $\alpha$ -tocopherol transfer protein ( $\alpha$ -TTP) actively takes part in transport of vitamin E in the hepatocytes (Qu et al., 2016). Vitamin K is one of the major components for production of blood clotting factors which is mostly stored in the liver (Simes et al., 2020). Iron and copper are two of the essential minerals in the human body. The iron level is tightly regulated by hepatic peptide hormone hepcidin. Secretion of hepcidin is stimulated by higher levels of iron storage, infection, and during the inflammatory responses. Hepcidin facilitates the uptake of extra iron and its internalisation, in order to increase its degradation. Liver is actively involved in the storage of copper. The copper metabolism is strongly dependent on the physiological needs (Doguer et al., 2018).

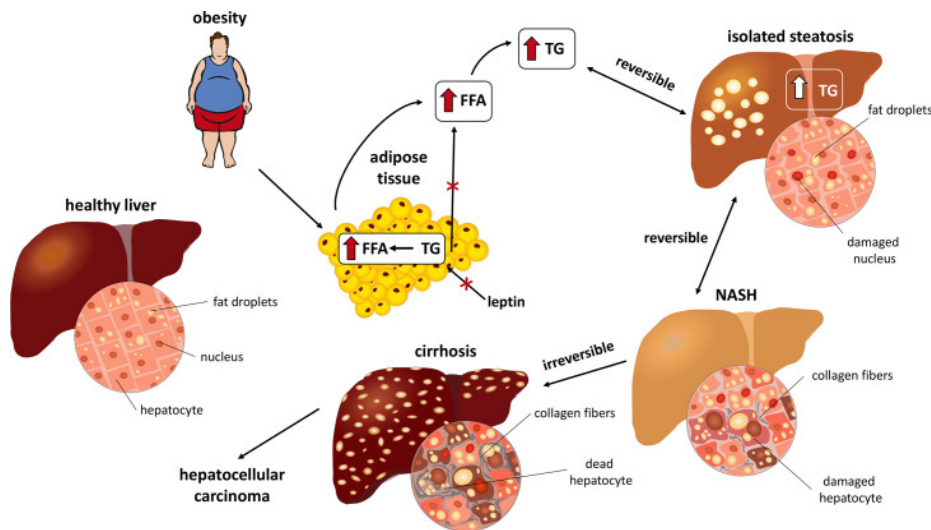
### **3. Non-alcoholic fatty liver disease (NAFLD)**

NAFLD is one of the most common types of liver disorders, that is characterised by the presence of steatosis in the hepatocytes. NAFLD is classified into two types based on its severity. The first type is the benign non-alcoholic fatty liver (NAFL) which is defined by the presence of steatosis. This type does not lead to the inflammatory response and permanent damage. The second type is the non-alcoholic steatohepatitis (NASH) which is more severe form of the NAFLD and is mainly accompanied by hepatocellular ballooning, lobular inflammation, and fibrosis. Long term inflammation alongside with the tissue fibrosis contribute to disease progression and manifestation of the liver cirrhosis. Cirrhosis is considered as the end-stage organ failure where tissue remodelling takes place. HSCs would actively participate in scar tissue formation that is composed of collagen type1 (Cobbina et al., 2017).

Insulin resistance is one of the major causes of the metabolic syndrome and it is responsible for the occurrence of type 2 diabetes and NAFLD. People with central obesity, low physical activity, elevated level of TAG and cholesterol, reduced level of high-density lipoprotein (HDL), and insulin resistance are at greater risk of NAFLD development. There are several mechanisms proposed to explain the pathogenesis of the NAFLD. However, some studies have shown that insulin resistance can have the greater impact (Cobbina et al., 2017). Apart from insulin resistance, genetic predisposition would play an important role in development of NAFLD. Carrying a missense mutation I148M of PNPLA3 (patatin-like phospholipase domain containing protein 3) gene is one of the major genetic risk factors. This genetic sequence gives rise to altered activity of enzyme which is responsible for the hydrolysis of TAG in hepatocytes as well as adipocytes (Romeo et al., 2008). Similarly, a E167K variant of TM6SF2 (transmembrane 6 superfamily 2) gene causes functional impairment of the protein and contributes to insulin resistance. As a result, it enhances the accumulation of TAG in the hepatocytes (Dongiovanni et al., 2015).

Abnormal fat deposition in the hepatocytes is the consequence of the combination of three factors, including diet, gut microbiota, and genetic factors. Enhancement of lipogenesis through the overactivity of lipogenic transcription factors such as sterol regulatory element binding protein-1 c (SREBP1c) and peroxisome proliferator-activated receptor gamma (PPAR-  $\gamma$ ) would also contribute to the steatosis (Cobbina et al., 2017).

Steatosis induces the hyperactivity of NF- $\kappa$ B which is involved in production of proinflammatory cytokines including IL-1, IL-6, and TNF- $\alpha$  which subsequently activate Kupffer cells (Anderson et al., 2008). Steatosis can negatively influence the function of intracellular organelles, particularly mitochondria. Impaired mitochondrial function is linked with the elevated level of FA (fatty acid) oxidation which in turn increases the production of ROS (reactive oxygen species). Oxidative stress is one of the complications of elevated level of ROS which can lead to hepatocyte necrosis (Cobbina et al., 2017).



**Figure 3:** The progression of the NAFLD (adopted from Kořínková et al., 2020)

### 3.1. NAFLD and type 2 diabetes mellitus

There is a strong correlation between NAFLD and type 2 diabetes mellitus (T2DM) since insulin resistance is known as the major cause of both pathological conditions. Some studies have suggested that patients with T2DM are at greater risk of development of NAFLD and vice versa. Insulin is a peptide hormone that is secreted from pancreatic B-cells. Insulin has a strong anabolic effect which enhances the formation of glycogen, increases the protein synthesis and storage of TAG in the adipose tissue (Tanase et al., 2020). Insulin resistance is characterised by the situation where insulin is not able to sufficiently apply its roles through the insulin

receptors in the human body (Kitade et al., 2017). Insulin resistance is linked with hyperlipidaemia and elevated level of blood glucose (Ighbariya et al., 2017). Obesity is one of the most important risk factors for the development of insulin resistance. In physiological state, adipose tissue expansion is accompanied by the proper angiogenesis and participation of adipogenic precursor cells which minimizes the risk of insulin resistance, whereas in abnormal adipose tissue expansion, enlargement of existing adipocytes takes place without sufficient angiogenesis, which can increase the risk of insulin resistance. One of the physiological functions of insulin is suppressing the lipolysis in adipose tissue. However, insulin resistance results in elevated level of free fatty acids (FFA) in the circulation and consequently it enhances the FFAs uptake by the hepatocytes. Increased delivery of FFAs to the liver can distort the normal lipid balance within the liver and causes steatosis (Sakurai et al., 2021).

### **3.2. Liver endothelial dysfunction in NAFLD**

Generally, NAFLD is accompanied by the pathological changes in microvascular system in the liver, which directly influences the blood flow in the sinusoids. Some studies have noted that decreased sinusoidal blood flow is directly related to the severity of steatosis. Additionally, hepatic steatosis can be linked with the increased intrahepatic vascular resistance (Pasarin et al., 2017). Impaired intrahepatic microcirculation can cause the lack of sufficient tissue oxygenation (Ijaz et al., 2005). Nitric oxide (NO) is one of the important vasodilatory and anti-inflammatory agents which is produced by the nitric oxide synthase (Cyr et al., 2020). Some studies have suggested that administration of L-arginine (precursor of nitric oxide) to diet-induced steatotic rats can improve the blood flow through the hepatic microcirculation and subsequently enhances the tissue oxygenation (Selzner et al., 2000).

Fibrogenesis is a complex process with the participation of different hepatic cells, including Kupffer cells, HSCs, and hepatocytes. Angiogenesis along with fibrogenesis can simultaneously occur (Bocca et al., 2015). Impaired sinusoidal endothelial cells (SECs) would have a great impact on the progression of the hepatic steatosis. During the liver fibrosis, the normal vascular architecture of the liver is distorted. This is mainly accompanied by the formation of abnormal vascular interconnections (Francque et al., 2012).

Long term hepatic steatosis can alter the structure of sinusoidal endothelial cells. SECs undergo vascularization and they acquire a pro-inflammatory, pro-vasoconstrictive, pro-thrombotic, and pro-angiogenic properties which gives rise to endothelial dysfunction.

Based on some studies, endothelial dysfunction occurs before the development of liver fibrosis, and it leads to the faster progression of the disease. In the physiological state SECs would inhibit the pathological activity of HSCs, since they have a great impact on the initiation of liver fibrosis. Overall, damaged SECs contribute to series of events, including stimulation of Kupffer cells, activation of NF- $\kappa$ B, decreased production of NO, and higher synthesis of pro-inflammatory cytokines. Each of these events would have a vital role in the disease progression and manifestation of liver fibrosis (Lafoz et al., 2020).

## **4. Inflammatory markers and liver**

### **4.1. NF- $\kappa$ B and liver inflammation**

NF- $\kappa$ B is a nuclear transcription factor which plays a critical role in the regulation of inflammatory response. The role of NF- $\kappa$ B is not only limited to inflammatory processes. NF- $\kappa$ B actively takes part in cell survival and differentiation, regulation of important function of cellular interaction, and production of cytokines (Mussbacher et al., 2019). NF- $\kappa$ B is made of heterodimer or homodimer structure that is mainly considered as DNA binding protein and constantly participates in gene transcription (Xiao et al., 2005). There are several factors proposed for activation of NF- $\kappa$ B. Some of these factors are inflammatory cytokines, such as TNF- $\alpha$ , IL-1, and lipopolysaccharide (LPS, an important component of bacterial cell wall). Additionally, presence of CD40 ligand and DNA damage can contribute to activation of NF- $\kappa$ B (Seigner et al., 2017). The impact of NF- $\kappa$ B signalling pathway and its physiological role is strongly connected with the cell type, intracellular localization, and the extent of its activation. NF- $\kappa$ B acts as a double-edged sword since it activates the Kupffer cells when inflammatory response is required by inducing the production of proinflammatory cytokines. It enhances the gene expression associated with the cell survival to protect the hepatocytes from inflammation-induced apoptosis. Moreover, NF- $\kappa$ B enhances the expression of a gene responsible for formation of superoxide dismutase 2 (SOD2) that is involved in neutralising of ROS (Luedde et al., 2011). NF- $\kappa$ B participates in liver fibrosis by induction of Kupffer cells to promote inflammatory responses. Some studies have shown that reduced activity of NF- $\kappa$ B in Kupffer cells can slow down the progression of liver fibrosis (Son et al., 2007).

Stimulation of NF- $\kappa$ B in HSCs can also contribute to liver fibrosis. HSCs are one of the major contributors to the liver fibrosis since they can differentiate into myofibroblasts that actively participate in liver fibrosis. Wide range of proinflammatory cytokines can activate the NF- $\kappa$ B in HSCs. Activation of NF- $\kappa$ B through LPS-mediated toll-like receptor 4 (TLR4) stimulation can exhibit the profibrogenic impact of NF- $\kappa$ B in the HSCs which directly promotes hepatic fibrosis (Seki et al., 2007).

### **4.2. ICAM-1 and liver inflammation**

ICAM-1 is a cell surface glycoprotein which acts as adhesion molecule particularly during the inflammatory responses. Activity of ICAM-1 can contribute to the attraction of immune

cells to the sites of inflammation (Bui et al., 2020). In addition to the regulation of inflammation, ICAM-1 plays a vital role in the cellular interactions, as well as participation in intracellular signalling pathways in response to various stimuli (Harjunpää et al., 2019). There are several cell types, including endothelial cells, epithelial cells, and leukocytes that up-regulate the expression of ICAM-1 when they are induced by the inflammatory cytokines. The level of expression of ICAM-1, as well as its inducible factors, would differ in various cells. For instance, NF- $\kappa$ B is responsible for increasing the expression of ICAM-1 in endothelial cells when it responds to TNF- $\alpha$ , while ICAM-1 expression is enhanced in epithelial cells when they are stimulated by interferon (IFN) (Bui et al., 2020). Occurrence of inflammation is mostly accompanied by the interaction of leucocytes and endothelial cells where in both cases there is an up-regulation of ICAM-1. Adhesive effect of ICAM-1 is crucial for leukocytes being grabbed by endothelial cells and directing them to the sites of inflammation (Lyck et al., 2015). The soluble form of ICAM-1 can also be detected in some inflammatory diseases, which can be a significant biomarker of inflammation (Capra et al., 2000). There are some enzymes, including elastase, cathepsin, and metalloprotease which actively participate in enzymatic cleavage of ICAM-1 to form the sICAM-1 (Robledo et al., 2003).

As it was previously mentioned, the level of sICAM-1 is enhanced in the inflammatory diseases. Higher expressions of ICAM-1 and subsequently increased serum levels of sICAM-1 are detected in the hepatitis. Some studies have demonstrated that a negative detection of ICAM-1 was confirmed in a case of healthy hepatocytes without the presence of inflammation, whereas higher expression of ICAM-1 in hepatocellular membrane was found in a case of hepatitis. There is a direct correlation between ICAM-1 and serum level of alanine transaminase (ALT). Effective treatment of hepatitis can lower the serum level of ALT and ICAM-1 (Capra et al., 2000).

### **4.3. COX-2 and liver inflammation**

Cyclooxygenase (COX) is an important enzyme which is responsible for metabolising of arachidonic acid. There are two isoforms of cyclooxygenase, COX-1 and COX-2. COX-1 plays a crucial role in provision of prostaglandin during the physiological state (housekeeping role). COX-2 expression is mostly induced by the proinflammatory cytokines in the pathological state that is mainly accompanied by inflammation (Núñez Martínez et al., 2003). Metabolization of arachidonic acid with the aid of COX gives rise to formation of some



bioactive lipids, such as prostaglandins and thromboxanes which are known as prostanoids. Arachidonic acid derivatives possess a wide range of physiological/pathological roles, including vascular functions, regulation of gastric acid juice secretion, platelets activation, and inflammation (Martin-sanz et al., 2010).

Based on some studies, the normal adult hepatocytes should not be induced by the proinflammatory cytokines to increase the expression of COX-2 (Casado et al., 2001). Absence of COX-2 expression in the adult hepatocytes can be associated with the higher level of enhancer binding protein- $\alpha$  (C/EBP- $\alpha$ ). EBP- $\alpha$  is a transcription factor that inhibits the formation of COX-2 which would be normally produced upon cytokines stimulation (Callejas et al., 2000).

As opposite to adult hepatocytes, COX-2 expression is up-regulated in Kupffer cells in response to proinflammatory cytokines, where COX-2 actively participates in provision of the prostaglandins for the liver demands (Casado et al., 2001).

The impact of COX-2 in the liver inflammation has been an interest for many researchers. Therefore, numerous studies have been carried out to investigate the role of COX-2 in the liver inflammation. Genetically modified mice were used to determine the effect of prostaglandin in the liver fibrosis. Based on some of these studies, genetically modified mice whose hepatocytes were able to express a considerable number of COX-2 (COX-2 transgene) exhibited much higher protection against the inflammation-induced apoptosis. Administration of selective COX-2 inhibitor decreases the cytoprotective effect of prostaglandin in the hepatic injury due to suppression of COX-2 activity and significant fall in the prostaglandin synthesis. In contrast, there are some studies suggesting the opposite results. The point of contradictions between the outcomes of these studies are expressed by the genetic variability of the tested animals (Martin-sanz et al., 2010, Martin-sanz et al., 2017, Mayoral et al., 2008).

#### **4.4. HO-1 and liver inflammation**

HO-1 is a metabolic enzyme that is involved in the heme degradation. Heme degradation is mainly accompanied by the generation of iron, carbon monoxide (CO), and biliverdin (Ryter et al., 2021). There are two isoforms of heme oxygenase, HO-1 and HO-2. HO-1 is the inducible isoform of the enzyme, whereas HO-2 has relatively constant production (Maines et al., 1997). HO-1 expression is mostly stimulated by the factors that exert physical and chemical cellular stress such as ultraviolet (UV) radiation, hydrogen peroxide, and heavy metals (Keyse

et al., 1989). HO-1 plays a crucial role in the regulation of the iron homeostasis. Accumulation of non-metabolised heme tend to elevate the risk of oxidative stress. According to some studies, HO-1 deficiency can be directly linked with heme deposition and subsequently oxidative stress (Yachie et al., 1999). HO-1 exerts its cytoprotective role through inhibiting oxidative stress-induced cell apoptosis (Ryter et al., 2021). Elevated level of lipid peroxidation along with higher ROS generation can accelerate the development of liver damage. Some studies have suggested that induction of HO-1 activity can slow down the progression of the NAFLD and can have the preventive role from the steatohepatitis (Du et al., 2020). In addition to anti oxidative effect, HO-1 can play an important anti-inflammatory role. Some studies have noted that the lack of sufficient HO-1 expression can increase the susceptibility to chronic inflammation in mice (Vijayan et al., 2010). It has been demonstrated that slightly high HO-1 expression may normally occur in Kupffer cells, as well as splenic macrophages. Enhanced level of HO-1 suppresses the expression of pro-inflammatory cytokines, such as IL-1, IL-6, TNF- $\alpha$  (Immenschuh et al., 1999). Endothelial cells play a fundamental role in the mediation of inflammation. They contribute to the transmigration of leucocytes to reach the sites of inflammation. Some studies have shown that elevated activity of HO-1 decreases the leucocytes adhesion (Hayashi et al., 1999). Some studies have suggested that heme can act as a pro-inflammatory agent, with a greater inflammatory impact in HO-1 deficient animals (Wagener et al., 2001).

## **5. Liver fibrosis in animal models**

During the past several years, a lot of effort were dedicated to the study of the liver pathological conditions, its diagnostic methods, and the safe and effective treatment. Working with the animal models has been a great contribution to the elucidating of the pathology, diagnosis, and treatment of the liver diseases, particularly NAFLD. Based on the research scheme, there is a wide range of substances to be used to induce the different level of liver fibrosis in animal models (Yanguas et al., 2016).

### **5.1. Chemical-based models**

There are several chemical compounds that are known to have the hepatotoxic effect. Exposure of the animal models to these hepatotoxic materials can result in inducing liver damage and consequently liver fibrosis. Intraperitoneal injection of these chemical compounds is preferred than the other route of administration, such as inhalation or oral administration. Injection route of administration can assure the induction of effective fibrosis in a shorter period of time (Yanguas et al., 2016).

There is a strong correlation between the ethanol consumption and development of chronic liver diseases. Ethanol is metabolised in the liver by the aid of cytochrome P450 and alcohol dehydrogenase. Ethanol metabolism can lead to the formation of ROS, increased lipid peroxidation, and glutathione reduction which overall can accelerate the liver damage (Yanguas et al., 2016). Generally, mice are the most common model used for the evaluation of liver fibrosis due to high susceptibility to liver fibrosis. Ethanol can be administered through drinking water (Beier et al., 2010).

Carbon tetrachloride (CCl<sub>4</sub>) is one of the most common hepatotoxic compounds which is mostly used for the induction of liver fibrosis in rodents. CYP2E1 is the enzyme that is mostly involved in the biotransformation of CCl<sub>4</sub>. Metabolization of CCl<sub>4</sub> is accompanied by the generation of free radicals which causes the hepatocellular damage. In addition to subcutaneous administration of CCl<sub>4</sub>, other routes of administration are available, including the inhalation and oral administration (Yanguas et al., 2016, Basu et al., 2003).

Thioacetamide is also used to mimic the liver fibrosis in animal models. This compound requires enzymatic biotransformation to gain the toxic properties. Biotransformation of thioacetamide is mainly carried out by cytochrome P450 which gives rise to formation of thioacetamide sulphur dioxide. Hepatotoxic effect of thioacetamide sulphur dioxide is generally associated with the elevated level of lipid peroxidation and higher production of free radicals. Thioacetamide is administered subcutaneously or orally (Yanguas et al., 2016, Low et al., 2004).

Dimethyl nitrosamine (DMN) and diethyl nitrosamine (DEN) are among the most common compounds that are used for the induction of liver fibrosis in animal models. Biotransformation of these compounds results in massive ROS production that interact with the nucleic acids, proteins, and lipids. High production of ROS can cause the severe hepatocellular damage and subsequent liver fibrosis (Yanguas et al., 2016).

## **5.2. Diet-based models**

Some particular diets are employed to induce liver fibrosis in the animal models. Diet-based model would mainly be applied to rodents (Yanguas et al., 2016).

Methionine-deficient and choline-deficient diet (MCD) are frequently used for induction of steatohepatitis in animal models. This diet-induced liver damage does not completely reflect the pathological processes of the disease in the human body, since two major risk factors of NAFLD, including the insulin resistance and obesity, are not well pronounced in the animal models. MCD increases the release of FA from the adipose tissue and its delivery to the liver. Moreover, MCD stimulates higher TAG synthesis in the liver. Higher FA delivery and synthesis of TAG can lead to hepatic steatosis (Yanguas et al., 2016, Rinella et al., 2004).

High-fat diet has the advantage compared to MCD, since it causes the animal models to experience obesity and insulin resistance which simulates the pathological processes of NAFLD in the human body. On the other hand, this method would take relatively long time to induce the liver fibrosis (Yanguas et al., 2016).

## **5.3. Surgery-based model**

It has been shown that liver fibrosis can be induced by surgical ligation of common bile duct in rats. This is one of the common methods to mimic liver fibrosis in rodents. The

obstruction of bile ducts results in activation of some fibrogenic markers, such as tissue inhibitors of metalloproteinases (TIMP), alpha smooth muscle actin ( $\alpha$ -SMA), collagen 1, and transforming growth factor beta (TGF $\beta$ 1). These events would result in distortion of the normal anatomy of the liver, which eventually lead to liver fibrosis (Miyoshi et al., 1999, Georgiev et al., 2008).

#### **5.4. Genetically modified model**

Research studies on transgenic animal models have revealed a very useful information about the type of proteins and signalling pathways which are involved in pathological processes of liver fibrosis. Findings obtained from this method have contributed to discovery of new ways of treatment for the liver damage (Hayashi et al., 2011).

It has been demonstrated that mouse multidrug resistance-associated protein 2 (Mdr2) deficient mouse model develops primary sclerosing cholangitis (PSC) due to activation of fibrogenic and inflammatory markers. Mdr2 gene is responsible for synthesis of P-glycoprotein. P-glycoprotein facilitates biliary phospholipid excretion. Therefore, the lack of P-glycoprotein results in hepatocellular damage and portal inflammation which resembles primary sclerosing cholangitis in the human body (Yanguas et al., 2016).

#### **5.5. Infection-based model**

Infection-based model is one of the methods which is mainly used for determination of the role of immune system in the pathology of the liver fibrosis. Rodents would not be normally infected by hepatitis B virus (HBV). Genetically modified mice have higher susceptibility to development of liver fibrosis when they are exposed to HBV. Liver fibrosis is mainly manifested in transgenic mice due to their immune system being compromised. In this method, working with transgenic mice would contribute to the determination of the role of each cytokine in development of chronic liver inflammation (Yanguas et al., 2016).

## **5.6. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) model of liver fibrosis**

Analysis of early stages of liver diseases, including primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) require some animal models which manifest slow hepatic pathological alterations (Mariotti et al., 2018).

Constant exposure of the mouse to DDC can lead to hepatic injury which is mostly accompanied by the formation of Mallory-Denk bodies. Formation of Mallory-Denk bodies is also seen in alcoholic and non-alcoholic steatohepatitis. Using of DDC in mouse is beneficial since it is able to resemble the NAFLD in the human body. High DDC intake would increase the production of free radicals which is responsible for occurrence of oxidative stress. Oxidative stress is considered as one of the major causes of hepatic inflammation, hepatocellular necrosis, and liver fibrosis (Fickert et al., 2002).

DDC is not only used for induction of metabolic type of steatohepatitis. Chronic exposure to DDC elevates the biliary porphyrin release which results in intraductal obstruction. Hence, DDC model is also useful for the evaluation of xenobiotic-induced cholangiopathy (Fickert et al., 2007). Chronic DDC intake alters the physiological role of the cholangiocytes in a way that higher expression of profibrogenic and proinflammatory cytokines take place. Some studies have suggested that DDC-induced liver injury is mainly accompanied by the hepatocellular necrosis which stimulates the Kupffer cells activation. DDC-based models are useful for the evaluation of the progression of hepatic injury caused by the xenobiotics as well as the metabolic disorders (Mariotti et al., 2018).

## **6. Carotuximab**

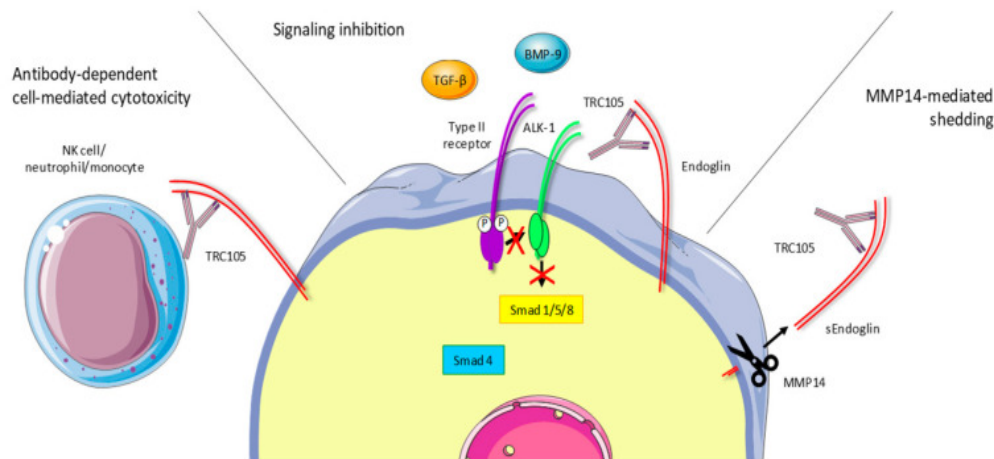
As previously mentioned, carotuximab is a monoclonal antibody that has been used for treatment of some oncological conditions. It has been demonstrated that carotuximab binds endoglin with a high affinity, which can inhibit its signalling pathways (Duffy et al., 2017).

### **6.1. Structure, function, and mechanism of action of carotuximab**

Endoglin (CD105) is a transmembrane glycoprotein which acts as major receptor for transforming growth factor- $\beta$  (TGF- $\beta$ ) ligands. Activation of endoglin pathways mediate important physiological processes, such as production of extracellular matrix, cell proliferation, and formation of new blood vessels. Angiogenesis plays a crucial role in progression of malignant tumours (Jeng et al., 2021). Hepatocellular carcinoma (HCC) carries a high mortality rate worldwide (Sayiner et al., 2019). It has been shown that proangiogenic properties of endoglin contributes to pathophysiology of HCC. Therefore, inhibition of endoglin can have a potential therapeutic effect on treating HCC. Studies have suggested that carotuximab has a partial effect on inhibition of angiogenesis in HCC which indicates its potential clinical application in the future (Jeng et al., 2021).

Carotuximab (TRC105) is a chimeric immunoglobulin 1 (IgG1) monoclonal antibody which offers anti-endoglin effect. Chimeric antibodies consist of multiple domains. These domains are mainly coming from different species. Typically, chimeric antibodies are made from combination of human and rodent antibodies (Duffy et al., 2015).

Carotuximab exhibits its effect through three main mechanisms. First, carotuximab can induce its effect through binding endoglin, which results in antibody-dependent cell-mediated cytotoxicity (ADCC). This phenomenon leads to cell apoptosis which is regulated by natural killer cells (NK), monocytes, and neutrophils. Additionally, carotuximab has an antagonistic effect on endoglin receptor. Therefore, it inhibits binding of BMP-9 to endoglin receptor. This would hamper the endoglin downstream signalling pathway. Furthermore, carotuximab can induce the enzymatic cleavage of transmembrane endoglin with the aid of MMP-14. This results in higher plasma concentration of soluble endoglin. Soluble endoglin acts as a trap for the ligands, which decreases the endoglin signalling in the human body (Liu et al., 2020)



**Figure 4:** *The mechanism of action of carotuximab (TRC105) (adopted from Liu et al., 2020)*

## 6.2. Carotuximab treatment

As previously mentioned, carotuximab can inhibit tumour progression through inhibiting angiogenesis. A number of clinical trials have investigated the therapeutic effect of carotuximab in oncology. The first clinical trial of carotuximab assessed its effect on treating prostate cancer. This study showed that approximately 50% of patients responded to the therapy. It has also been demonstrated that around 4% of the patients had a long response to the therapy (Liu et al., 2020).

It has been demonstrated that concomitant treatment of carotuximab and anti-vascular endothelial growth factor (VEGF) reduced the tumour size in renal cell carcinoma (Liu et al., 2020).

A clinical trial assessed the effect of carotuximab in herceptin receptor 2 (HER-2) negative breast cancer. Although this study showed that soluble endoglin level was raised in the responder group, the result of this study was not statistically significant, indicating the requirement for further clinical trials (Liu et al., 2020).

Another clinical trial has demonstrated that a combined treatment of anti-VEGF with carotuximab has a 32% response rate in patients with sarcoma. This study also illustrated that ICAM-1 level was significantly reduced in the responder group (Liu et al., 2020).



## **7. Aims of diploma thesis**

This study aimed to investigate the effect of carotuximab in a mouse model of DDC-induced hepatic fibrosis. Western blotting technique was performed to determine the relative expression of proteins involved in hepatic inflammation (NF- $\kappa$ B, ICAM-1, HO-1, COX-2) in three groups of mice: control group, a group fed 3,5-diethoxycarbonyl-1,4-dihydrocollidine diet (DDC), and a group fed 3,5-diethoxycarbonyl-1,4-dihydrocollidine diet treated with carotuximab (DDC+TRC).

## **8. Experimental part**

### **8.1. Methods**

#### **8.1.1. Animals**

Eighteen 3-month-old C57BL/6 male mice were recruited for this study. They were divided into three groups: control group (n=6), DDC (n=6), and DDC-TRC (n=6). These mice were accommodated with the 12 hours light/dark cycle. They were allowed ad libitum water consumption. Standard chow diet was introduced for the control group. However, DDC-supplemented diet was utilised for the DDC and DDC-TRC groups. DDC and control group were treated by intraperitoneal administration of saline solution (15 mg/kg) twice a week for a period of four weeks whereas DDC-TRC group were treated by intraperitoneal injection of TRC 105 (15 mg/kg) twice a week for the same period of time. All the mice participated in this study were weighed and sacrificed at the end of four weeks of treatment. Subsequently, blood samples were taken for the purpose of biochemical analysis of the liver samples. Animals were treated in line with the standards set by the ethical committee of the Charles university, Faculty of Pharmacy in Hradec Králové. All organised experiments were in accordance with the Czech Law No. 246/1992 Sb. (Date: October 27,2020)

#### **8.1.2. Biochemical analysis**

Alkaline phosphatase (ALP) is a transmembrane enzyme which is extensively distributed in mammalian cells. It has been demonstrated that elevated plasma level of ALP can be associated with the liver damage (Fernandez et al., 2007).

Measurement of ALP was performed using commercial Preventive Care Profile Plus test and VetScan2 (Abaxis Germany). For the purpose of biochemical analysis, blood samples were taken from the inferior vena cava. The profile was filled with 100 µl of each blood sample and placed inside the instrument. The results of ALP measurement were obtained after a few minutes.

### **8.1.3. Western blot analysis**

Western blotting is one of the most frequent laboratory techniques in biochemistry and molecular biology. This method is used for detection of a targeted protein in a sample mixture. The basic principles of the western blotting method have not been changed over time, nevertheless the reagents and imaging methods have improved in order to increase the sensitivity and extend the applicability (Taylor et al., 2014). This method is composed of several major steps, including separation of the targeted proteins, transfer of the proteins to a solid membrane, and using of appropriate primary and secondary antibodies for visualisation of the proteins which enables the further evaluation (Taylor et al., 2013). Separation of the proteins would take place according to the molecular weight of the proteins and with the aid of gel electrophoresis (Mahmmod et al., 2012). Western blotting method enables the precise analysis of the pattern of protein production in different physiological and pathological states.

#### **8.1.3.1. Sample preparation**

Liver tissues were extracted, and liquid nitrogen was recruited for freezing of the samples. The samples were stored in -80°C until homogenisation was achieved. After liver tissue homogenisation, preparation of total fraction and membrane fraction were performed. Bicinchoninic acid assay (BCA) was employed for the determination of the protein concentration. Each well of the prepared gel must receive the identical amount of the protein. Therefore, Milli-Q water was used for the dilution of the samples to have 25 µg of proteins in each well.

Mini-PROTEAN<sup>®</sup> Tetra Cell kit (*Bio-Rad laboratories, Inc., CA, USA*) comprising transparent stand and two green stands were properly assembled prior to gel preparation. For the preparation of each gel, two glass plates with different size (shorter and longer) were recruited. The shorter glass plate was placed on the longer one which has the elevated part on both sides. A narrow space was formed upon putting two glass plates together which was sufficient for applying the gel. The glass plates were watchfully inserted in the green stand and then were placed on the transparent stand. Transparent stand contains a clasp which contributes to appropriate fixing of the green stand and prevention of the leaking of the gel content.

### 8.1.3.2. Gel preparation

#### 1. Separating gel

Preparation of a separating gel would require the mixing of several components comprising Milli-Q water, separating gel buffer, acrylamide-Bis solution, sodium dodecyl sulphate (SDS), ammonium persulfate (APS), and tetramethyl ethylene diamine (TEMED). The value of each component is determined based on the gel percentage. The percentage of each gel is directly associated with the molecular weight of a particular protein. 7.5% gel was employed for the detection of ICAM-1, NF- $\kappa$ B, and COX-2, while GAPDH and HO-1 detections were performed in a 10% gel (Table 1). Required components with respect to the gel percentage were mixed in a beaker appropriately. Polymerization was commenced upon addition of TEMED. Thus, a fast working flow was required to avoid possible errors. The prepared gel mixture was pipetted in the narrow space between the two glass plates. Air bubbles were removed and the space above the gel mixture was covered by isobutanol. 30-60 minutes was recommended as the polymerization time, and it was advised to preserve a small amount of the gel solution in a beaker to ensure about the progression of the polymerization. When the polymerization was completed the isobutanol was removed and the space above the gel was rinsed using Milli-Q. The water used for rinsing was dried with the aid of filter paper.

Table 1: *Composition of separating gel*

Components	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

## 2. Stacking gel

For preparation of stacking gel, the required components were properly mixed in a beaker in respect to the Table 2. After preparation of stacking gel solution, it was instantly applied to the space above the polymerized separating gel in a way that was overlapped with the edge of shorter glass plate. A plastic comb was immediately implanted in the unpolymerized stacking gel. Due to the insertion of the plastic comb, a part of the stacking gel solution was poured out. Thus, a small amount of stacking gel solution was again added to both sides of the plastic comb. 30-60 minutes were recommended as the polymerization time, and it was advised to preserve a small amount of the gel solution in a beaker to ensure about the progression of the polymerization.

Table 2: *Composition of stacking gel*

Components	5% gel
Milli-Q water	6.150 ml
Stacking gel buffer	2.500 ml
Acrylamide-Bis solution	1.250 ml
10% (W/V) SDS	0.100 ml
10% APS	0.030 ml
TEMED	0.015 ml

### 8.1.3.3. Application of samples on gel

Dilution of the samples was carried out using the specified amount of the sample and Milli-Q water in Eppendorf tubes. Equal volume of sample buffer was added to each tube containing sample (6  $\mu$ l sample+ 6  $\mu$ l sample buffer). Centrifugation of the samples at 5000 rpm was carried out for 45 seconds. Then the samples were heated for 5 minutes in a water bath which was preheated up to 95°C following by 30 seconds centrifugation.

Preparation of running buffer was performed since it was needed for electrophoresis. 100 ml of running buffer (10x Tris/glycine/SDS, *Bio-Rad Laboratories, Inc., CA, USA*) was diluted up to 1000 ml using Milli-Q water.

The glass plates containing the prepared gel were carefully removed from the green stand and were placed in Mini-PROTEAN Tetra Cell attachment in an orientation that smaller glass plates were facing inward. Then it was transferred to the electrophoretic chamber which was appropriately assembled. The formed space between glass plates was filled with the running buffer. While wells were covered by the running buffer, the plastic comb was pulled out gently and watchfully. After removing of the plastic comb, marker and samples were applied into the wells. The first well was filled with 10  $\mu$ l of a marker with the known molecular weight (Precision Plus Protein <sup>TM</sup>Dual Colour Standards, *Bio-Rad Laboratories, Inc., CA, USA*) which was considered as a reference. After filling of the first well with the marker, each well was filled with 10  $\mu$ l of each sample consecutively from left to right. Samples were applied into the wells by a proper pipetting technique in which the tip of the pipette was placed in the space between the two glass plates that was covered by the running buffer and the content was injected into each well. The electrophoretic chamber was filled up to the mark of 2 gels with the running buffer.

#### 8.1.3.4. Gel electrophoresis

The chamber was locked with a cap containing electrodes. The colour code (red to red, black to black) was carefully followed when the chamber was connected to the power source. The power source instrument was adjusted according to Table 3. Since gel electrophoresis procedure was accompanied by temperature enhancement and heating up of the instrument, as a result of that both sides of the chamber were surrounded by the ice pads in order to prevent from too much heating.

Table 3: *Gel electrophoresis conditions (2 gels)*

Voltage	200 V
Electric current	120 mA
Time	35 minutes

### **8.1.3.5. Blotting**

#### **1. Preparation of membranes**

Membrane activation was fulfilled before the electroblotting of the proteins. A polyvinylidene difluoride (PVDF) membrane (Immobilon®-P PVDF membrane, *Sigma-Aldrich Inc., MO, USA*) was utilised for the protein transfer. PVDF membrane was cut according to a pattern of (6.5 cm\*9.5 cm). Both sides of the membrane were covered by blue papers. The membrane was watchfully pulled out with the aid of tweezer and was put in a methanol bath followed by shaking for 30 seconds. Then the membrane was transferred to the bath of transfer buffer which was placed on a shaker for a duration of 25 minutes.

#### **2. Preparation of transfer buffer**

Protein transfer from the gel to the membrane was carried out in the medium of transfer buffer. 100 ml of transfer buffer (10x Tris/Glycine/Buffer for Western blots and Native Gels, *Bio-Rad Laboratories, Inc., CA, USA*) were diluted with the 700 ml of Milli-Q water then the mixture was further diluted with 200 ml of methanol. Since an exothermic reaction takes place between the transfer buffer and methanol, it was recommended to avoid a direct dilution of the transfer buffer with the methanol. Hence, the transfer buffer was first diluted with Milli-Q water and then methanol was added.

#### **3. Wet method**

Wet method of electroblotting was recruited for this thesis using Mini Trans-Blot® Cell kit (*Bio-Rad Laboratories, Inc., CA, USA*). Soaked black sponges and thick filter papers were employed to form a so-called sandwich. Before preparation of the sandwich, black sponges and the filter paper were moistened using the transfer buffer. First black sponges were placed on the black side of the cassette which exhibits the negative pole. Then, thick filter papers were situated on the black sponges.

The glass plates were watchfully pulled out of the electrophoretic chamber. The glass slide was gently removed, and the gel was placed in the centre of the thick filter paper. PVDF membrane, which was previously activated, got positioned on the top of the gel. It was highly advisable not to move the membrane when it was fixed on top of the gel. Another layer of thick

filter papers was placed on the top of the membrane. A roller was used for removing of air bubbles properly. Last layer of the sandwich which was the black sponges were situated on the thick filter paper to complete the structure of the sandwich. Then the cassette was closed, and it was transferred to a rack with respect to the colour code that the black side of the cassette was facing the black side of the rack and the transparent side (exhibiting the positive pole) of the cassette was oriented toward the red side of the rack. The prepared rack was situated in an electroblotting chamber. The chamber was filled with the transfer buffer up to the mark. Electrodes were connected to the power source with respect to the colour code (red to red and black to black) and then the power source was adjusted according to Table 4 for the process of the protein transfer.

**Table 4: *Electroblotting conditions (2 membranes)***

Voltage	140 V
Electric current	300 mA
Time	90 minutes

When the process of electroblotting was completed, the membrane was carefully taken out of the sandwich, and it was placed in a bath of transfer buffer.

#### **4. Blocking, application of antibody, and detection**

TBS-T was prepared for the purpose of membrane washing and provision of blocking buffer. For preparation of the TBS-T, 100 ml of 10× concentrated Tris buffer saline (TBS) was utilised to be mixed with 900 ml of ultra-pure water. 1 ml of Tween<sup>®</sup> 20 (*SERVA Electrophoresis GmbH, Germany*) was added to the mixture and magnet stirrer was used for the proper mixing of the solution. The resulted solution is known as the TBS-T.

The membrane was taken out of the bath of transfer buffer and was positioned in a way that the marker was situated on the left side. The membrane was cut into a few strips based on the molecular weight of the desired proteins and was marked by pencil. The molecular weight of the evaluated proteins is shown in Table 5.



Table 5: *Molecular weight of the proteins*

Protein	Molecular weight (kDa)
<b>HO-1</b>	28
<b>GAPDH</b>	37
<b>NF-<math>\kappa</math>B</b>	60-65
<b>COX-2</b>	64-70
<b>ICAM-1</b>	85-95

Glyceraldehyde -3-phosphate dehydrogenase (GAPDH) was employed to play the role of reference for the applied samples.

Blocking buffer (TBS-T-5% milk) was prepared using 5 g of low-fat milk powder (Blotting-Grade Blocker, *Bio-Rad Laboratories, Inc., CA, USA*) which was dissolved in 95 ml of TBS-T. To minimize the possible errors and avoid of any non-specific reactions between the membrane and the antibody, membrane was introduced to the blocking buffer for 1 h on a shaker at room temperature.

### **5. Incubation of membranes with primary and secondary antibodies**

Protein detection on the membrane is mainly conducted through applying the primary and secondary antibodies. Primary antibody binds the particular band on the membrane which represents the specific protein (Kurien et al., 2015). Dilution of the Primary antibody was carried out using the blocking buffer for a purpose of protein detection based on Table 6. Then, the membranes were put in specific boxes while the protein side of the membranes were facing upwards. The primary antibody was applied all over the membranes and they were immediately transferred to a shaker for 1 hour at room temperature. Then, the membranes were transferred to a fridge for an over-night stay. Membrane washing was performed on the next day using TBS-T. Membranes were washed 5 times and each time 10 minutes. Secondary antibody, which is mainly found in a conjugated form with a specific enzyme, binds the primary antibody causing the formation of a complex which contributes to the staining of the band and consequently identification of the protein (Kurien et al., 2015).

Blocking buffer was used for the dilution of the secondary antibody to the desired concentration (Table 7). Secondary antibody, which was conjugated with horseradish

peroxidase (HRP), was used for an hour incubation of the washed membranes at room temperature. Membrane washing was again fulfilled for 5 times, each time 10 minutes.

Table 6: *Primary antibodies dilution*

Primary antibody	Producer	Catalogue number	Host	Dilution
<b>GAPDH</b>	Cell Signalling Technology (MA, USA)	2118	Rabbit	1:8000
<b>ICAM-1</b>	R&D System	AF796	Goat	1:500
<b>NF-<math>\kappa</math>B</b>	Abcam (Cambridge, UK)	ab16502	Rabbit	1:1000
<b>HO-1</b>	Abcam (Cambridge, UK)	ab13248	Mouse	1:2000
<b>COX-2</b>	Proteintech (IL, USA)	12375-1-AP	Rabbit	1:200

Table 7: *Secondary antibodies dilution*

Protein	Secondary antibody	Producer	Catalogue	Dilution
<b>GAPDH</b>	Anti-rabbit	Cell Signalling Technology (MA, USA)	7074S	1:10000
<b>ICAM-1</b>	Anti-goat	(Sigma-Aldrich Inc, MO, USA)	A5420	1:5000
<b>NF-<math>\kappa</math>B</b>	Anti-rabbit	(Abcam, Cambridge, UK)	ab6112	1:4000
<b>HO-1</b>	Anti-mouse	Sigma-Aldrich	A9917	1:4000
<b>COX-2</b>	Anti-rabbit	(Abcam, Cambridge, UK)	ab6112	1:1000

### 8.1.3.6. Chemiluminescence detection

Pico (Super Signal west Pico Plus chemiluminescent Substrate), (*Thermo Fisher scientific Inc., IL, USA*) and Femto (Super Signal West Femto Maximum Sensitivity Substrate), (*Thermo Fisher scientific Inc., IL, USA*) were recruited as the chemiluminescent substrates. For preparation of each substrate, two reagents were used. Reagents number 1 and 2 were mixed with 1:1 ratio (500 µl reagent 1+ 500 µl reagent 2). The membranes were placed on a plastic sheet and based on the type of protein and required sensitivity, a chemiluminescent substrate was chosen and applied all over the membrane. The reaction time could vary for each protein (Table 8). The reagent was drained from the membrane and was directly placed in a plastic cassette. Air bubbles were removed using tissue and the plastic cassette was put in ChemiDoc instrument. The exposure time could be adjusted if it was required (Table 8).

The final Western blot images were obtained and quantified by ImageLab imaging software version 6.0.1 (Bio-Rad). The gels were loaded with identical amount of proteins, which was confirmed by the immunodetection of GAPDH. Statistical evaluation of the results was carried out using GraphPad Prism 8 software.

Table 8: *Reaction time of chemiluminescent substrates*

Protein	Substrate/Time	Exposure time
GAPDH	Pico/ 3 min	30 sec
ICAM-1	Pico/ 4 min	45 sec
NF-κB	Pico/ 4 min	20 sec
HO-1	Pico/ 3 min	70 sec
COX-2	Femto/ 2 min	35 sec

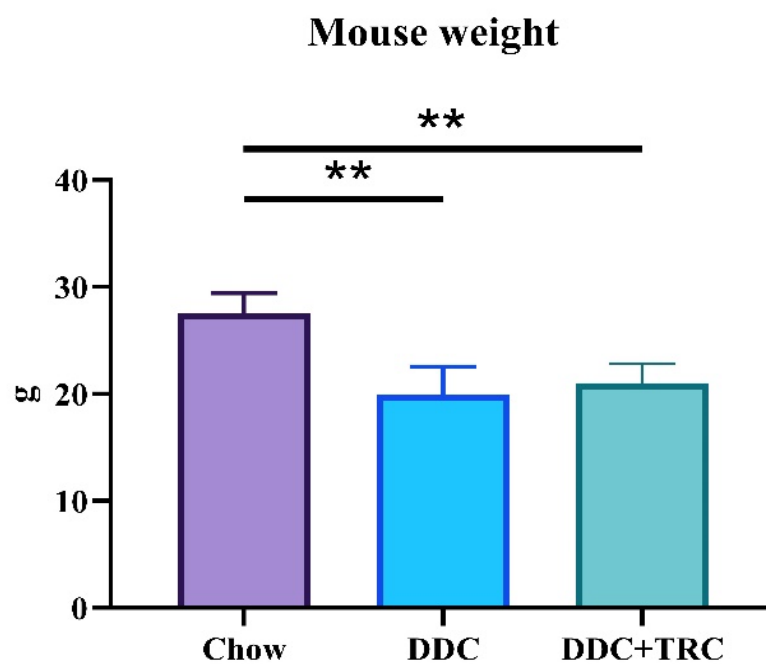
### 8.1.4. Statistical analysis

GraphPad Prism 8.0 software (*San Diego, CA., USA*) was utilised for carrying out of all analyses. Kruskal-Wallis test was recruited to evaluate the comparisons between different groups and verify the significance. A difference of  $P < 0.05$  was considered statistically significant.

## 9. Results

### 9.1. The effect of DDC diet on mouse weight

Mice that were fed with DDC diet showed a significant reduction in body weight (~23%) compared to mice fed with a chow diet at the end of 4 weeks. Similar body weight reduction was seen in DDC+TRC group (Figure 5).

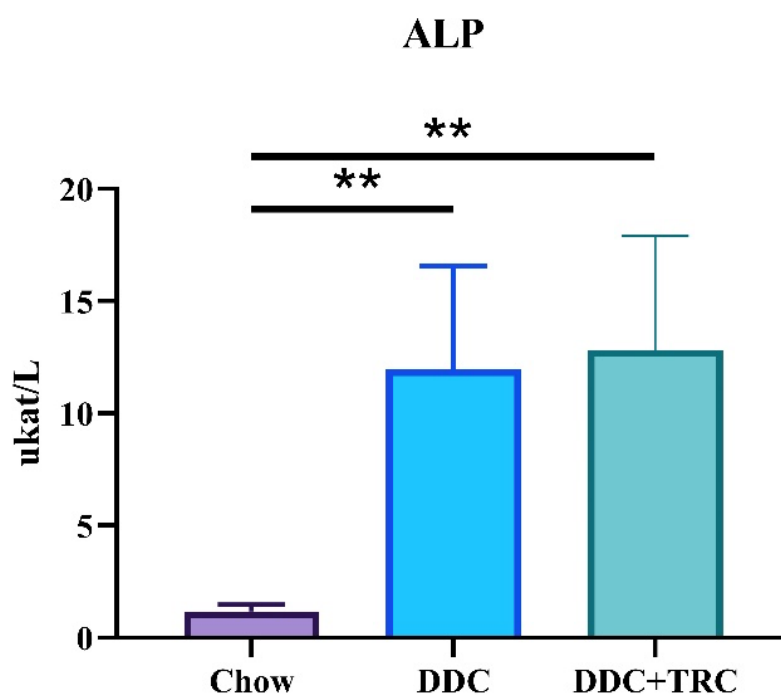


**Figure 5.** DDC diet effects on body weight. The data are presented as mean  $\pm$  S.E.M, ( $n = 6$ ).

\*  $p < 0.05$  \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , using the Kruskal–Wallis test.

## 9.2. The effect of DDC diet on liver enzyme

The plasma level of alkaline phosphatase (ALP) was measured in order to confirm the liver injury in mice fed DDC diet. There was a significant elevation of plasma levels of ALP in DDC and DDC+TRC group compared to chow diet group (Figure 6), suggesting liver impairment.



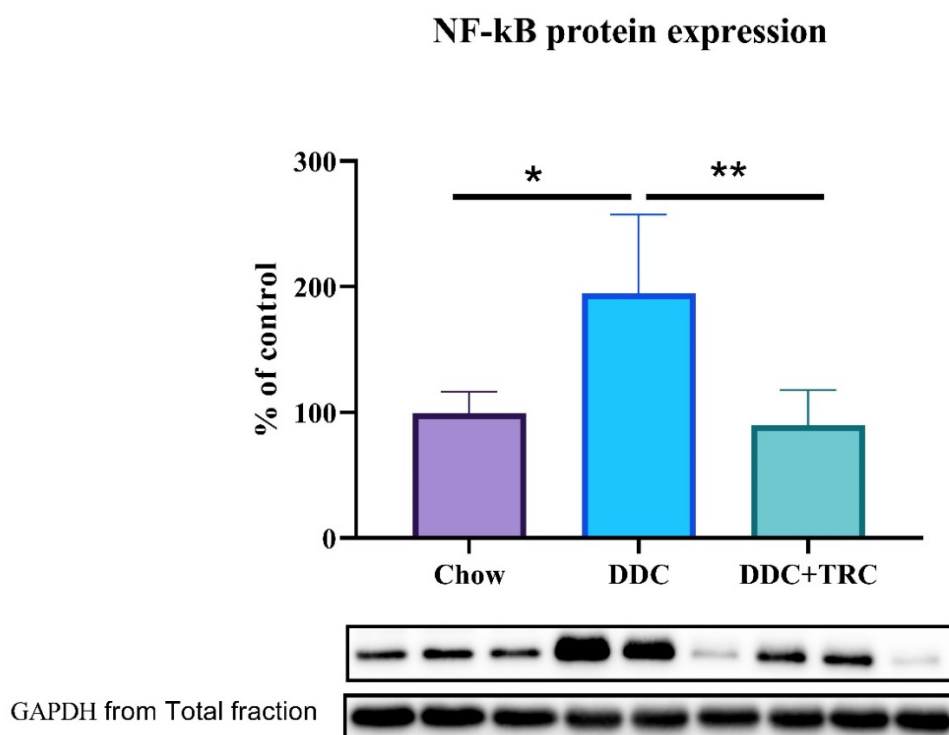
**Figure 6.** DDC diet effect on the plasma level of alkaline phosphatase (ALP). The data are presented as mean  $\pm$  S.E.M, ( $n = 6$ ). \*  $p < 0.05$  \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , using the Kruskal–Wallis test.

### **9.3. The effect of DDC diet and carotuximab treatment on the expression of inflammatory biomarkers in the liver**

Western blot analysis was used to determine the expression of inflammatory markers in the liver, such as nuclear factor kappa-light chain-enhancer of activated B cells (NF- $\kappa$ B), intracellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), and heme oxygenase-1 (HO-1).

### 9.3.1. The effect of DDC diet and carotuximab on NF- $\kappa$ B protein expression

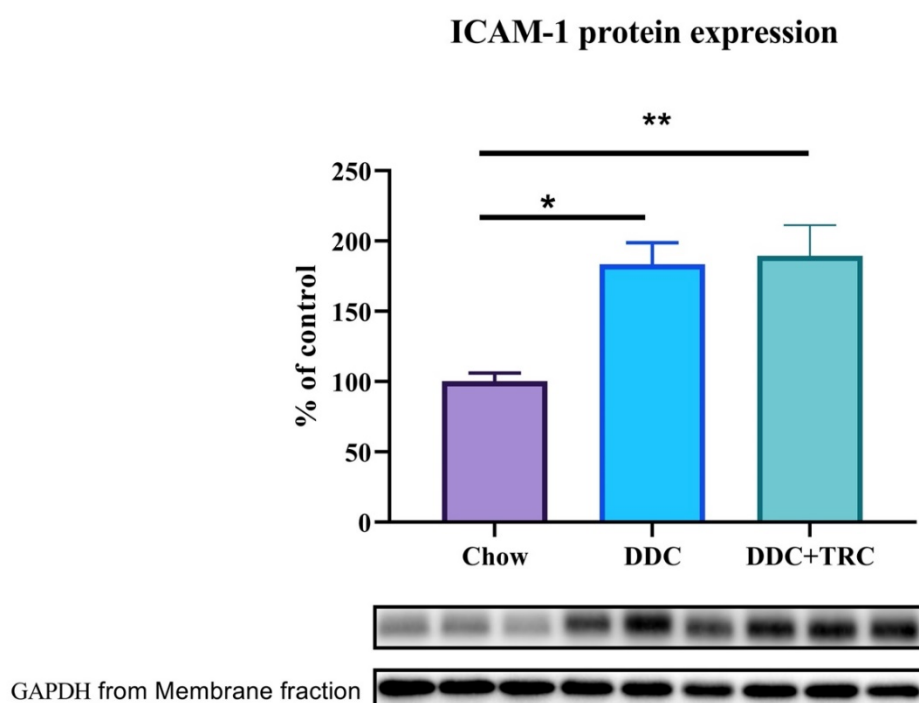
Western blot analysis demonstrated a significant increase in the level of NF- $\kappa$ B protein expression in DDC group compared to control group. Moreover, the expression of NF- $\kappa$ B was significantly reduced in DDC group treated with carotuximab compared to DDC group (Figure 7). As shown in Figure 7, DDC+TRC group has a similar protein expression of NF- $\kappa$ B as the control group.



**Figure 7.** *NF- $\kappa$ B 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  $\pm$  S.E.M, (n = 6). \*  $p < 0.05$  \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , using the Kruskal–Wallis test.*

### 9.3.2. The effect of DDC diet and carotuximab on ICAM-1 protein expression

ICAM-1 protein expression was measured in liver samples to detect the effect of the DDC diet and carotuximab impact on mice liver. As shown in Figure 8, Western blot analysis demonstrated a significant increase in the level of ICAM-1 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 ICAM-1, when compared to DDC group (Figure 8).

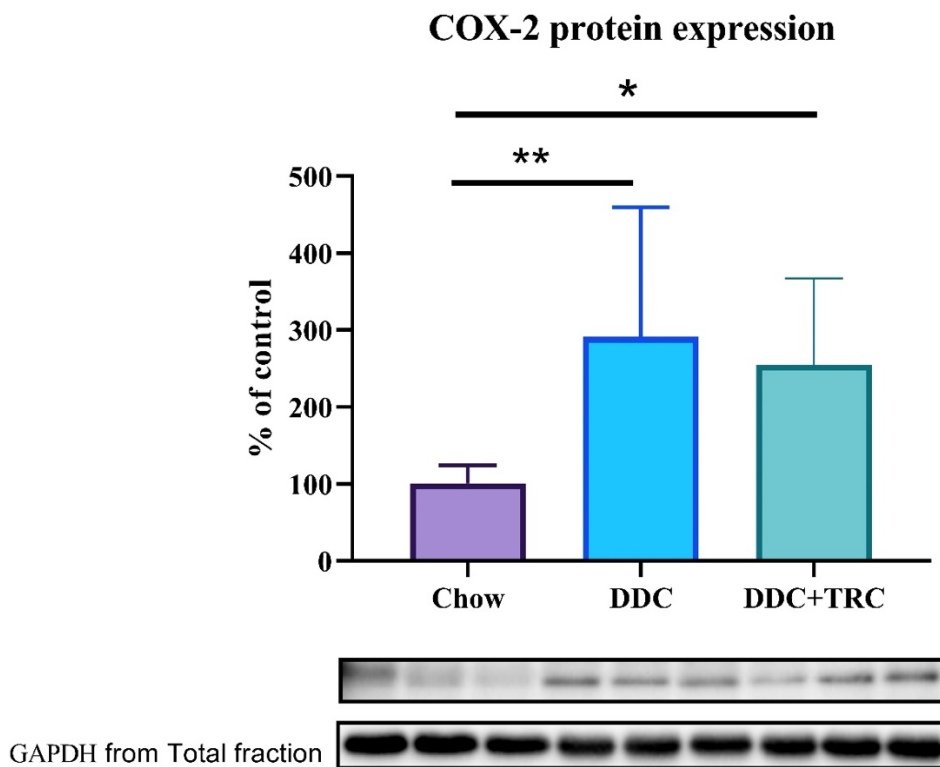


**Figure 8.** *ICAM-1* protein expression in mice liver. Densitometric quantification of immunoreactive bands (top panel: densitometric analysis, control = 100%) was recalculated to the GAPDH signal (bottom panel: representative immunoblots). The data are presented as mean  $\pm$  S.E.M, ( $n = 6$ ). \*  $p < 0.05$  \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , using the Kruskal–Wallis test.



### 9.3.3. The effect of DDC diet and carotuximab on COX-2 protein expression

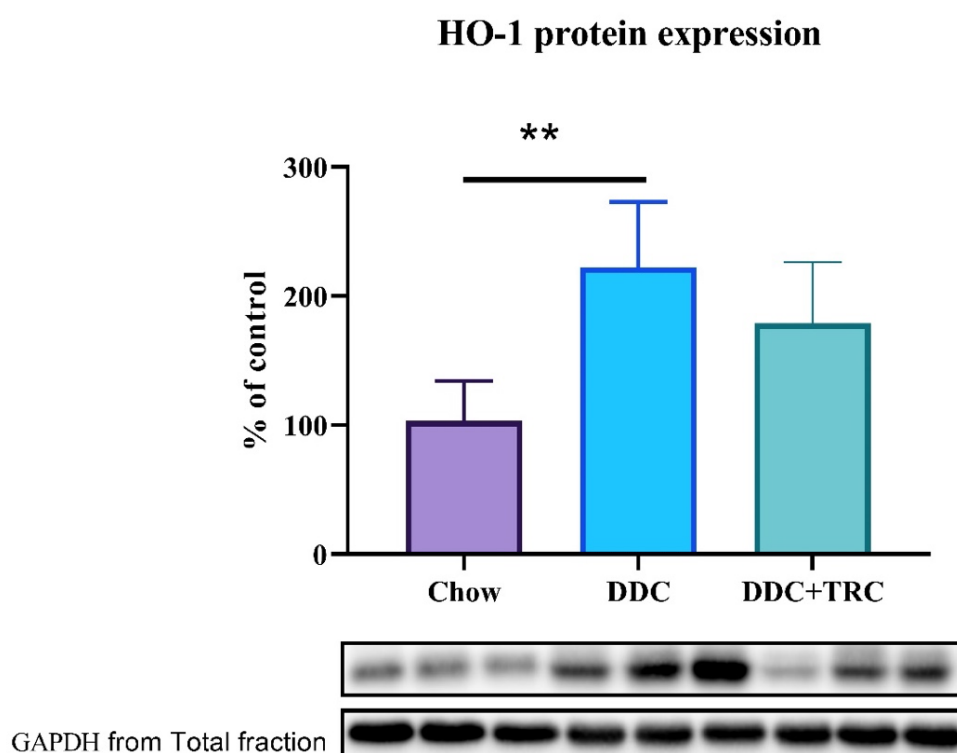
Western blot analysis revealed a significant increase in expression of COX-2 in the DDC fed mice compared to the control group, suggesting inflammation in the liver of mice fed with the DDC diet (Figure 9). Additionally, there was no significant difference in COX-2 expression between DDC group and DDC+TRC group (Figure 9).



**Figure 9.** COX-2 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  $\pm$  S.E.M, ( $n = 6$ ). \*  $p < 0.05$  \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , using the Kruskal–Wallis test.

### 9.3.4. The effect of DDC diet and carotuximab on HO-1 protein expression

Western blot analysis illustrated that the expression of HO-1 was significantly increased in DDC group compared to control group. There were no significant differences in the level of HO-1 between DDC and DDC+TRC group (Figure 10).



**Figure 10.** *HO-1* 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  $\pm$  S.E.M, ( $n = 6$ ). \*  $p < 0.05$  \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , using the Kruskal–Wallis test.

## 10. Discussion

The liver is the biggest internal organ in the human body, and it is involved in many physiological processes, such as metabolism of carbohydrates, lipids, and proteins. Additionally, it participates in detoxification and metabolism of endogenous and exogenous compounds, including drugs and hormones. It also plays an important role in the heme synthesis, degradation, and excretion of bilirubin. The liver actively takes part in metabolism of cholesterol and bile synthesis. Liver acts as a storage site for many vital vitamins and minerals (Hen et al., 2016, Toleikis et al., 2020, Rendic et al., 2018, Haines et al., 2020, Chiang et al., 2018, Roth et al., 2018, Blaner et al., 2016).

There are different types of cells in the liver that are involved in its diverse physiological activities. Kupffer cells are considered as the resident macrophages in the liver which play an important role in mediating the hepatic immune response. These cells are actively involved in eliminating the pathogens and initiation of the immune response. Kupffer cells participate in production of proinflammatory cytokines such as IL-1, IL-6, and TNF- $\alpha$  (Tanwar et al., 2020).

Hepatic stellate cells (HSCs) are another type of liver cells which reacts to inflammation. These cells undergo differentiation to form the myofibroblasts which contribute to the formation of scar tissue and liver fibrosis (Higashi et al., 2017).

Non-alcoholic fatty liver disease (NAFLD) is one of the most common types of liver disorders which is characterized by the presence of steatosis in hepatocytes. There are two types of NAFLD based on their severity. Isolated presence of hepatic steatosis, which does not lead to serious inflammatory response and permanent damage, is known as benign non-alcoholic fatty liver (NAFL), whereas non-alcoholic steatohepatitis (NASH) is a more severe form of the NAFLD which results in hepatic inflammation and formation of fibrous tissue. Long term inflammation and chronic insult to the liver results in alteration of its architecture and formation of the scar tissue. This permanent damage is known as liver cirrhosis which is the terminal stage of the disease and can lead to hepatic failure (Cobbina et al., 2017).

Animal model studies have provided a valuable modality for research on hepatic pathophysiology, particularly NAFLD. These studies greatly contributed to gain a better understanding of the processes linked to the liver fibrosis and potential avenues for diagnosis and treatment of these conditions (Yanguas et al., 2016).

There is a wide range of techniques for inducing the liver fibrosis in animal model studies. 3,5-diethoxycarbonyl-1,4-dihydrocolidine (DDC) in a diet was used to induce liver fibrosis in

mouse during this study. DDC can cause hepatic damage by increasing the production of free radicals that can lead to oxidative stress-induced cellular injury. These free radicals also participate in hepatocellular inflammation, necrosis, and liver fibrosis. DDC induced mice model of liver damage mimics NAFLD in human. Therefore, this model is useful to gain a better understanding of NAFLD and potential avenues for its treatment (Fickert et al., 2002).

DDC diet was used for a period of 4 weeks in this study. This study shown that DDC diet elevated the level of alkaline phosphatase (ALP) which indicates the hepatocellular injury (see Figure 6 in the result chapter) (Fernandez et al., 2007).

Endoglin (CD105) is a transmembrane glycoprotein that serves as auxiliary receptor for TGF- $\beta$  signalling. Activation of TGF- $\beta$  signalling pathways mediates important physiological processes, such as extracellular matrix production, cell proliferation and angiogenesis (Jeng et al., 2021). Previously, it has been demonstrated that endoglin and TGF- $\beta$  signalling pathway are involved in liver inflammation and fibrosis (Jeng et al., 2021).

Carotuximab (TRC105) is a chimeric monoclonal antibody (IgG1) that exerts anti-endoglin effects. Some studies have shown the potential of carotuximab in treatment of cancers via inhibition of angiogenesis in tumour tissue (Duffy et al., 2015, Liu et al., 2020).

In order to investigate the role of carotuximab in liver inflammation, mice were divided into 3 groups: control group (chow diet), DDC group, and DDC+TRC group. This study hypothesized that carotuximab reduces the inflammation in DDC induced liver inflammation in mice.

NF- $\kappa$ B is a transcription factor that plays a vital role in the regulation of inflammatory response. Additionally, NF- $\kappa$ B participates in cell survival and differentiation, cellular interactions, and cytokines production (Mussbacher et al., 2019). It has been shown that activation of NF- $\kappa$ B in hepatic stellate cells (HSC) will result in their differentiation into myofibroblasts that can subsequently lead to liver fibrosis (Seki et al., 2007).

ICAM-1 is a glycoprotein on the cell surface which acts as an adhesive molecule in inflammatory reaction. ICAM-1 activity can recruit immune cells to the site of inflammation (Bui et al., 2020). Additionally, ICAM-1 is also involved in the regulation of inflammatory response and plays a crucial role in cellular interactions (Harjunpää et al., 2019).

COX-2 is an enzyme that takes part in the metabolism of arachidonic acid which results in formation of some bioactive lipids, including prostaglandin and thromboxane that are known as prostanoids (Núñez Martínez et al., 2003). Proinflammatory cytokines increase the expression of COX-2 during inflammation (Martin-sanz et al., 2010).

HO-1 is a metabolic enzyme that is involved in heme degradation and plays a vital role in iron homeostasis. It has been shown that cellular stress increases the expression of HO-1 (Ryter et al., 2021). Furthermore, it has been demonstrated that induction of HO-1 activity can halt the progression of NAFLD and can have a preventable role in development of steatohepatitis (Du et al., 2020).

As previously mentioned, NAFLD in its serious form is accompanied by chronic liver inflammation which results in liver fibrosis. In order to assess the effect of DDC diet and carotuximab on liver inflammation in mice, the level of inflammatory markers, such as NF- $\kappa$ B, ICAM-1, COX-2, HO-1 was evaluated by Western blot method.

Western blotting is one of the most common laboratory techniques in biochemistry and molecular biology. This method is used to detect the target protein in the mixture. this method allows the precise quantification of a particular protein (Taylor et al., 2014).

This study demonstrated that mice fed DDC diet had elevated levels of inflammatory biomarkers, including NF- $\kappa$ B, ICAM-1, COX-2, HO-1. Therefore, we confirmed the results of previous studies, that DDC diet in mice induces liver inflammation. Furthermore, the impact of carotuximab on potential lowering of these inflammatory biomarkers was not proved consistently. Carotuximab reduced the level of NF- $\kappa$ B in DDC+TRC group compared to the DDC group (see Figure 7). However, it did not exert the same impact on the levels of ICAM-1, COX-2, HO-1. In fact, there was no significant difference in the levels of ICAM-1, COX-2, HO-1 between DDC group and DDC+TRC group (Figure 8, 9, 10). Therefore, further studies are required to gain a clearer picture of the effect of carotuximab on liver inflammation.

## **11. Conclusion**

Carotuximab (TRC105) is a monoclonal antibody that has shown some effect in cancer treatment. TRC 105 exerts anti-endoglin effect, from that its anti-inflammatory and anti-fibrogenic effects have been hypothesised. In our study, we considered its potentially anti-inflammatory effect in a mouse model of DDC-induced hepatic inflammation. It was shown that carotuximab lowers the elevated levels of NF- $\kappa$ B after DDC diet. However, it did not have a significant effect on the levels of ICAM-1, COX-2, HO-1 in DDC-fed mice. Thus, further detailed studies are suggested to investigate the role of carotuximab in hepatic inflammation.

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