An-Najah National University
Faculty of Graduate Studies

THE EFFECT OF BILE ACID SIGNALING PATHWAY ON LUNG IMMUNE CELLS IN LIVER FIBROSIS MICE MODEL

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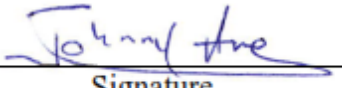
Dr. Jhonny Amer Supervisor	 Signature
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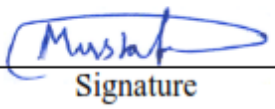
Dr. Mustafa Ghanem Internal Examiner	 Signature

Dedication

This research is dedicated to all the hard-working, self-made scientists who are passionate about seeking knowledge. To anyone who sought to learn new skills and improve himself despite all the circumstances. To my colleagues in the Gaza Strip who unfortunately couldn’t accomplish their graduation projects. And to all the young girls who dream about science as a profession, the world needs science, and science needs women!

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Last but not least, to my professor and supervisor Dr. Jhonny Amer, my greatest appreciation for all your time, wise bits of advice, and understanding. Mentors like you, are the ones I seek as academic role models.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

THE EFFECT OF BILE ACID SIGNALING PATHWAY ON LUNG IMMUNE CELLS IN LIVER FIBROSIS MICE MODEL

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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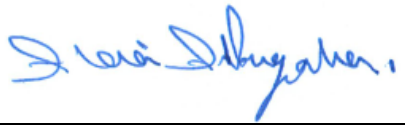
Signature:	
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Abstract

Background: Patients with liver diseases are at high risk for the development of acute respiratory distress syndrome (ARDS). The mechanisms by which the liver participates in the pathogenesis of acute lung injury are not understood.

Objectives: Our study aims to assess for mediators that could interfere with liver-lung axis via studying the involvement of bile acids (BAs) signaling in the lung in a mouse model of liver fibrosis.

Methods: C57BL/6J mice were induced for liver fibrosis through i.p injection with carbon-tetrachloride (CCl_4) for 2 weeks (as an acute model) and 6 weeks (as a chronic model) (n = 12). Serum, BALF, and lung tissue samples were collected on sacrificing day. ELISA was used to detect levels of sRAGE and inflammatory cytokines (IL-1β, IL-4, IL-6, TNF-α) in BALF samples, and BAs in both serum and BALF. Real-time PCR was used to quantify markers of lung oxidation (GPx, GSH: GSSG, MDA, SOD), injury (SP-D1), and fibrosis (α-SMA, MMP-9, GFAP). Flow cytometry evaluated isolated resident lung NKs for activation and viability, through the expression of CD107a marker and NTCP. Isolate alveolar epithelial cells (AEC) type I and II were assessed for apoptosis.

Results: BAs concentrations were linearly correlated with liver fibrosis severities in serum and BALF. It was found that markers of inflammation, oxidative stress, injury, and fibrosis were significantly increased in both the acute and chronic models of liver fibrosis compared to the control group. Isolated resident lung NK cells exhibited a significant increase in the expression of BA-specific transporter, NTCP, which was associated with a reduction in the expression of CD107a maker; the key indicator of NKs activation and functionality. Moreover, AECs type I and II demonstrated a substantial increase in their apoptosis rate, which was correlated with liver fibrosis severity.

Conclusion: BAs, by-products of cholesterol generated in the liver, were found in high concentrations in the lungs of mice with liver fibrosis. These data were associated with an elevation in oxidative stress markers in the lung, and impairment of resident lung NK cells with overexpression of NTCP on their surface. BAs insults on NK cells may partly contribute to the acute lung injury (ALI) pathogenesis and AECs apoptosis. Our data suggest BAs as a valuable approach in treating and diagnosing of liver fibrosis and pulmonary complications.

Keywords: Liver fibrosis, resident lung NK cells, BAs, BALF.

Chapter One

Introduction and Theoretical Background

1.1 Background

Liver disorders, either alcohol-related or non-alcoholic fatty liver disease (NAFLD) are responsible for 2 million deaths each year, accounting for 3% of global mortalities (1). NAFLD is restricted in 2 forms: NAFL (including steatosis), and non-alcoholic steatohepatitis (NASH), with fibrosis being the most critical histological feature, developing into cirrhosis (2) and eventually hepatocellular carcinoma (HCC); the most common form of liver cancer (75 – 80%) and the 16^{th} most common cause of deaths per year worldwide (3).

1.1.1 Liver Fibrosis - Main Causes and Risk Factors

Liver fibrosis (LF) is a molecular and cellular wound-healing response triggered by various factors that cause hepatic injury (4). Alcohol consumption and metabolic syndrome (represented by insulin resistance, high-fat diet, diabetes, elevated blood pressure, hormonal changes, oxidative stress, and inflammation) are the leading causes of LF in Western and industrial countries (3, 5); on the other hand, viral hepatitis (mainly type B and C) is the primary cause in many Asian, Western Pacific and African regions (2). Other factors include certain health conditions (such as primary sclerosing cholangitis and autoimmune diseases), in addition to some toxins (drug-induced liver injury) (1), and genetic factors (3).

1.1.2 Pathophysiology of LF

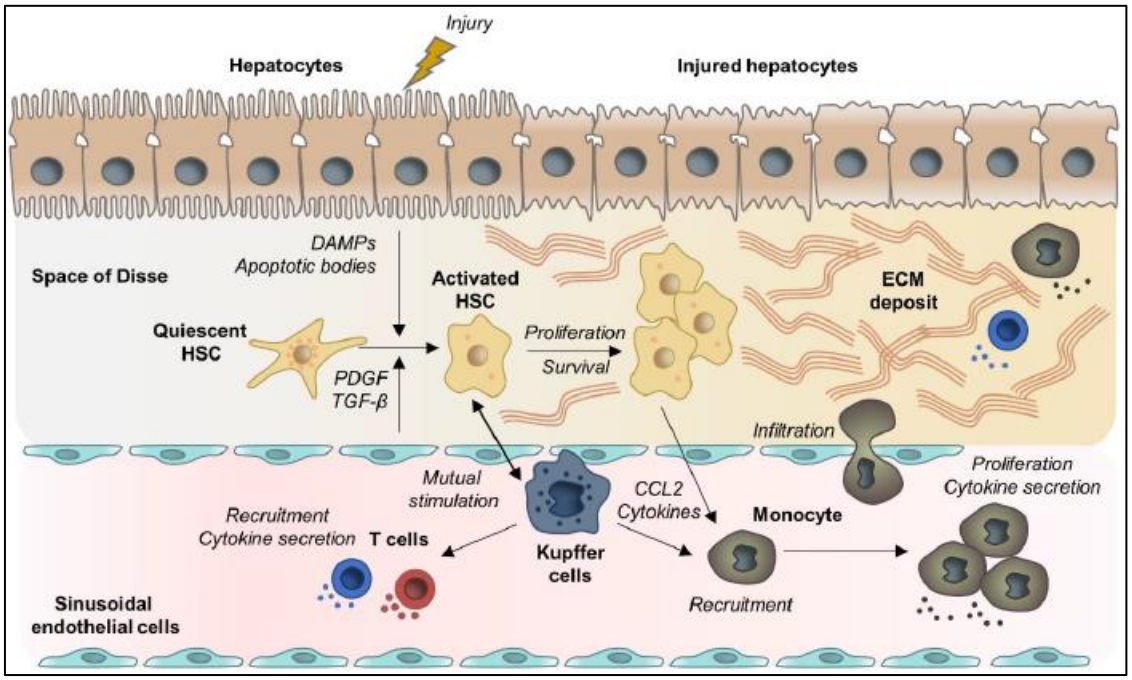
Chronic hepatic injury causes the release of apoptotic bodies, damage-associated patterns (DAMPs), and several cytokines (mainly tissue growth factor beta1 (TGFβ1), platelet-derived growth factor (PDGF), and interleukins (IL1, IL6)) leading to the activation of hepatic stellate cells (HSCs); the critical step in LF (4). Typically, HSCs are quiescent non-proliferative star-shaped cells with ample storage of retinoid lipid droplets and vitamin A(6) however, once activated, they undergo morphological changes, proliferation, contractility, and chemotaxis as they migrate to the site of injury to initiate tissue repair by differentiating into myofibroblasts; the primary source of extracellular matrix (ECM) in the damaged liver (7). In addition, aHSCs lose their lipid droplets and

vitamin A content while constantly producing several ECM components (including type I collagen, alpha-smooth muscle actin (α-SMA), and matrix Metalloproteinases (MMPs)(8).

Collagen production is not the only response of aHSCs to profibrogenic stimuli; they can also stimulate a cellular immune response by releasing inflammatory mediators (such as TGF-β1, IL8, and IL10) that activate lymphocytes, neutrophils, and macrophages, the key players in LF pathogenesis (9) (Figure 1).

Figure 1

Mechanistic concepts for liver fibrosis



Note: Roehlen, Crouchet, & Baumert, (2020).

LF can be reversible if the injury-stimulating factor is removed, as long as the liver is not at the advanced cirrhotic stage. This is accomplished by a cascade of events represented by reduced cytokines, fibrous scars, and myofibroblasts, as well as elevation in collagenase activity. In addition, macrophages undergo phenotype changes from pro-fibrogenic to anti-fibrotic, terminating HSCs activation and causing a lack of ECM components; moreover, the anti-fibrotic macrophages secret MMPs (especially MMP9), leading to degradation and phagocytosis of ECM (4, 6). However, if the injury-causing factor remains, continual HSCs activation will cause disturbances between ECM formation and dissolution. Accumulation of ECM leads to collagen deposition and fibrous

scar formation, affecting hepatocytes quantity (cell count) and quality (cell behavior and function), and eventually, liver failure (8).

1.1.3 Complications of LF

Besides liver cancer (10), several extrahepatic complications are associated with mortality in LF patients, such as cardiovascular diseases (CVDs) (11) and abnormal thyroid function (12). LF can also impair insulin clearance by the liver, leading to insulin resistance that may develop into diabetes (hepatogenous diabetes) (13). Many LF patients develop osteoporosis, which is connected to several mechanisms that cause impaired bone mineralization and microstructure, leading to a reduction in bone mass and density, which explains their experience with bone pain, that might eventually lead to fractures (14).

Another major complication of LF is portal hypertension, mainly due to the increased intrahepatic vascular resistance to portal blood flow as a result of the massive hepatic structural changes caused by fibrosis (15). These circadian dynamic changes are associated with complex events, including electrolyte abnormality (mainly hyponatremia) (16), splenomegaly (17), and brain dysfunction (hepatic encephalopathy) due to toxins accumulation, leading to coma (18), hepatorenal syndrome, characterized by decreased glomerular filtration rate (GFR) (19), systemic inflammation (20), and hemostasis disturbances; affecting the coagulation system, and increasing the risk of bleeding, brushing, or clot formation (21). Thus, detection and staging are crucial in these patients' management and treatment decisions.

1.1.4 Staging and Diagnostic Tools

The golden standard method for LF assessment is liver biopsy, where a specific classification system determines the stage of fibrosis using scores from 0 (no fibrosis) to 4 or 6 (Cirrhosis) (Table 1). After that, another scoring system is used to classify the severity of the determined fibrosis stage and predict mortality. This score (from 5 to 15) is then calculated with the patient`s lab results and clinical factors, and the severity is classified into (A, B, or C) (22).

Table 1

Primary liver fibrosis classifications

Diagnosis and classification	No Fibrosis	Early (periportal) fibrosis	Late (bridging) fibrosis	Cirrhosis
Fibrosis score & illustration	0 – Normal connective tissue 1 – Enlarged, fibrotic portal expansion	2 – Periportal fibrosis, with or without portal-portal septa	3 – Bridging fibrosis, but no obvious cirrhosis	4 - Regenerative nodules encircled by fibrous septa, probably or definite cirrhosis

Note: Berumen, Baglieri, Kisseleva, & Mekeel, (2021).

Despite being the most precise, this invasive procedure has many limitations, such as pain, bleeding, and sampling errors. The biopsies only represent a small portion of the liver, in addition to intra-observer and interobserver variations. Therefore, most clinicians rely on noninvasive methods for LF assessment, such as imaging techniques and laboratory blood tests (23).

The bile acid (BA) profile is one of several biomarkers used for LF determination and monitoring. Both its synthesis and metabolism are markedly disrupted in these patients, leading to elevation in its circulatory and fecal levels, with gut microbiota implicated (24).

1.1.5 Bile Acid Synthesis

Bile acids (BAs), the major lipid component of bile (along with phospholipids, cholesterol, proteins, and bilirubin), are amphipathic molecules synthesized in the pericentral hepatocytes from cholesterol by the rate-limiting microsomal enzyme cholesterol 7α-hydroxylase (CYP7A1) (25), creating the primary BAs (cholic acid (CA) and chenodeoxycholic acid (CDCA)), which are later conjugated to either glycine or taurine mainly by two enzymes, BA CoA synthase (BACS) and BA-CoA-amino acid N-acetyltransferase (BAAT) (24).

The resulting hydrophilic conjugated BAs are secreted from the liver into bile to be concentrated and stored in the gallbladder (26). During the meal, the entero-hormone cholecystokinin stimulates the gallbladder to release bile through the biliary tract into the duodenum to facilitate the emulsification and absorption of dietary fats, cholesterol, and fat-soluble vitamins (25).

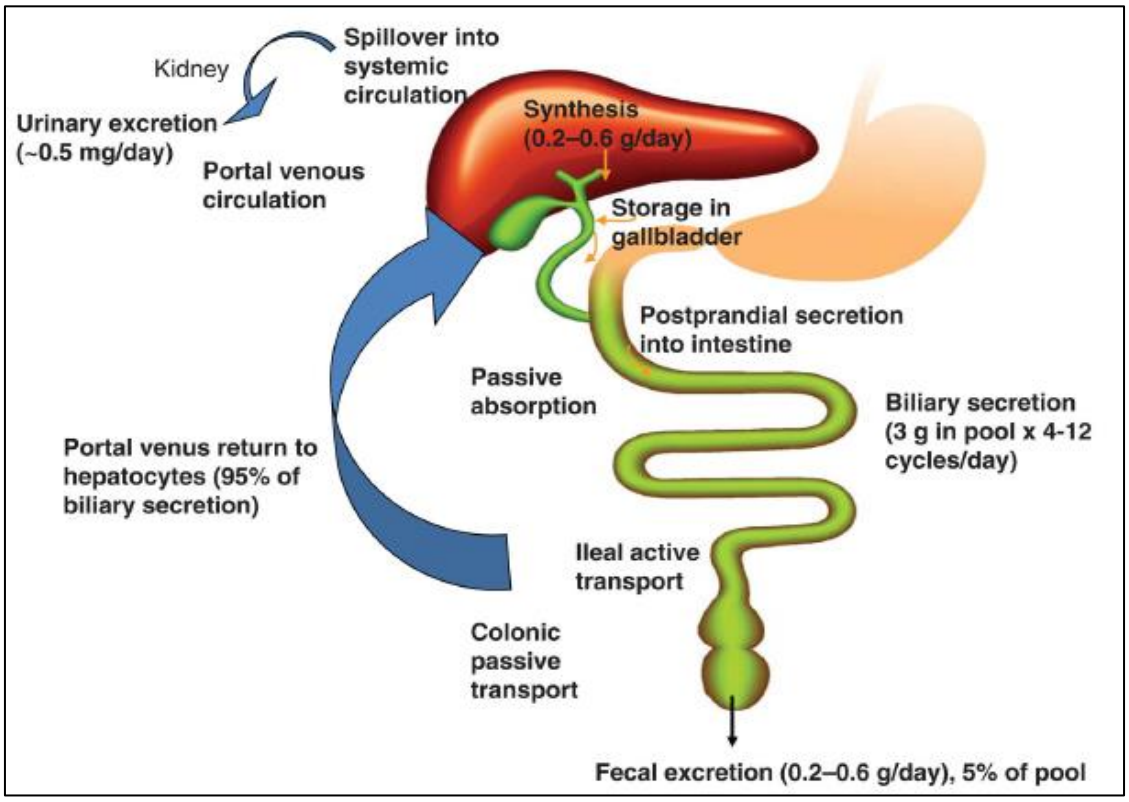
After that, 95% of the BAs are actively reabsorbed in the terminal ileum by carrier-mediated transport across the membrane of the intestinal epithelial cells and return to the liver through the portal vein, leading to negative feedback inhibition of BAs synthesis (26). However, some BAs (5%) escape the intestinal reabsorption and enter the colon, where they undergo deconjugation, dehydrogenation, and dehydroxylation by the resident gut microbiota and transform into secondary and more hydrophilic BAs, such as deoxycholic acid (DCA) and lithocholic acids (LCA) (24), which are passively reabsorbed through the portal circulation into the liver; where all BAs are recycled to be secreted to the biliary tract; completing the so-called enterohepatic circulation, an exchange system between the liver and the gut that regulates nutrients absorption, distribution, and metabolism (27) (Figure 2).

1.1.6 BAs Transport

The transport of BAs is maintained by a specialized system of proteins localized on the membrane of liver and intestinal cells (28). In the portal vein, BAs bound to albumin, and the complex passes through the fenestrae to reach the space of Disse, where it should dissociate for the BAs to flux through the hepatocytes, as albumin undergoes conformational changes once it's bound to the basolateral (sinusoidal) side of the hepatocytes (29).

Figure 2

Enterohepatic Circulation of bile acids



Note: Di Ciaula, et al., (2017).

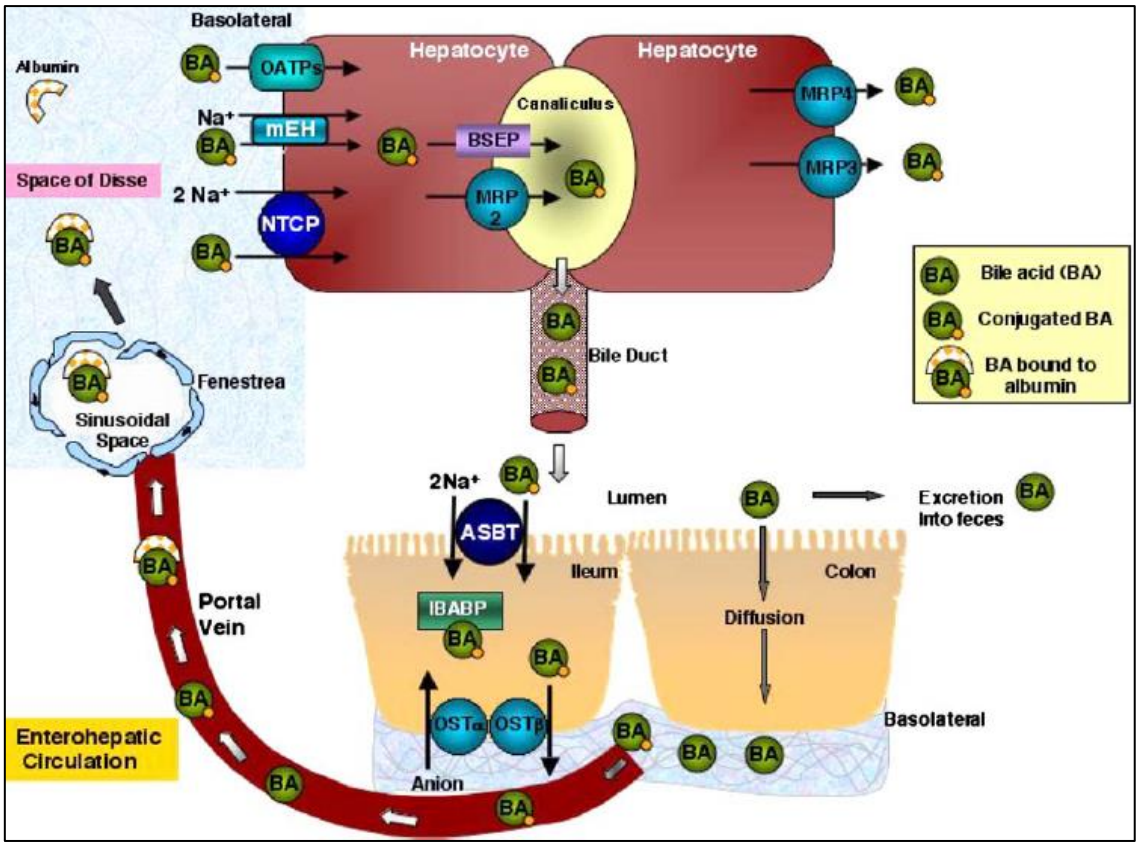
Since the liver cell has an impermeable membrane, BAs flux should be an active process against a concentration gradient (30). Sodium mainly maintaines the first step of BAs clearance via Na^+-dependent taurocholic cotransporting polypeptide (NTCP), which has an electrogenic nature and can bind to both conjugated and unconjugated BAs, but with higher affinity to the conjugated form; this uptake is driven by an inwardly Na^+ gradient accomplished by Na^+/K^+ ATPase (31). Even though hydrophobic unconjugated BAs can be transported by passive diffusion, most of their uptake is maintained by basolateral Na^+-independent BA transporters (ex. Organic Anion Transporting Polypeptide (OATP)), where BAs are exchanged with an intracellular anion, such as $HCO3^-$ or glutathione (GSH) (29).

Once inside the hepatocyte, BAs move intracellularly to the apical (canalicular) site for their secretion into the bile for emulsifying dietary lipids (30); this occurs via two ATP-dependent means of transport: BAs Export Pump (BAEP) and Multidrug Resistant Protein 2 (MRP2) (31). In the distal ilium, BAs are actively absorbed by Apical Na^+-dependent BA Transporter (ASBT), then move intracellularly across the enterocyte by

Ileal BA Binding Protein (IBABP), and eventually efflux to the portal blood by Organic Solute Transporter α and β (OSTα/OSTβ) (28) (Figure 3).

Figure 3

Hepatocellular transport of bile acids



Note: Alrefai, & Gill, (2007).

1.1.7 BAs Receptors

BAs are highly effective signaling molecules that regulate essential cellular and metabolic processes, such as cholesterol homeostasis, intestinal lumen absorption of lipids, cholesterol, and fat-soluble vitamins, and increasing insulin sensitivity (32). They also activate signaling pathways that regulate electrolyte absorption and apoptosis of intestinal and colonic epithelia, affecting both the function and integrity of these cells (29).

All these functions are regulated by acting on a specific family of membrane and nuclear receptors known as BA-activated receptors. The two central characterized receptors are the nuclear receptor Farnesoid X Receptor (FXR), and the G Protein-Coupled Bile Acid Receptor 1 (GPBAR1) also known as Takeda G Protein-Coupled Receptor 5 (TGR5) (33).

When FXR binds to BA, it regulates its synthesis and transport by stimulating the enterokine Fibroblast Growth Factor (FGF), which flows through the portal vein to the liver to inhibit CYP7A1, causing downregulation of BA production (feedback inhibition) (34). In addition, FXR indirectly suppresses NTCP gene transcription after BAs elevation; this is maintained by stimulating the expression of Short Heterodimer Partner (SHP) in the hepatocytes, which in turn blocks the stimulatory effect of either retinoic acid receptor and retinoid X receptor (RAR/RXR) heterodimer or glucocorticoid receptor on NTCP Promoter (32).

On the other hand, BAs bind to TGR5 in plasma, acting on tissues beyond the enterohepatic circulation. This binding regulates energy expenditure from the diet and maintains lipid and glucose homeostasis (35). Unlike FXR, TGR5 has an anti-inflammatory, anti-fibrotic effect. Thus, its binding to BA suppresses inflammatory immune responses (34).

1.1.8 BAs and Gut Microbiota

Due to their central role, BAs synthesis, secretion, and intestinal reabsorption must be balanced (36). This homeostasis is achieved by the interaction between BAs and intestinal microbiome, where BAs exert direct control on gut microbiota through binding to FXR; this binding stimulates Adenosine monophosphate (AMP) production, suppressing intestinal microbial overgrowth (37). On the other hand, intestinal microbiota regulates BAs pool metabolism, composition, function, and circulation by modulating the formation of secondary BAs (24).

This correlation strongly and positively affects both adaptive and innate (cellular) immune responses since several BA nuclear (FXR) and cell surface receptors (TGR5) are expressed by several immune cells, such as monocytes and macrophages, dendritic cells (DCs), natural killer cells (NKs), and NK T cells (38). Activation of BA receptors in these cells causes an inhibitory response, which suppresses the assembly of inflammasomes, downregulates the expression of pro-inflammatory cytokines in macrophages and DCs, inhabits the pro-inflammatory ability of monocytes, and activates their differentiation into DCs (characterized by their poor IL-12 and TNF-α production), in addition to downregulating the differentiation of Th17 meanwhile up regulating regulatory T cells differentiation; causing the activation of anti-inflammatory factors (39).

TGR5 activation suppresses the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in both the liver and intestine and shifts the macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory) phase (38). On the other hand, FXR stimulation suppresses the activation and expression of several inflammatory genes (40). All of these facilitate mucosal inflammation, protect the intestinal barrier integrity, and maintain intestinal epithelial homeostasis by strengthening tight junctions between epithelial cells, suppressing their apoptosis, and stimulating stem cell renewal (39). Experimental studies on mice that lack any of these receptors have shown inflammation development when treated with infectious agents, indicating BAs role in controlling immune response (36).

1.1.9 Complications associated with BAs Abnormalities

Alterations in BAs metabolism, such as high-fat diet, consumption of alcohol and some drugs (antimicrobial), disruptions in sleep and circadian rhythm (hormonal changes), and metabolic disorders (ex. Obesity), all can lead to intestinal dysbiosis, an imbalance in the gut microbial community leading to qualitative and quantitative alterations in its activities, and gut barrier dysfunction (40). This shifts the balance between primary and secondary BAs, impairs enterohepatic circulation, and simulates mitochondrial oxidative stress, which initiates a cascade of immune responses (41).

The resulting inflammation eventually leads to several metabolic disorders, such as diabetes, dyslipidemia, and inflammatory bowel diseases (IBD) (e.g. Crohn`s disease, ulcerative colitis) (42), in addition to cardiovascular diseases (e.g. Atherosclerosis, and extrahepatic vascular dysfunction) (43). BAs can also induce tumorigenesis in several organs, such as esophageal, gastric, hepatic, breast, prostate, and ovarian cancers (44). Therefore, several bile-acid-activated signaling pathways have become attractive therapeutic targets for inflammation and metabolic disorders (45).

As BAs profile gets affected in LF patients, abnormalities in BAs quality or quantity can also lead to several hepatic disorders, including LF (42); a typical example is the need for liver transplantation in patients with biliary atresia for long-term survival (46). Research studies on mouse models of LF induced by carbon tetrachloride (CCl_4) and high-fat diet have shown the increased hepatic concentration of BAs (47), which induces pro-fibrogenic signaling, promotes HSC proliferation, and activates protein expression of

fibrosis-related markers, exacerbating liver fibrogenesis (48). In addition, BAs can apply a detergent action on membrane lipid components, generating oxidative stress in the mitochondria and endoplasmic reticulum, and inducing cell injury in several organs, such as the lungs (49, 50), suggesting that LF can have severe complications in lung health.

1.2 Problem Statement

BAs abnormalities in LF may cause lung injury. This severe health condition leads to persistent alveolar injury and activation of lung fibroblasts (51) as the induced fibrotic response progresses in the lung. In this case, it causes impaired functional gas exchange, respiratory failure, and even lung cancer (52), the second most common cancer and the first leading cause of cancer deaths worldwide (53).

1.3 Study Significance

This Study is essential for a better understanding of the liver-lung axis, as filling this gap will inspire a new research direction, stimulate funding in this field, and provide valuable insights into new diagnostic targets and therapeutic strategies for clinical intervention for liver fibrosis and lung injury.

1.4 Study Objectives

1.4.1 General Aim

This study aims to determine the presence of bile acids in the lungs and their contribution to mediating phenotypic changes in lung resident NK cells in a mice model of liver fibrosis.

1.4.2 Specific Aims

Our objective was to establish a potential crosstalk between the liver and the lungs in terms of BAs, by focusing on the following aspects:

- Analyzing the alterations of BAs profile in both serum and BALF in cases of LF.
- Examining the impact of BAs on the factors of oxidation, injury, inflammation, and fibrosis in pulmonary cells.
- Assess respiratory efficiency by measuring sRAGE levels.
- Determining the effect of BAs on the activity of pulmonary NK immune cells

obtained from liver fibrosis mice model via flow cytometry.

- Evaluating the correlation between BAs and lung functionality by estimating the rate of apoptosis of AEC types I and II.
- Investigating BAs implication in lung injury in LF cases through estimating NTCP expression on lungs-resident NK cells via flow cytometry.

1.5 Research Question and Hypothesis

1.5.1 Research Question

- How does LF affect BAs profile?
- Is there a connection between LF and lung injury?
- What are the impacts of BAs on respiratory health and sufficiency in LF cases?
- Is there an association between NK cells activity and lung functionality?
- What is the relationship between BAs levels and the activity of lung NK cells in LF models?

1.5.2 Study Hypothesis

1.5.2.1 Alternative (research) Hypothesis

- A correlation exists between changes in BA levels, and respiratory sufficiency in LF models.
- There`s a relationship between NK cells activity, and lung functionality in LF models.
- NK cells are central in the biochemical pathway linking LF to lung injury.

1.5.2.2 Null Hypothesis

- No correlation exists between BAs level changes and respiratory sufficiency in LF models.
- There`s no relationship between NK cells activity and lung functionality in LF models.
- NK cells are not a primary factor in the biochemical pathway linking LF to lung injury.

1.6 Literature Review

1.6.1 Liver-Lung Interaction

Although the liver and lung are located in two different cavities separated by the diaphragm, the currently available data on liver-lung interaction shows that some diseases can affect both organs, such as cystic fibrosis, and α1-antitrypsin deficiency (54), in addition to COVID-19 infection, where several studies indicated that patients with metabolic dysfunction-associated liver disorders (MAFLD), such as type 2 diabetes, obesity, hypertension, and cardiovascular diseases had a high prevalence of COVID-19 and a tendency to develop a severe type of respiratory infection with the need for ICU hospitalization. Other studies indicated that liver injury could be exacerbated in patients with MAFLD once infected with COVID-19 (55).

Clinical studies have shown that liver disorders, especially those that include fat accumulation, such as NAFLD, are considered multisystemic diseases. The resulting insulin resistance, lipogenesis, intestinal microbiota alterations, oxidative stress, and systemic inflammation can all lead to many disorders, and are often associated with respiratory complications independent of any pre-existing lung disease (56). Such as asthma, chronic obstructive pulmonary disease (COPD), pleural effusion, α1-antitrypsin deficiency, hepatopulmonary syndrome (HPS), portopulmonary hypertension (PPH), hepatic hydrothorax, and even pulmonary cancer. All of which may further exacerbate the hepatic condition, causing hepatic hypoxia and eventually hepatic failure (57). In addition, NAFLD in COPD patients might contribute to cardiometabolic complications, like congestive heart failure and coronary artery disease. Explaining the need for pulmonary evaluation before liver transplantation (54).

To better understand liver pathogenesis in lung disorders, an experimental study was conducted where mice were injected with bleomycin (BLM). This glycopeptide antibiotic can cause pulmonary toxicity and fibrogenesis, despite its benefits in treating cancers (53). Mice were sacrificed, and the lungs and livers were collected for further analysis. The detected histological damage was caused by BLM-induced oxidative stress, and mediated by an inflammatory response of immune cells secreting interleukins and chemokines, stimulating TGF-β and other fibrotic factors in the lung. Mice livers have shown changes in microenvironment and biochemistry, including an increased cell size,

ECM deposition, and high expression in proliferation proteins and oxidation markers (ex. Glutathione). BLM is inactivated by BLM hydrolase in the liver, if the inflammatory condition of the lungs (the first and directly affected organ by BLM) might chronically damage the liver. Suggesting systemic sclerosis (SSc) patients with pulmonary disease should care for their liver (58).

Another example of the liver-lung interaction is the shared typical general response in both organs to injury damage, specifically if it`s alcohol-related. Resident macrophages found in both the liver and lung play a crucial role in maintaining inflammatory responses, such as an indication of cytokine, including IL-6, IL-8, TNF-α, TNF-β, and granulocyte/macrophage colony-stimulating factor (GM-CSF). This stimulates oxidative stress, which contributes to tissue injury and organ dysfunction. In addition, tissue remodeling gets activated, followed by the deposition of ECM components, like fibronectin and collagen. All of which impairs host defense mechanisms (59).

Mortality in acute lung injury patients is almost 100% in the presence of hepatic failure (55). However, it`s not well understood; several studies have indicated that the interdependence between the liver and lung is via mediators released from the gut (59).

1.6.2 Gut-Lung Axis

Lung and gut share a common embryological origin, as several respiratory aspects were reported in IBD patients. Therefore, when treating these patients with TNF-α inhibitors, there`s a high risk of developing tuberculosis infection. In addition, several intestinal function alterations, such as impaired permeability, have been observed in cases of asthma and COPD (60). This connection between the lung and intestines is described as lung-gut crosstalk, and it involves several potential mechanisms. COPD and asthma cases exert an overexpression of MMP-9 and other proteases that can digest collagen, elastin, and fibronectin. The contribution of formed autoantibodies and abnormal lung microbiome affects the lung and gut's mucosal integrity, stimulates inflammatory lymphocytes, and exacerbates the pathogenesis of pulmonary and intestinal disorders(61).

Bilirubin is an essential contributor to the lung-liver crosstalk, and it`s positively related to lung function outcomes. Independent of cigarette smoking, bilirubin levels appear to be depleted in patients with COPD due to oxidative stress exposure (62).

The gut-lung axis is bidirectional and unclear. However, studies suggest it may be related to gut microbiota dysbiosis, epithelial barrier dysfunction, increased intestinal permeability, immune system stimulation, and inflammatory cascade inhibition (60). Dysbiosis of the gut microbiota increases gut permeability and promotes the translocation of pathological bacteria, it can also stimulate inflammation, exacerbating COPD. On the other hand, systemic hypoxia and oxidative stress in COPD can lead to intestinal dysfunction. Leaky gut, a dysfunction in the intestinal mucosal barrier causes neutrophil infiltration, epithelial shedding, and increased intestinal expression of TNF-α, IFN-γ, and IL-8. The immunopathology of IBD and COPD is characterized by elevation in bone marrow cells, suggesting similarities in the immune system between the lung and gut (63).

One of the most vital of evidence of lung-gut linkage is the increased risk of developing pulmonary diseases in older people. The aging process is characterized by genomic, cellular, metabolic, and immunological hallmarks, all associated with structural and functional changes in the microbiota of several organs, including the gut and lungs. These aspects are affected by the quality and quantity of diets, as malnutrition is one of the factors that cause dysbiosis and gut-lung axis alterations. Therefore, it`s essential to target food intake in these populations, using probiotics-containing foods, to maintain lung health (64).

Studies show that a high-fiber diet is associated with better lung function and reduces the risk of COPD. Since fibers are not absorbed by the intestine, this suggests the role of the gut-liver-lung axis in activating the COPD pathogenic pathway. Dietary fibers alter bowel microbiota, enhancing the bacterial synthesis of immune-modulating compounds; short-chain fatty acids (SCFA), which can pass through circulation from the gut to the bone marrow and promote the maturation of hematopoietic stem cells, that can migrate from the bone marrow to the lungs, playing a role in regulating lung immunity and inflammatory response (65). In addition, SCFAs pass the portal circulation to the liver, the critical organ for immune regulation and the primary source of acute phase proteins, such as C-reactive protein (CRP) and IL-6. In cases of gastrointestinal abnormality, these proteins stimulate the innate immune response by activating neutrophils and macrophages, causing systemic inflammation that can exert damage to the coronary arteries. Lungs further up-regulate the innate immune response through the spell-over of inflammatory cytokines, elevating the pro-inflammatory state and contributing to the

development of COPD and lung cancer, supporting the evidence of the gut-liver-lung axis (66).

Human and experimental IBD models exhibit significant lymphatic alterations, such as obstruction, edema, and angiogenesis, supporting the involvement of the lymphatic system in intestinal inflammation, where immune cells and inflammatory cytokines formed in the gut can transport through the lymphatic system to the heart and lungs (67).

Studies on bacterial-infected mice have shown a red, swollen spleen with elevated inflammatory cytokines (68). Since the spleen is the center of the initiation of adaptive immune responses, and gut microbiota is involved in spleen development and function, this suggests that the spleen also contributes to the gut-lung axis (69). Both lab and clinical data on their mechanisms and practical applications must be involved.

1.6.3 BAs and Lung

Several studies have explained the contribution of liver damage in the development of acute respiratory distress syndrome (ARDS); as liver injury causes changes in acute phase proteins expression, and elevation in plasma levels of proinflammatory cytokines (IL-1, IL-6, TNF-α, …), gut bacterial products, procoagulant and vasoactive factors, and bilirubin in the circulation and lungs. These mediators change the hepatic and pulmonary microbiota and stimulate tissue inflammation and oxidative stress. All these responses mediated by the liver-gut-lung axis contribute to lung injury and multiorgan dysfunction (70). On the other hand, RDS can also be associated with high BA levels. An experimental study on pregnant mice with obstructive cholestasis (OCP) has shown an elevation in BAs profile in both maternal and fetal organs, including lungs, leading to infant respiratory distress syndrome (iRDS) and structural changes in lung tissue, such as peribronchial edema, the collapse of alveolar spaces and deposits of hyaline material in the alveolar lumen, and infiltration of lung tissue by inflammatory cells (71).

Lacking specific translational studies and cross-disciplinary awareness made it challenging to understand bile acid-induced lung injury mechanisms in the past. These mechanisms are represented by the interaction with the secretory phospholipase A2 pathway, surfactant structure and function, the biological effects on inflammation and local immunity, and direct cellular toxicity (50). In addition, the direct impact of BA

signaling on both gut microbiota and host response is the underlying principle of cystic fibrosis pathogenesis (72).

A prospective study enrolled 10 neonates delivered from intrahepatic cholestasis of pregnancy, affected by respiratory distress syndrome requiring mechanical ventilation (intrahepatic cholestasis of pregnancy group) and 2 control groups. The first control group consisted of 20 infants with respiratory distress syndrome delivered from pregnancies without any sign of intrahepatic cholestasis of pregnancy (respiratory-distress-syndrome group), while the second group included 20 neonates with no lung disease who were ventilated for extrapulmonary reasons (no-lung-disease group). In the first 24 hours of life, BA and pH in the bronchoalveolar lavage fluid (BALF) and serum were measured. BAs were detected in the BALF of all of the infants in the intrahepatic cholestasis of pregnancy group but were absent in the 2 control groups, however, BALF pH was not different among the 3 groups. Infants in the intrahepatic-cholestasis-of-pregnancy group had significantly higher serum BA levels than those in both of control groups (73). These findings allow us to hypothesize that BA has a role in causing pneumonia by reaching the lung after uptake from the circulation, indicating that BA profile is a meaningful predictor of lung disease progression (74).

The common bile duct ligation (CBDL) model of experimental mice has significantly contributed to understanding the disease. The common bile duct is isolated and ligated during this surgical procedure, leading to LF. One week later, researchers noticed portal hypertension, intrapulmonary vascular dilations (IPVDs), abnormal gas exchange causing widened alveolar-arterial oxygenation P(A-a)O2 gradient, and eventually hypoxemia. All these features are described as hepatopulmonary syndrome (HPS), which affects 10-30% of liver disease patients who require liver transplantation (mainly cirrhosis, portal hypertension, acute inflammatory liver disease, and altered blood flow between the liver and lung) (75).

HPS Pathogenesis is a complex interaction between the liver, the gut, and the lungs, contributing to vasodilation, shunt formation, and alveolar dysfunction. Advanced liver diseases are associated with elevation of BA circulation, which induces Endothelin-1 (ET-1), a classic potent vasoconstrictor; however, ET-1 can also stimulate nitric oxide (NO) synthase and increase NO levels, leading to vascular relaxation. The resulting gas

exchange defects and arterial pulmonary pressure elevation explain the increased heart failure risk and high mortality rates in HPS patients (76). In addition, increased BAs levels cause gut bacterial translocation and overgrowth, combined with endotoxemia due to decreased hepatic clearance capacity. In Animals with experimental HPS, these toxins can reach the pulmonary circulation, inducing the release of chemotactic factors, immune cells recruitment, and eventually, pulmonary inflammation (75).

Aerodigestive diseases, such as gastroesophageal reflux (GOR) have been linked to end-stage lung disorders, with BAs being implicated, as patients in these cases have shown higher BAs levels in their BALF (77). However, the exact mechanism needs to be better understood. An experimental study was conducted to evaluate the effect of BAs on immortalized human bronchial epithelial cell line (BEAS-2B). Morphologically and physiologically, these cells represent an excellent model for assessing airway epithelial studies, as they can secrete several cytokines while maintaining epithelial cell structure in vitro. Cultured BEAS-2B cells were injected with primary and secondary BAs, and after 48-h incubation, cells suspension was collected to measure cytokines concentration using ELISA. Finally, cells viability was measured using CellTiter-Blue and MTT assays. Results have shown an elevation in inflammatory cytokine IL6, IL8, and granulated-macrophage colony-stimulating factor (GM-CSF). In addition to increased cell death (reduced viability), compared to the controls (78). These findings suggest that GOR leads to BA aspiration, which damages airway epithelia, inducing the release of pro-inflammatory cytokines and leukocyte infiltration, leading to lung injury and chronic respiratory diseases, such as pneumonitis (77).

Aspiration of gastrointestinal content is linked to bad outcomes after lung transplantation cases (79). Several cohort studies have examined the BALF of patients before and after lung transplantation (they served as their own controls), for unknown causes, to identify the biomarkers and metabolic changes associated with aspiration. Patients with BA and pepsin in their BALF showed higher rates of lung injury, bronchitis, and pulmonary inflammation. Since BA is correlated to other biomarkers, immune activation was also measured. Patients who had GOR after lung allograft had elevated levels of T-cell granzyme B, chemoattractants (CXCL9, CXCL10), Interleukins (IL-1α, IL1β, IL-8, 12p70), and chemokine (C-C motif) ligand 5 (CCL5).

Meanwhile, the receptor levels for advanced glycation end products (RAGE) were reduced (80). These findings suggest that the presence of BA in the lung is a risk factor for subsequent chronic graft failure, as aspiration weakens pulmonary epithelial barrier function. Studies linking aspiration to decreased levels of surfactant protein A (SP-A), increasing inflammation (81), and pseudomonas colonization, which was noticed clinically in these patients (79), support this.

One of the key contributors to the exacerbation of pulmonary disorders, such as cystic fibrosis (CF), COPD, asthma, and bronchitis, is the inflammation induced by several pulmonary microbes, including pseudomonas aeruginosa (81). It has been suggested that BA is a crucial trigger for the P. aeruginosa inflammatory effect developed in these patients. When BAs reach the lungs, as in GOR cases, they stimulate the production of IL-6 in pulmonary epithelia, triggered by FXR and hypoxia-inducible factor (HIF)-1. BA signaling also reorganizes bacterial behavior, suppresses microbial cellular reparation, and induces antibiotic resistance. These factors lead lung microbes to switch from an acute virulent lifestyle to a chronic phase infection (82).

Individuals with high body mass index (BMI) usually suffer from asthma, which may require hospitalization in some cases. Obesity induces pro-inflammatory cytokine production, and immune cell activation. In addition to generating oxidative stress, however, the exact mechanism must be fully understood. Experts suggest that BAs are contributing factors in this pathological process (83).

For further investigation, a study was conducted on a group of 4-week-old C57BL/6 male mice fed a high-fat diet (HFD) for 12 weeks. The obese study group was sensitized with house dust mite (HDM), an allergen that stimulates pulmonary sensitization and allergic airway disease (AAD). On the other hand, the obese control group only received adjuvant to avoid forming AAD. On days 14-18, some mice of the (obese-AAD) group received nitro-oleic acid (NO2-OA), an inflammatory and metabolic regulator, three hours before the HDM challenge. A computer-controlled small-animal mechanical ventilator assessed pulmonary function. After sacrificing, blood samples and BALF were collected for BAs measurements. Qualified pathologists performed histopathological assessments to determine pulmonary inflammation and fibrosis. Both obese groups have developed asthma. However, the HDM-sensitized group has shown higher levels of inflammatory

markers and BAs in BALF and serum, in addition to airway inflammation and abnormal pulmonary mechanics, compared to the obese control group (84).

When comparing the two AAD groups, the NO2-OA group has shown a significant decrease in pulmonary resistance and elastance, with a reduction in tissue damage and airway hyperresponsiveness. However, expression of inflammatory mediators was not suppressed after NO2-OA administration. On the other hand, NO2-OA group had lower levels of BA in the lungs compared to the other AAD group (without NO2-OA administration). This is explained by the role of NO2-OA in suppressing the expression of the hepatic enzyme CYP7A1, and inducing FXR to regulate BAs synthesis and conjugation. Since nitroalkene gave good respiratory outcomes through BAs regulation, this suggests the contribution of BAs in airway dysfunction in obese individuals (85).

An experimental study on human alveolar epithelial cells (AECs), that were taken by surgical lung biopsy, and incubated with BAs has shown a dramatic decline in cells viability and increased intracellular ROS levels. BAs can directly promote fibrosis in AECs by stimulating lung fibroblast cells migration, type I collagen and fibronectin secretion, and α-SMA activation. In addition, BAs can indirectly activate lung fibroblast by stimulating pro-fibrotic factors and cytokines, such as TGF-β1, GM-CSF that were detected using ELISA. Both direct and indirect BA-induced lung fibrosis pathways were maintained through FXR. Western blot results showed that pulmonary cellular FXR expression strongly increases in fibrosis more than in normal lungs. However, the exact role is still unclear (86).

The presence of acids in lung tissue is associated with some clinical features, such as neonatal bile acids pneumonitis (NBAP) in neonates born to mothers with intrahepatic cholestasis of pregnancy (ICP). Human data have shown a risk of developing respiratory failure 2-3 times higher than the control. In addition, BAs were higher in BALF, amniotic fluid, and maternal and cord blood (87).

Animal experiments show a strong correlation between maternal circulating BAs levels, BAs-induced production of secretory phospholipase by alveolar macrophages, and the degree of alveolar injury. Mice subjected to common bile duct ligation with lung injury, characterized by local inflammation, neutrophil infiltration, and angiogenesis, were followed by gram-negative mice having a significant amount of TNF-α (BA switch

microbiota from less to more pathogenic) causing endotoxemia, systemic inflammation, and more severe organ dysfunction. TNF-α is a key activator for sPLA2 transcription(88).

Research studies have investigated the mechanisms where BAs induce damage to various organs, including lungs; these are represented by:

a. Interaction with phospholipase A2 pathway and secretion increment from lung cells; as sPLA2 can hydrolase surfactant phospholipids and regulate the first step in the inflammatory cascade (50).

b. Effect on surfactant function and structure, which regulates immune defense (BA immunomodulation) (81).

c. Biological effects of BAs on Inflammation and local immunity. TGR5 –BA interaction causes inflammatory reactions in various organs, and stimulates pro-fibrotic mediators (41).

d. Direct cellular toxicity and chemical properties of BAs as a solubilizer of biological membranes. Adding BAs to cultured pneumocytes altered cell permeability by increasing intracellular calcium levels (49). Furthermore, FXR inhibitors suppress BA cytotoxicity in pnemocytes (41).

Chapter Two

Methods

2.1 Study design

An experimental study was conducted at the central research lab at An-Najah National University, Nablus – Palestine, on LF animal model. 12 weeks old C57BL/6J male mice received care according to the university`s ethical regulations and NIH guidelines. This genetically modified strain is the most widely used among mice to examine hepatic studies, its high sensitivity provides faster and more desirable results. Male mice were used to avoid the confounding effects of the female reproductive cycle and other variables that may interfere with experimental outcomes (84). The institutional animal care ethical committee approved all animal protocols, and the mice were housed in a barrier facility.

In order to establish a LF model, 12 mice were intraperitoneally (IP) injected with pure carbon tetrachloride (CCl_4; Sigma, C-5331), 0.5 per gram of body weight. CCl_4 is a powerful hepatotoxic organic agent, widely used to induce hepatic disorders in experimental animal studies, such as LF, however, CCl_4 has a damaging effect on other organs as well, therefore, IP injection was preferred in this study, as it allows quick reabsorption of large volumes of substances, and focuses on the liver area, unlike the lungs which mostly get affected by CCl_4 through inhalation (89). The CCl_4 was diluted in maize (corn) oil at a ratio of (1:9). Mice were divided into 3 groups (4 mice in each); the first group received IP injection twice a week, for 6 weeks, to cause chronic LF. In the last 2 weeks, the second group got an IP injection to cause acute LF. The third group, which was injected with corn oil, represented the control group (Table 2). During the injection period, mice were regularly assessed for their weight.

Table 2

Mice IP injection plan

Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Expected Final Result
I	Corn oil	Corn oil	Corn oil	Corn oil	Corn oil	Corn oil	No LF (Control Group)
II	✘	✘	✘	✘	✔ CCl_4	✔ CCl_4	Acute LF (ALF)
III	✔ CCl_4	✔ CCl_4	✔ CCl_4	✔ CCl_4	✔ CCl_4	✔ CCl_4	Chronic LF (CLF)

On sacrificing day (2 weeks after the last injection), all mice were administered anesthesia by inhalation of 5% isoflurane for 10 seconds, and ultimately underwent cervical dislocation. Lungs were extracted from each mouse and kept at (-80˚c).

2.2 Study population

This study was performed in vivo, on a population of LF animal (mice) model.

2.3 Study sample

A sample of 12 weeks old C57BL/6J male mice was classified into 3 groups, in each:

A. The control group (treated with corn oil).

B. The acute LF group (2-week CCl_4-IP injection).

C. The chronic LF group (6-week CCL_4-IP injection).

2.4 Study Duration and Setting

The Injection period in the university`s animal research unit was 6 weeks until we got 3 study groups (normal, acute LF, chronic LF). After sacrificing, experiments were held for 10 months in the central research lab at the Faculty of Medicine and Health Sciences, to investigate the effect of LF on the lungs.

2.5 Study Variables

Mice:

1. Age (weeks).
2. Weight (gram).
3. Gender (male/female).

Serum test:

1. BA level (μM).

BALF tests:

1. BA level (μM).
2. sRAGE (pg/ml).
3. IL-1-β (pg/ml).

4. IL-4 (pg/ml).
5. IL-6 (pg/ml).
6. TNF-α (pg/ml).

RT-PCR:

1. Superoxide dismutase (SOD) (Fold Changes).
2. Malondialdehyde (MDA) (Fold Changes).
3. Glutathione peroxidase (GpX) (Fold Changes).
4. Glutathione reduced Form (GSH).
5. Glutathione disulfide (oxidized) form (GSSG).
6. Surfactant Protein-D (SPD-1) (Fold Changes).
7. αSMA (Fold Changes).
8. Glial Fibrillary Acidic Protein (GFAP) (Fold Changes).
9. MMP-9 (Fold Changes).

NK-isolation and Flow Cytometry:

1. CD107a (%).
2. NTCP (%).
3. Alveolar epithelial cells I (AECs I) apoptosis (%).
4. Alveolar epithelial cells II (AECs II) apoptosis (%).

2.6 Serum BAs Levels

On Sacrificing day, whole blood samples were withdrawn and left at RT for 30 minutes, then centrifuged at 3500 rpm for 10 minutes at 4˚c. The supernatant was collected and stored at -80˚c until use. Serum levels of BAs were detected and quantitated using Trinity Biotech bile acids reagents for enzymatic determination of BAs concentration kit (Biotest; 450A) according to the manufacturer's instructions (Abcam; USA).

2.7 Bronchoalveolar Lavage Fluid (BALF) Aspiration

On sacrificing day, anesthetized mice were placed supine, then the thoracic and abdominal regions were disinfected with 70% ethanol. Using a scalp, we made a skin incision near the trachea, then opened the skin to expose the salivary glands, which were separated with forceps. A surgical suture was positioned over the trachea; then, a puncture was applied using a 25G needle; after that, we inserted a BALF needle with 1 ml of sterile, ice-cold PBS. We gently pulled back the syringe to aspirate the instilled PBS; about 700-900 µl of BALF were extracted and collected in a tube on ice. After repeating the process 3 times for mouse, BALF samples were centrifuged at 3000 rpm/4˚c/5 min, and the supernatant was saved at -80˚c for ELISA.

2.8 BAs Detection in BALF

BAs levels in serum and BALF were detected and quantitated using Trinity Biotech bile acids reagents for enzymatic determination of BAs concentration kit (Biotest; 450A) according to the manufacturer's instructions (Abcam; USA).

2.9 Soluble Receptor for Advanced Glycation End Product (sRAGE) Detection in BALF

sRAGE levels in BALF were detected and quantitated using sRAGE Elisa kit (Biotest; MBS766075) according to the manufacturer's instructions.

2.10 Luminex MAGPIX tests

A multiplexed sandwich enzyme-linked immunosorbent assay-based technology (Cat# MHSTCMAG-70K; R&D Systems) was used to simultaneously determine the concentration of multiple cytokines (IL-6, IL-4, IL-1β, TNF-α) in BALF. Samples from each group of mice were analyzed as instructed by the kit.

2.11 RNA isolation, cDNA preparation, and real-time PCR

“Total cellular of 2 µg/ µl RNA (purity 98%) determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was isolated from mice lung tissue samples, using 2 ml of TRI reagent (Bio Lab; Cat# 90102331). The samples were centrifuged (1,400 rpm) for 15 minutes at 4°C to collect RNA supernatant. For RNA precipitation, the supernatant of each sample was transferred to a new microcentrifuge tube, and 0.5 ml of isopropanol (Bio Lab; Cat# 16260521) was added and incubated at

25°C for 10 minutes. The tubes were then centrifuged (12,000 rpm) for 10 minutes at 4°C, the supernatants were removed, and one milliliter of 75% ethanol was added to the pellets before centrifugation (7,500 rpm) for 5 minutes. The pellets were air-dried at room temperature for 15 minutes, 50 µl of DEPC was added, and the samples were heated for ten minutes at 55°C. cDNA was prepared with a High-Capacity cDNA Isolation Kit (R&D; Cat# 1406197)."

"Real-time PCR was performed with TaqMan Fast Advanced Master Mix (Cat# 4371130, Applied Biosystems) to quantify SOD, MDA, GSH, GSSG, SPD-1, αSMA, GFAP, and MMP-9 gene expressions with normalization to the expression of the housekeeping gene GAPDH. Cycling conditions for the Thermofisher one-step RT-PCR kit involved RT step for 30 minutes at 50 °C and denaturation for 15 minutes at 95 °C. Further, the reaction mixture was incubated for 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min followed by 72 °C for 10 min. Data analysis was analyzed using QuantStudio™ 5 Real-Time PCR System (Cat# A34322, Applied biosystem)."

2.12 Tissue-resident lung NK cell isolations from lung tissues

"Under deep ether anesthesia, mice were euthanized by isoflurane, USP 100% (INH), then the lung was removed, and a part of them was transferred to a Petri dish that contains 5 ml DMEM medium (Biological industries; Cat# 01-055-1A). The tissues were thoroughly dissected by stainless steel mesh, the cells were harvested with the medium and added to 50 ml tubes containing 10 ml DMEM, and then carefully cells were transferee to new tubes that contained Ficoll (Abcam; Cat# AB18115269). Tubes were centrifuged for 20 minutes at 1,600 rpm at 20°C. The supernatant in each tube was transferred to a new tube for another centrifuge for 10 min, at 1,600 rpm at 4°C. After the second centrifuge, the pellet in each tube was suspended in 1ml of DMEM for the NK isolation kit (StemCells; Cat# 19665)".

2.13 Flow Cytometry

"All used antibodies were incubated with the isolated NK lung cell suspensions (1:100) at 4°C for 45 minutes; cells were washed 2X PBS with 1%FCS before the secondary antibody (1:100) at 4°C for 45 minutes if required. Primary mouse antibodies used are anti-CD45 (ab10558, abcam), anti-CD3 (ab33429, abcam), anti-NK1.1 (ab289542, abcam), anti-CD107a (lysosomal-associated membrane protein-1 (LAMP-1) (ab24170,

abcam), and anti-NTCP (GTX17693, GeneTex). Isotype IgG labeled with the relevant fluorochrome was used as a control for each antibody".

"Before flow cytometry analysis, cells (106/100 µl) were assessed for their viability measured by propidium iodide (PI) (A35110, R&D systems). PI-negative cells were considered viable, and the mean viability rate of the cells was 92.7±1.5%. Apoptosis was evaluated with annexin-V (A35110, R&D systems) staining. Early apoptotic cells were defined as annexin-V+PI- cells, and late apoptotic cells were defined as annexin-V+PI+ cells. All stained cells were examined on a flow cytometer (BD LSR Fortessa™, Becton Dickinson, Immunofluorometry Systems) and analyzed by FCS Express 7 by De Novo Software for Flow Cytometry".

2.14 Statistical Analysis

Statistical differences were analyzed with two-tailed unpaired Student's t-test (for comparison between two groups) or one-way analysis of variance (one-way ANOVA with Newman-Keuls post-tests among multiple groups) in GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

2.15 Ethical Approval

This Animal study was reviewed and approved by the institutional review board (IRB), the scientific research committee of the public health department, and the scientific research board of the faculty of graduate studies at An-Najah National University (IRB approval letter – Appendix). Ref: Mas. Feb. 2022/1. Data access was limited to only research team members and was stored in a password-secured computer.

2.16 Funding Information

Our research was funded by the university research institution with 1000$ to cover all study tools and lab equipment used for sample collection, processing, and analysis.

Chapter Three

Results

3.1 CCl4-induced liver damage causes an elevation in BAs levels in both serum & BALF

Liver fibrosis of an acute and chronic mouse model was performed, and serum and BALF samples were withdrawn from each mouse group to measure BA levels using an ELISA kit.

Both acute and chronic LF mice groups showed significantly exacerbated serum BAs levels ($P < 0.0001$) after CCL_4 IP injection, resulting from liver injury (Fig 4-A). The acute LF group had a 15 ± 2.85 µM BAs serum level. On the other hand, it was 20 ± 4.26 in the chronic LF group. At the same time, the naïve mice group had a serum BAs level of 2 ± 0.76. Since elevated serum BAs levels are one of the significant hallmarks of liver injury, these data indicate the damaging effect of CCl_4 injection on liver health.

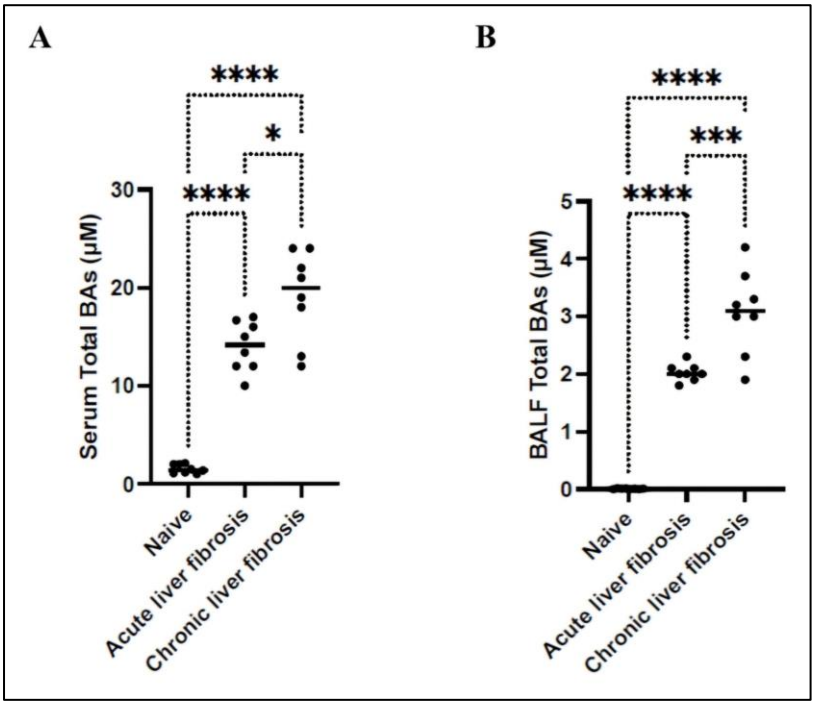
To explore if this liver damage can cause harmful effects on the lungs through abnormal circulating BAs, BALF samples were also collected from the 3 mice groups to detect BAs levels by ELISA kit. Both acute and chronic LF mice groups showed significantly higher BALF BAs levels than the control ($P < 0.0001$) after CCL_4 IP injection (Fig-4B). The acute LF group had a BAs level of 2 ± 0.125 µM in their BLAF samples, while in the chronic LF mice group, BALF BAs levels were 3 ± 0.756 µM. These results suggest that elevated BAs levels, caused by liver pathology, can pass through the circulation and reach the respiratory system. Therefore, further experiments had to be performed to investigate the effect of BAs on the lungs.

3.2 Elevated BAs levels stimulate oxidative damage in the lungs

Real-time PCR test was performed on lung tissue cells to measure the markers of oxidative damage induced by BAs. Fig (5-A) shows the changes in glutathione peroxidase (GPx) levels among the 3 mice groups after CCl_4 injection. GPx is one of the primary antioxidants in the body, which converts reduced glutathione (GSH) to its oxidized form, glutathione disulfide (GSSG), in order to get rid of ROS, and control the pro-inflammatory process in the liver and lungs. Our results show a reduction by 1.43-folds in the acute, and 5-folds in the chronic LF mice groups in GPx levels, compared to the naïve group ($P < 0.05$), ($P < 0.001$), respectively.

Figure 4

BAs levels in serum (4-A) and BALF (4-B) in the 3 mice groups, naïve, acute, and chronic LF. BAs concentrations were measured using an ELISA kit (Biotest; 450A)



Note: (4-A) shows the distribution of serum BAs level for naive, acute (2-weeks CCl4), and chronic (6-weeks CCl4) liver fibrosis. (4-B) shows BALF BAs concentrations for naive, acute (2-weeks CCl4) and chronic (6-weeks CCl4) liver fibrosis.
(Each experiment was repeated three times and data was represented as mean ± SD. [*p = 0.05, **p = 0.01, ***p = 0.005, and ****p = 0.0001].

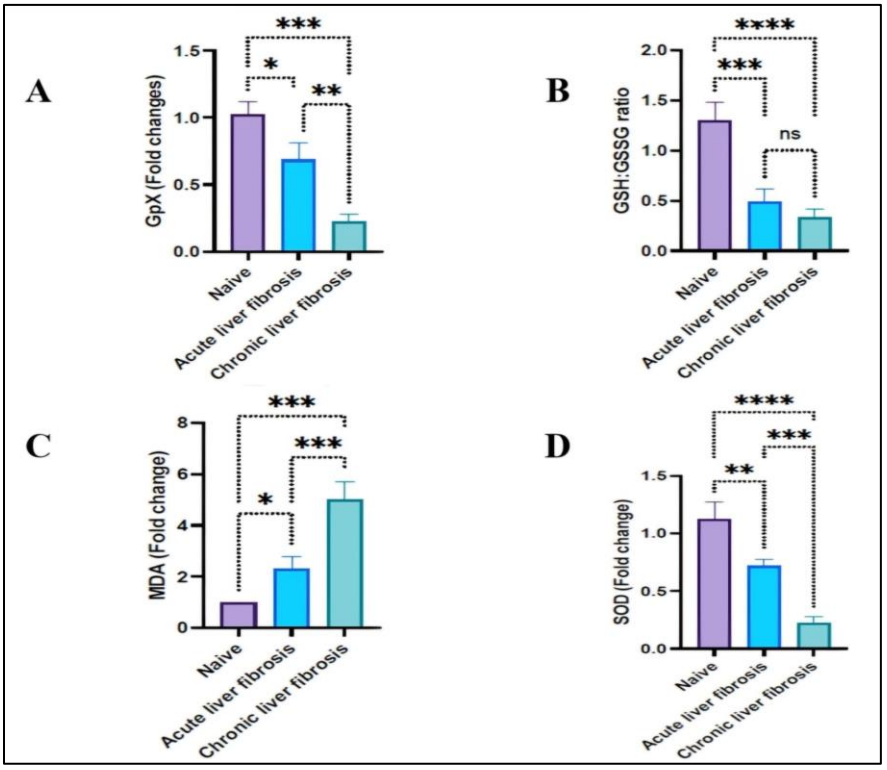
Additionally, GSH:GSSG ratio was measured as a marker for respiratory pathology. Fig (5-B) shows a reduction in GSH:GSSG ratio by 2.6-folds in the acute, and 3.71-folds in the chronic LF mice groups, compared to the naïve group ($P < 0.001$), ($P < 0.0001$) respectively. This significant reduction in GPx and GSH:GSSG ratio in both LF groups after CCl_4 injection, is explained by increased consumption, due to the oxidative imbalance stimulated by BAs.

rt-PCR results have also shown an increase in malondialdehyde (MDA), an oxidative agent that results from the oxidation of lipid components in the cellular membrane. The acute LF mice group had an elevation by 2.3 folds in their MDA expression compared to the naïve (control) group ($P < 0.05$). In comparison, MDA expression in the chronic LF group was 5.2 folds higher than naïve mice group ($P < 0.001$) (fig 5-C). This significant elevation in MDA expression, in both LF mice groups after CCl_4 injection, indicates exacerbated BA-induced oxidative damage of respiratory cells, which can further stimulate pulmonary inflammation.

All these oxidative stress events need to be managed to prevent lethal complications, such as lung injury, and fibrosis. One of the major pulmonary defense agents is superoxide dismutase (SOD), which regulates respiratory oxidation and attenuates inflammation. Therefore, rt-PCR was performed to detect SOD levels. Results have shown a decline in SOD expression by 1.57 folds in the acute LF group, and by 5.5 folds in the chronic LF mice group, compared to the naïve group ($P < 0.01$) ($P < 0.0001$) respectively (Fig 5-D). This significant reduction in SOD levels is explained by its overconsumption, due to oxidant: antioxidant disturbances.

Figure 5

Oxidative markers in the lungs of the 3 LF mice groups, naïve (control), acute (2-weeks CCl4), and chronic (6-weeks CCl4)



Note: Tested markers include GPx (5-A), GSH:GSSG ratio (5-B), MDA (5-C), and SOD (5-D). Both antioxidants GPx and SOD expressions decreased over time after CCl_4 injection, followed by a decline in GSH:GSSG ratio, indicating their overconsumption, due to the oxidative imbalance caused by abnormally elevated levels of BAs. This oxidative stress damages respiratory cells; increasing the release of several oxidative agents; such as MDA. Expression was detected and quantified using real-time PCR.
(Each experiment was repeated three times and data was represented as mean ± SD. [*p = 0.05, **p = 0.01, ***p = 0.005, and ****p = 0.0001].

3.3 Pulmonary injury stimulates overexpression of surfactant protein D (SP-D), as a protective response

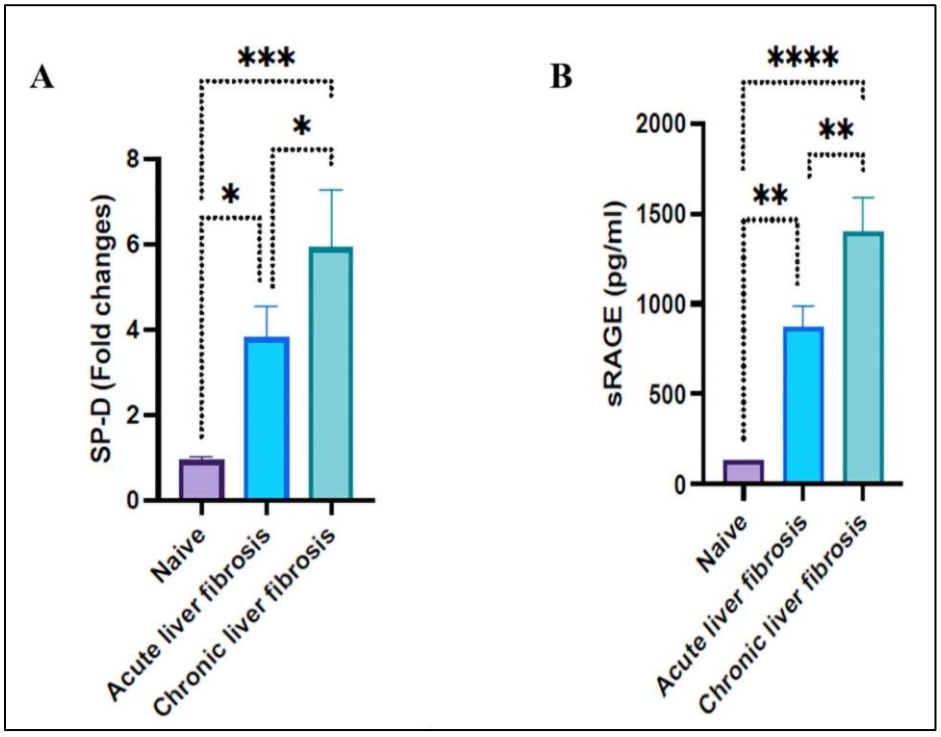
All this oxidative stress caused by abnormally elevated BAs levels, can cause lung injury, and alveolar damage, therefore we performed rt-PCR on lung tissues of the 3 mice groups, to detect surfactant protein D (SP-D), which is usually released by pulmonary cells for attenuating alveolar collapse. Our results show that SP-D expression increased by 4.88 in the acute, and by 8.63 in the chronic LF mice groups, compared to the control ($P < 0.05$) ($P < 0.001$) respectively (Fig 6-A). Since SP-D release is highly induced in respiratory disorders, as a protective response, this indicates exacerbated oxidative damage in the lungs caused by BAs in LF cases.

3.4 Exacerbated inflammatory injury of lung tissue increases the expression of soluble receptor for advanced glycation end products (sRAGE)

In order to confirm lung damage, we detected the levels of soluble receptor for advanced glycation end products (sRAGE) in BALF samples of the 3 LF mice groups using an ELISA kit (Biotest; MBS766075). Results showed that sRAGE was 7.29 folds higher in the acute (875 ± 125 pg/ml), and 11.67 folds higher in the chronic (1400 ± 225 pg/ml) LF mice groups, compared to the control (120 ± 5 pg/ml), ($P < 0.01$) ($P < 0.0001$) respectively (Fig 6-B). Since sRAGE is mainly expressed in the lungs as a result of injury and inflammation, in order to regulate immune response, this significant elevation in its expression in both acute and chronic LF mice groups indicates severe damage in the lungs of these mice, and uncontrolled immune response, due to BAs aspiration.

Figure 6

Exacerbated inflammatory injury of lung tissue increases the expression of soluble receptor for advanced glycation end products (sRAGE)



Note: Fig (6-A) shows how the expression of SP-D (a marker of lung injury) is elevated in the acute (2-weeks CCl_4), and chronic (6-weeks CCl_4), compared to the naïve (control). Expression was detected and quantified using real-time PCR.

Fig (6-B) shows the elevation of sRAGE (a key regulator of immune response in the lungs) levels in the acute (2- weeks CCl_4), and chronic (6-weeks CCl_4), compared to the naïve (control). sRAGE were measured using ELISA kit (Biotest; MBS766075).

(Each experiment was repeated three times and data was represented as mean ± SD. [*p = 0.05, **p = 0.01, ***p = 0.005, and ****p = 0.0001].

3.5 Inflammatory cytokines levels were elevated in BALF samples of CCL4-injected mice

Since BAs aspiration is strongly related to respiratory oxidation, we performed an ELISA test on BALF samples extracted from the 3 mice groups in order to measure the levels of the most common inflammatory cytokines (IL-1β, IL-4, IL-6, and TNF-α), and detect the effect of BA-induced pulmonary damage on inflammatory immune response.

Our results show a significant elevation of all the tested inflammatory cytokines in the acute and chronic LF mice groups, compared to the control (Fig 7). IL-1β, which is strongly associated with structural lung damage, was 37.5 folds higher in the acute (375 ± 150 pg/ml), and 58 folds higher in the chronic (580 ± 120 pg/ml) LF mice groups compared to the control (10 ± 2.5 pg/ml), ($P < 0.001$) ($P < 0.0001$) respectively. Indicating the level of pulmonary destruction in these mice.

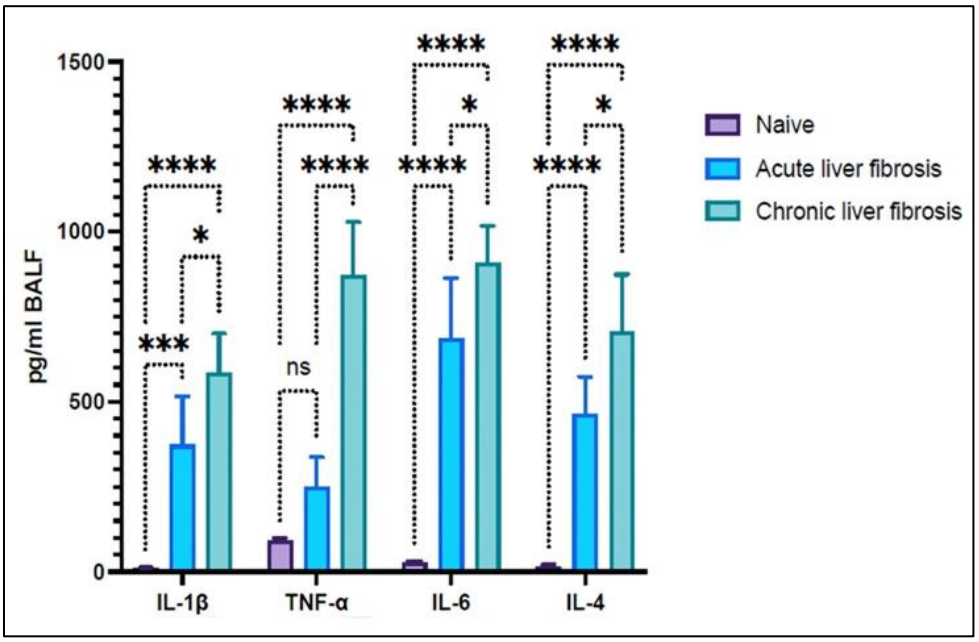
IL-4, which can regulate the activation of both B and T immune cells, in addition to fibrosis, was significantly elevated in the acute (by 22.5 folds) (450 ± 120 pg/ml), and the chronic (by 35 folds) (700 ± 175 pg/ml) LF mice groups, compared to the naïve group (20 ± 10 pg/ml), ($P < 0.0001$ in both). Indicating lethal pulmonary inflammation, and exacerbated collagen deposition.

Another measured immune response regulator was IL-6, which showed a significant elevation by 28 folds in the acute (700 ± 175 pg/ml), and 36 folds in the chronic (900 ± 150 pg/ml) LF mice groups, compared to the naïve mice group ($P < 0.0001$ in both). Indicating lung injury, and uncontrolled inflammatory immune response.

TNF-α, one of the key mediators in cellular immune response, apoptosis, and even tumorigenesis, was also detected in mice BALF samples. Where it was 2.5 folds higher in the acute group (250 ± 100 pg/ml) (non-significant), and 8.75 folds higher in the chronic (875 ± 175 pg/ml) LF mice groups ($P < 0.0001$), compared to the control.

Figure 7

Pro-inflammatory cytokines were measured in BALF samples of the three mice groups



Note: All of IL-1β, TNF-α, IL-6, and IL-4 showed to be significantly elevated in BALF samples of both the acute (2- weeks CCl_4), and chronic (6-weeks CCl_4) LF mice groups, compared to the naïve (control).

(Each experiment was repeated three times and data was represented as mean ± SD. [*p = 0.05, **p = 0.01, ***p = 0.005, and ****p = 0.0001].

3.6 Tissue damage in the lungs activates growth factors and fibrosis markers

BA-induced oxidative stress can cause tissue injury. Therefore, we performed real-time PCR on lung tissue samples, taken from the 3 LF mice groups to detect the expression of growth factors and fibrosis markers, which are usually activated and released for injury healing response.

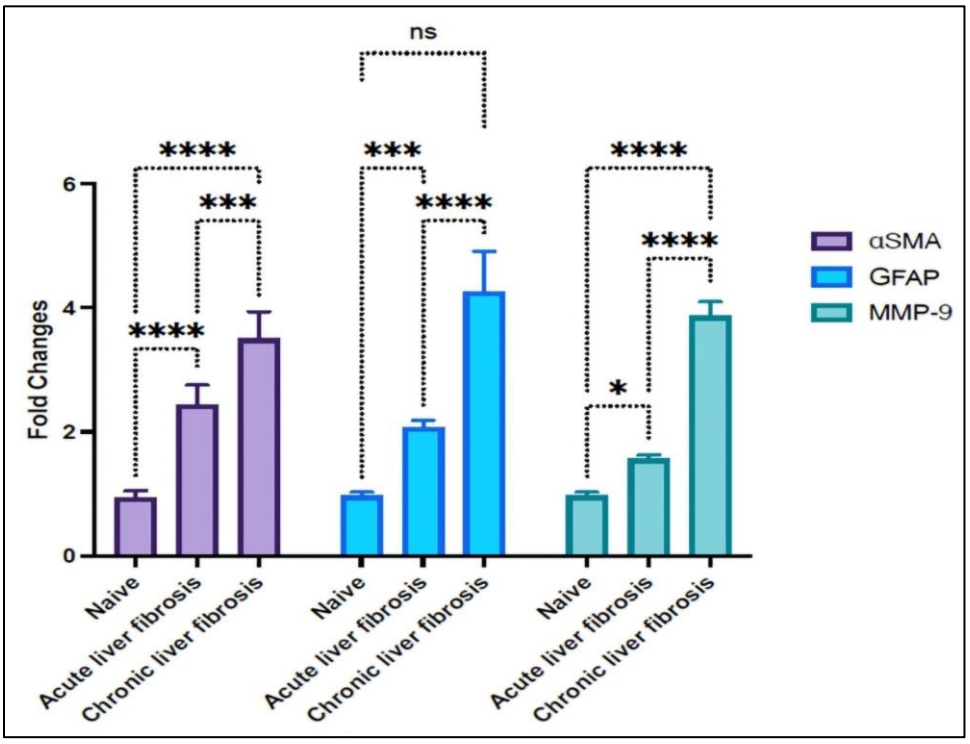
PCR results revealed a significant upregulation in the expression of fibrosis factors in the acute and chronic LF mice groups, compared to the control (Fig 8). MMP-9, which regulates inflammation and injury response by controlling inflammatory cytokines, fibroblast differentiation, and the synthesis of collagen and ECM proteins, was overexpressed by 1.71 folds in the acute, and 4.57 folds in the chronic LF mice groups, compared to the control ($P < 0.05$) ($P < 0.0001$) respectively. This significant elevation in MMP-9 levels, caused by oxidative stress, suggests bronchial inflammation and fibrosis, and exacerbated collagen deposition in the ECM.

The expression of α-SMA showed to be upregulated by 2.86 folds in the acute, and 4 folds in the chronic LF mice groups, compared to the control ($P < 0.0001$ in both). Since α-SMA is the primary indicator of myofibroblast activation and differentiation, this significant elevation in its expression indicates lung injury and fibrosis, in addition to pulmonary tumorigenesis.

We also noticed a significant upregulation in the expression of glial fibrillary acidic protein (GFAP), which regulates cytoskeletal structure, respiratory innervation, and the synthesis of collagen and ECM proteins. GFAP was 2.4 folds higher in the acute group ($P < 0.001$), and 4.86 folds higher in the chronic LF mice group (non-significant), compared to the control. Since GFAP is highly expressed in myofibroblasts during oxidative inflammation and injury; these results indicate that experimental mice had pulmonary inflammation, collagen accumulation, and respiratory failure.

Figure 8

Growth factors and fibrosis markers detected from lung tissue samples of the three mice groups



Note: α-SMA, GFAP, and MMP-9 markers showed to be significantly overexpressed in lung tissue samples of both the acute (2- weeks CCl_4), and chronic (6-weeks CCl_4) LF mice groups, compared to the naïve (control). Expression was detected and quantified using real-time PCR.

(Each experiment was repeated three times and data was represented as mean ± SD. [*p = 0.05, **p = 0.01, ***p = 0.005, and ****p = 0.0001].

3.7 The activity of lung resident NK cells was declined after aspiration of high levels of BAs

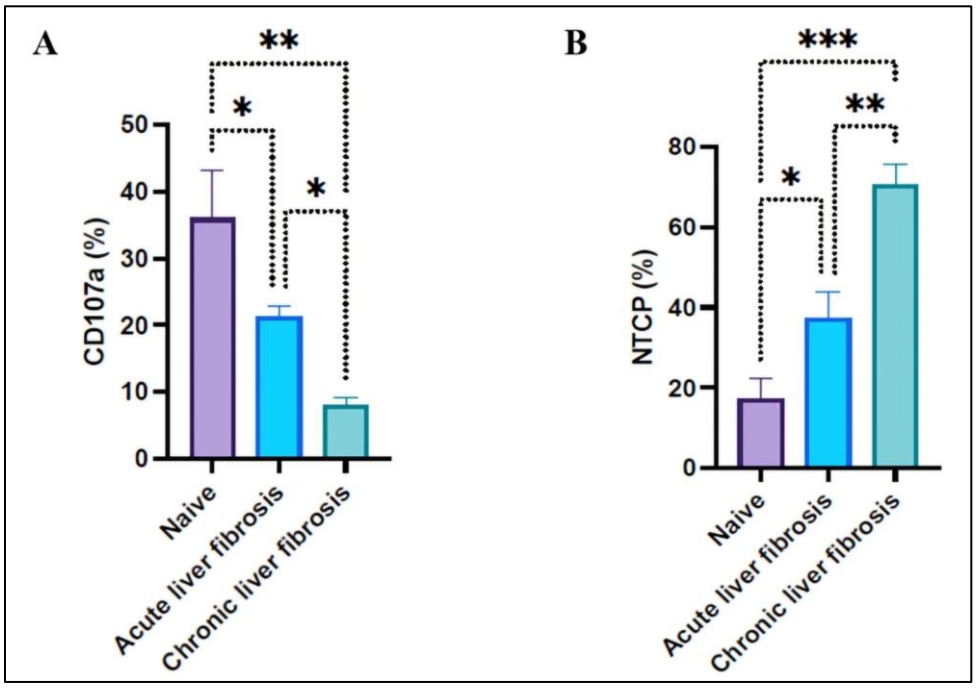
The immune system plays a crucial role in the tissue healing process; therefore, regulating the immune system is essential for an efficient injury-healing response. One of the primary immune response regulators in the lungs is tissue-resident natural killer cells (trNK). In our study, we isolated trNK from the lung tissue of the 3 LF mice groups, in order to be examined via flow cytometry. Lung NK usually become activated in pulmonary disorders; such as injury, allergies, inflammations, and tumorigenesis; however, when we detected the percentage of the expressed cluster of differentiation (CD107a) on lung NK, which is a key indicator of NK activation and functionality, we noticed a decline by 1.7 folds in the acute, and 4.5 folds in the chronic LF mice groups, compared to the naïve group ($P < 0.05$) ($P < 0.01$) respectively (Fig 9-A). Indicating a decline in lung NK efficiency in cases of LF.

To ensure that this reduction in NK activity was caused by the elevated levels of BAs aspirated in the lungs, we detected the levels of expressed NTCP on lung NK via flow cytometry, Our results showed an elevation in the percentage of NTCP by 2.11 folds in the acute, and 4.11 folds in the chronic LF mice groups, compared to the Naïve group ($P < 0.05$) ($P < 0.001$) respectively (Fig 9-B).

These data undoubtedly indicate the effect of BAs signaling pathways on lung NK cells activity in patients with LF. The aspiration of high levels of BAs caused by LF stimulates the expression of NTCP (BAs-specific transports) on NK cells. This increases BAs uptake inside pulmonary cells; inducing exacerbated lung tissue damage, and respiratory impairment.

Figure 9

Flow cytometry examination of NK cells, isolated from lung tissue of the three LF mice groups, acute (2- weeks CCl4), chronic (6-weeks CCl4), and naïve (control)



Note: Fig 9-A: Flow cytometry results show a significant decline in CD107a expression (the key indicator of NKs activation and functionality) on NK cells, and hence; a decline in lung NK activity, in both acute and chronic LF, compared to the control.

Fig 9-B: Flow cytometry results show a significant upregulation in the expression of NTCP (BA-specific transporter) on lung NK in both acute and chronic LF, compared to the control. Indicating an increased BAs uptake in pulmonary cells

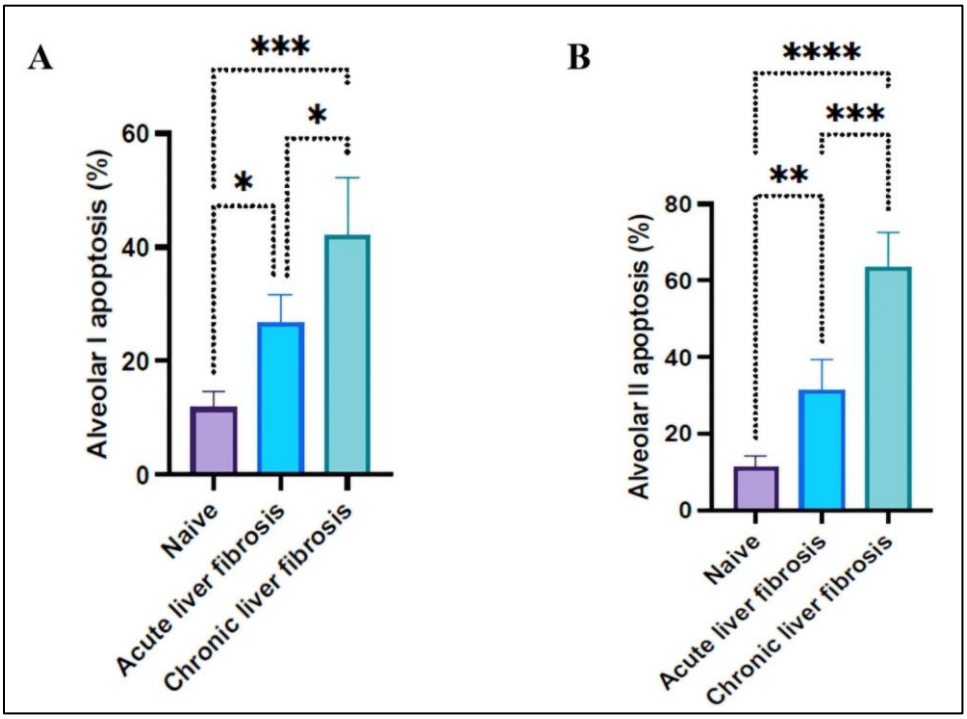
(Each experiment was repeated three times and data was represented as mean ± SD. [*p = 0.05, **p = 0.01, ***p = 0.005, and ****p = 0.0001].

3.8 BAs toxicity induces apoptosis of AEC

In order to determine the level of respiratory damage caused by BAs aspiration in LF cases, we examined AECs types I and II via flow cytometry; to estimate the level of apoptosis in these cells. Results have shown that both AECs I and II were highly affected during BAs aspiration (Fig 10), where the acute LF mice group showed elevated levels of apoptosis by 2.45 folds in AECs I, and 3.25 folds in AECs II, compared to the controls ($P < 0.05$) ($P < 0.01$) respectively. On the other hand, the chronic LF mice group had an elevated apoptosis rate by 3.81 folds in AEC I, and 6.5 folds in AECs II, compared to controls ($P < 0.001$) ($P < 0.0001$) respectively. Indicating the direct cytotoxic effect of BAs on pulmonary cells, and the consequent exacerbated inflammation, and respiratory failure.

Figure 10

Level of apoptosis in AEC I



Note: (Fig 10-A) and AECs II (Fig 10-B). both cell types were extracted from the 3 LF mice groups, acute (2- weeks CCl_4), chronic (6-weeks CCl_4), and naïve (control). Apoptosis levels were measured using flow cytometry (described in materials and methods – chapter 2).

(Each experiment was repeated three times and data was represented as mean ± SD. [*p = 0.05, **p = 0.01, ***p = 0.005, and ****p = 0.0001].

Chapter Four
Discussions and Conclusions

4.1 Discussion

4.1.1 Introduction

An experimental study was conducted at the central research lab at An-Najah National University, Nablus – Palestine, on LF animal model. 12 weeks old C57BL/6J male mice (the most widely used strain among mice to examine hepatic pathology) received care according to the university`s ethical regulations and NIH guidelines. The institutional animal care ethical committee approved all animal protocols, and the mice were housed in a barrier facility.

In order to establish a LF model, 12 mice were intraperitoneally (IP) injected with pure carbon tetrachloride (CCl_4; Sigma, C-5331), 0.5 per gram of body weight. CCl_4 is a powerful hepatotoxic organic agent, widely used to induce hepatic disorders in experimental animal studies, such as LF, however, CCl_4 has a damaging effect on other organs as well, therefore, IP injection was preferred in this study, as it allows quick reabsorption of large volumes of substances, and focuses on the liver area (89). CCl_4 was diluted in maize (corn) oil at a ratio of (1:9). Mice were divided into 3 groups (4 mice in each); the first group received IP injection twice a week, for 6 weeks, in order to cause chronic LF. In the last 2 weeks, the second group got an IP injection to cause acute LF. The third group, injected with corn oil, represented the control group. During the injection period, mice were regularly assessed for their weight.

On sacrificing day (2 weeks after the last injection) all mice were administered anesthesia by inhaling 5% isoflurane for 10 seconds, and ultimately underwent cervical dislocation. Lungs were extracted from each mouse and kept at (-80˚c) for further laboratory investigations.

This study came to identify the hypothesis that has been conducted, and to clarify the effect of BAs signaling pathways on the lungs, in cases of LF. Several assessments were conducted to determine if liver injury could affect pulmonary cells. There might be a link between LF and lung disease. To check for lung damage, we performed several tests in order in to detect biomarkers of oxidation, injury, inflammation, and fibrosis, in addition

to specific proteins released from damaged lung cells. Furthermore, BAs levels were measured in both serum and BALF. NK immune cells were isolated from lung tissue, and went through flow cytometry with AECs types I and II to detect their viability and function. By doing all these tests, we hope to understand how LF is connected to respiratory complications. The elevated levels of BAs due to liver damage, might help explain the lung injury and respiratory failure that occurs in LF patients.

4.1.2 BAs profile assessment in serum and BALF

Since the liver is the origin of BA synthesis and regulation, any hepatic abnormality will result in elevated serum BA levels (24), due to the inability of hepatocytes to remove BA from portal blood, which may further exacerbate liver damage (25). Indicating the role of BAs in the diagnosis and prognosis of LF patients (26).

When elevated levels of BA pass through the circulation, they get up taken in the lungs, and accumulate in BALF (73). Aspiration of BAs damages airway epithelia, stimulates lung injury, and activates the release of inflammatory cytokines (ex. IL-6, IL-8) (77). In addition, BA causes cell damage through disrupting cell membrane ionic balance and permeability, leading to mucosal breakdown, and affecting the production of surfactant proteins and phospholipids (79). All of which weakens epithelial barrier function. Therefore, BA profile is valuable for the prognosis of structural and chronic pulmonary diseases, and for reflecting the lung allograft microenvironment (80).

In addition, elevation of BA levels stimulates the expression of its regulatory transporter (NTCP) and receptor (FXR) in both liver and lung tissues (28). NTCP expression also increases in lung NK cells, affecting immune response (32).

4.1.3 Oxidation

One of the fatal complications of BAs disturbances in liver fibrosis is oxidative stress (49). Toxic bile salts can pass from the liver through circulation and reach several organs, including the lungs (50). Alveolar epithelial cells are highly sensitive to injury, therefore, BAs retention in pulmonary cells can induce the production of free radicles (FR) and reactive oxygen species (ROS) that peroxidase lipids in cellular membrane, creating several oxidizing agents as malondialdehyde (MDA) (90). Resulting in the release of pro-inflammatory mediators, such as IL-6 and TNF-α. This can induce the immune response

of T-cells and macrophages, and further stimulate the secretion of other inflammatory cytokines (91). All of which exacerbates necrosis, apoptosis, and DNA damage, leading to numerous diseases, such as malignancies (90).

The Liver is the key organ of metabolism and extraction, and since it is often exposed to various xenobiotics and therapeutic agents, it has an antioxidation system that scavenges FR to maintain homeostasis (1). Glutathione peroxidase (GPx) is one of the main anti-oxidative enzymes that converts reduced glutathione (GSH) to the oxidized form, glutathione disulfide (GSSG) in order to get rid of ROS, and control the pro-inflammatory process in the liver and lungs (92).

The ratio of GSH:GSSG can be used as a marker for oxidative stress. In a resting cell, the molar GSH:GSSG ratio exceeds 100:1. However, under respiratory pathological conditions, oxidant/antioxidant imbalance occurs, where ROS levels increase above the steady state, and GSH stores in pulmonary cells become consumed, therefore, the GSH:GSSG ratio declines to 10:1, and even 1:1 (93).

Inflammatory lung diseases are characterized by inflammation, that causes cell damage. Normally, this process is a protective response to remove the injury cause, and stimulate tissue repair. When the damaging agent (elevated levels of BAs) still exists, imbalance occurs, leading to chronic inflammation (94).

Another major defense against oxidative stress, found in abundance in the lung is superoxide dismutase (SOD), which catalyzes superoxide to hydrogen superoxide (H2O2) and water (95). SOD is usually found in increased levels in cases of lung injury and neutrophil inflammation for blocking neutrophils recruitment and number in BALF (96). It has also an important role in regulating the oxidation in pulmonary fibrosis, via protecting alveolar collagen from oxidation, inhibiting the expression of chemoattractants for inflammatory cells, and downregulating the overproduction of inflammatory cytokines, such as TGF-β (95). However, disruption of the oxidant/antioxidant balance causes depletion of SOD, increasing mice sensitivity to oxidative stress, and enhancing ECM damage and lung injury (94).

4.1.4 SP-D and lung injury

Exacerbated inflammation causes lung tissue injury, disrupting gas exchange and lung function (97). One of the protective responses to this oxidative damage is the overexpression of the pulmonary surfactant protein D (SP-D), a complex mix of lipids and proteins secreted in to the alveolar space by several pulmonary cells, mainly AECs II (98). SPD plays a main role in diminishing surface tension in the alveoli, preventing their collapse, and maintaining lung surfactant homeostasis and alveolar structure (97).

Lung injury and inflammation stimulate the release of SPD, and elevate its levels in both BALF and serum, suggesting SPD role as a biomarker for pulmonary damage (99). Damage-associated molecular patterns (DAMPs) stimulate several inflammatory pathways, including toll-like receptor 4 (TLR-4) that can be expressed on the surface of several inflammatory cells, where it can be recognized by, and bound to the carbohydrate recognition domain (CRD) of SPD, suppressing the activation of many inflammatory signaling pathways (98). In addition, SPD maintains alveolar epithelial integrity and protects it from injury, by downregulating pulmonary macrophages and fibrocytes number, pro-fibrotic cytokines expression (TNF-α, TGF-β, IL-6), and fibrotic lung remodeling in response to injury (99).

In vivo and in vitro experiments have shown that mice with decreased or muted SPD had activated fibrotic response, with an increased number of lung laden-lipid foamy macrophages (FMs), and elevated MMP-9 levels. In addition to a severe airway inflammation, similar to that in COPD and influenza patients (98).

SPD also serves as an antioxidant, inhibiting ROS formation and collagen deposition, and suppressing MMP-9 production by alveolar macrophages. In addition to its critical role in lipid homeostasis, by suppressing laden-lipid FMs (100). Furthermore, SPD targets microbial pathogens that colonize the lungs, by binding to them and activating their uptake by phagocytic cells. All of which helps in regulating innate immune response, and clears the respiratory tract from infectious pathogens (97). Therefore, further clinical studies should be performed on the role of SPD as a non-invasive diagnostic tool, and a prognostic predictor for worse outcomes and mortality risk in several respiratory disorders (100).

4.1.5 sRAGE and lung inflammation

Soluble receptor for advanced glycation endproducts (sRAGE) is a pro-inflammatory recognition receptor (PPR), that is highly expressed in lung tissue, mainly in the basal membrane of alveolar epithelial cells (AECs) types I and II. In addition to vascular and airway smooth muscle cells, neurons, and some immune cells (101). sRAGE signaling pathway has a significant number of functions, however, it`s well-known for its role in the initiation, amplification, and maintenance of immune response. In addition to cell-to-cell adhesion, participating in leukocytes and other inflammatory cells recruitment to inflamed tissue (102). sRAGE expression is upregulated in various cell lines in pathological inflammatory conditions, such as diabetes, vascular and neurodegenerative diseases, and even cancers (101).

Since sRAGE is mainly expressed in lung tissue, and is involved in AECs proliferation and migration, in addition to remodeling of pulmonary vasculature, studies have indicated its role in several pulmonary diseases, including asthma and allergic reactions, COPD, lung injury, pulmonary fibrosis, and tumorigenesis. Therefore, it is suggested that sRAGE be used as a biomarker for diagnosis, prognosis, and response to treatment of these patients (102).

The role of sRAGE in pulmonary diseases, especially fibrosis is confusing and contradictory. Several studies on patients and animal models of lung fibrosis have shown that overall sRAGE expression is decreased in plasma and BALF (103). Some of these studies revealed that RAGE blocking in mice led to the proliferation and migration of ACEs and lung fibroblasts when exposed to fibrotic agents, implying the protective role of RAGE on lung`s health and attenuating damage (102). Other studies suggest that sever pulmonary fibrosis leads to a decline in sRAGE, due to injury and loss of AECs I in these cases, since these cells are the major site for RAGE expression (101).

On the other hand, several research studies on both humans and animals have indicated the implication of RAGE in fibrosis development in the liver, kidney, heart, and lungs (103). In vitro, stimulation of fibrosis has shown elevated sRAGE expression. In addition, RAGE-null mice were protected from fibrosis, showed lower production of cytokines and inflammatory mediators, such as MMP9, and TGF-β, and had better survival rates compared to controls (102). Clinical investigations on adult and pediatric patients with

asthma have revealed elevated levels of sRAGE and TNF-α in their sputum samples. This can be explained by the fact that RAGE signaling contributes to ROS formation, which stimulates the secretion of IL-4, activates T cells and eosinophils, and exacerbates fibrosis (103). Although alveolar sRAGE expression decreases due to injury, sRAGE signaling may still promote inflammation and fibrosis (101). Further research on RAGE therapeutic effects should be performed.

4.1.6 Lung/BALF inflammatory cytokines

Aspiration of BAs causes injury of AECs I and II, leading to a significant release of IL-1β, IL-4, IL-6, and TNF-α. All of these, in addition to ROS, are crucial for creating chronic inflammation, the initial and most important phase of carcinogenesis (104). Both inflammatory and tumor cells release cytokines and chemokines, which eventually leads to airway epithelial cells damage and death. Making these interleukins the target for therapy and clinical diagnosis (78).

Oxidative stress activates caspase-1, resulting in the secretion of IL-1β from pulmonary macrophages, which exacerbates lung injury. IL-1β is strongly associated with structural lung diseases, where its elevated release stimulates neutrophilic inflammation, even in the absence of any microbial pathogens, causing airway mucus obstruction, and hypoxic necrosis of AECs (105). In addition, IL-1β receptor can activate angiogenesis, tumor development, and metastasis by stimulating multiple growth factors (104). In vivo research on mice showed that blockage of IL-1β by caspase-1 inhibitors has clinical benefits for the treatment of patients with lung injury with poor prognosis (105).

IL-4 has immunostimulatory properties, as it`s the key activator of B-cells function, chemotaxis of eosinophils, and T-cells differentiation. However, its conflict activities make it hard to predict its role in pulmonary injury (81). A study conducted on 2 groups of bleomycin-treated mutant mice, where IL-4 was deficient in the first group, and highly expressed in the second one. At the early stages of the study, IL-4 deficient mice showed lethal pulmonary inflammation, early collagen synthesis, and high mortality rates, compared to the second group (106). Indicating the anti-inflammatory, immunosuppressive, and healing role of IL-4 in early inflammation; as it limits the early recruitment of T-cells, suppresses the production of pro-inflammatory mediators (ex. IL-6, IL-8, TNF-α), and stimulates the production of IL-1 receptor antagonist (104).

On the other hand, later stages of the study had undesirable results, IL-4 deficient mice showed less inflammation than the highly expressed group, indicating the pro-fibrotic role of IL-4 in chronic inflammation. In vitro studies showed that IL-4 can regulate fibroblast functions; including, chemotaxis, proliferation, and collagen synthesis (106). However, IL-4 is not directly responsible for exacerbated ECM deposition, it is suggested that it regulates the expression of several pro-fibrotic mediators, in addition to growth factors such as TGF-β, which further stimulate fibroblast excretion (104).

IL-6 is an important multifunctional pro-inflammatory cytokine, that plays a crucial role in regulating immune response and inflammation, by activating monocytes, T-cells differentiation, and B-cells maturation (82). The role of IL-6 in lung injury is biphasic, a study conducted on mice injected with IL-6 neutralizing antibodies, has shown that mice initially had higher BALF macrophages, activated lung fibrosis, and apoptosis of AECs; when treated with an injury-causing factor, compared to the later stages of the study; where mice showed a decline in fibrosis activity (107). This indicates the anti-inflammatory role of IL-6 at the early stages of lung injury; as its signaling regulates surfactant homeostasis, and protects AECs, in addition to suppressing the pulmonary cytokine network, as a host defense mechanism against pathogens (104).

IL-6 has also anti-fibrotic properties; through regulating the cellular lipid content of respiratory epithelia. However, elevation of IL-6 at chronic stages of oxidative lung damage stimulates respiratory inflammation, by activating the synthesis of acute phase proteins; leading to pulmonary fibrosis (107). Moreover, IL-6 regulates hematopoiesis and oncogenesis, therefore, exacerbated activation of IL-6 receptor can activate growth differentiation in tumor cells; leading to lung cancer (82).

TNF-α is an early-phase inflammatory cytokine and a death ligand, that plays a crucial in the pathogenesis of acute lung injury. Alveolar macrophages-derived TNF-α is a key mediator for pulmonary fibrosis, through activating caspase 8 and P55, which stimulate death signaling; causing AECs dysfunction and apoptosis, pulmonary fibrosis, and edema (108). All of that is assessed by the elevated levels of sRAGE in BALF (104). TNF-α is also involved in the immune response associated with cancer progression (108).

All these cytokines were shown to increase during severe lung diseases in both serum and BALF, but with higher levels and earlier elevations in BALF (79). They serve not only

as biomarkers for diagnosis of inflammation, but also for distinguishing between benign and malignant stages; avoiding unnecessary invasive diagnostic procedures, and enhancing overall survival through disease management (81).

4.1.7 Lung fibrosis

BAs-mediated oxidative damage causes injury of pulmonary cells, where they stimulate the synthesis and release of various cytokines and growth factors, including TGF-β (109). This process activates ECM proteins, and the proliferation of fibroblasts, which synthesize new collagen fibrils; maintaining tissue repair and homeostasis (4). However, prolonged aspiration of BAs in elevated levels causes repetitive epithelial injury; leading to an abnormal response to damage (72). Uncontrolled activation of fibroblasts causes excessive collagen deposition in the ECM, and stimulates epithelial-mesenchymal transition (EMT); a process by which epithelial cells lose their cellular polarity; due to suppressed expression of E-cadherins, and become migratory mesenchymal cells (4). This is the hallmark step in pulmonary fibrosis; a chronic irreversible inflammatory response, characterized by pulmonary epithelial cells death, lung stiffness, shortness of breath, alveolar capillary damage, and eventually, respiratory failure (51). Several cytokines play a crucial role in this pathological fibrogenesis, such as MMP-9, α-SMA, and glial fibrillary acidic protein (GFAP), making them an excellent diagnostic and therapeutic target (80).

MMP-9 is a zinc-dependent endopeptidase expressed in AECs and pulmonary macrophages under the control of ECM proteins, growth factors, and cytokines; including TNF-α and IL-1β (110). At the beginning of lung injury, MMP-9 serves as an anti-fibrotic agent, regulating inflammation and injury response by degrading collagen I and III, and ECM proteins. On the other hand, MMP-9 becomes pro-fibrotic during later stages of injury, as it activates EMT, fibrotic migration, inflammatory cells recruitment, and T-cells maturation. In addition to stimulating a strong cascade of cytokines; including TGF-β, which can also upregulate MMP-9 expression and fibrosis (fibrogenic feedback loop) (111). This disturbed balance between activating and suppressing pulmonary fibrogenesis causes defected collagenolysis, leading to ECM accumulation, and lung disease (110).

Clinical investigations have shown elevation in MMP-9 levels in BALF samples of patients with pulmonary fibrosis (111). In vivo experiments revealed that MMP-9-

deficient mice were protected from bronchial inflammation and fibrosis after being injected with bleomycin, compared to the control group that showed overexpression of MMP-9 in macrophages (110). Due to its crucial role in exacerbating lung damage, blocking MMP-9 is a therapeutic approach, that attenuates pulmonary fibrosis by downregulating inflammatory cytokines, ROS, and collagen synthesis (111).

TGF-β regulates cellular growth and metastasis, its molecular signaling activates EMT in lung injury via several pathways; mainly by upregulating the expression of α-SMA, a biomarker for alveolar myofibroblasts activation and differentiation (112). Patients with respiratory disorders, such as acute lung injury, pulmonary fibrosis, and COPD had high levels of α-SMA detected in their serum and urine samples (109). Injecting mice with TGF-β, has revealed an upregulation in α-SMA, in addition, IL- 1 was also detected in lung myofibroblasts of these mice; indicating its role in activating α-SMA (112). Several studies have supported the association between α-SMA and lung cancer, as fibroblasts were found in tumor microenvironment of pulmonary adenocarcinoma patients, with highly expressed α-SMA. Showing increased metastasis and contractility, and poor prognosis, compared to normal fibroblasts (109). Making it a novel biomarker for measuring activated fibroblasts, and a therapeutic target; as blocking the expression of α-SMA attenuates EMT in pulmonary fibrosis (112).

GFAP is a type III intermediate filament protein, responsible for the cytoskeletal structure of glial cells (113). GFAP is highly expressed in myofibroblasts during oxidative injury and inflammation, where it activates ECM proteins, and collagen synthesis (114). Chronic inflammation exacerbates GFAP expression, and leads to pathological accumulation of collagen in fibrotic lesions, this occurs in various diseases, such as spleen, liver, hematopoietic, and pulmonary disorders. In addition to several types of cancers, multiple sclerosis, and neurodegenerative disorders, such as Alzheimer, and Parkinson diseases (86). GFAP is also expressed in pulmonary glial cells, regulating autonomic innervation of lungs, bronchial smooth muscles, and airway blood vessels (114). Neuronal GFAP can be activated by various inflammatory cells, causing either attenuation or exacerbation of pulmonary inflammation, depending on the type of the activated nervous receptor. Indicating the useful role of GFAP in both diagnosis and therapy (113).

4.1.8 Reduction in the activity of lung tissue-resident NK cells

BAs aspiration causes lung cells damage, and disturbs pulmonary microenvironment, making it more susceptible to severe inflammation, and even tumorigenesis. In order to retain hemostasis, an immediate immune defense is needed, but without leading to excessive inflammation (44). Natural killer (NK) cells are innate cytotoxic lymphocytes, representing the first line of defense against tumor and inflammatory cells, as well as intracellular invasive pathogens. These abnormal cells, in addition to inflammatory cytokines, are the key activators for NK cells (115). NK cells express several activating and inhibitory receptors, that control the secretion of many chemokines, such as TNF-α, IFN-γ, and GM-CSF, allowing them to interact with other immune cells, such as T-lymphocytes. NK cells can also secrete several cytokines such as perforin, and granzyme B, which mediate cytotoxicity (116).

NK cells play a pathological role in activating allergies and inflammatory sensitization, such as stimulating IgE-mediated allergic reactions. On the other hand, they have a protective role in attenuating inflammation, by activating apoptosis of eosinophils (115). Indicating the contradictory role both regarding the number (percentage), and function (cytotoxic efficiency) of NK cells; where they can either respond immediately to the involved pathogen and clear it, or cause uncontrolled inflammation and pathological damage (116). Clinical investigations have revealed that asthmatic children have a higher number and cytotoxicity of NK cells, compared to healthy controls, even after therapy. On the other hand, other studies showed a decline in NK cells percentage in serum and BALF, in addition to impaired cytotoxicity in asthmatic patients, compared to their healthy controls (117). In vivo experiments on mice with induced asthma showed no change in the total number of NK cells in the lungs, however, selectively immature NK cells were noticed. Furthermore, the depletion of NK cells at the peak of inflammation delayed the clearance of pathological immune cells (116).

In addition to being a protective immune response against inflammatory conditions, such as asthma, COPD, and microbial infections, NK cells have also anti-tumor properties in several organs, including lung cancer, and HCC; through activating adaptive T-cells, where NK-T cells cooperation suppresses tumor growth (118). An experimental study on NK-deficient mice injected with cancer cells, showed a high tumor burden compared to control mice, indicating NK`s crucial role in controlling tumor burden. However, late

stages of cancer are characterized by NK dysfunction, mainly due to impaired viability. Therefore, a decline in NK% is an indicator of, not only severe inflammation, but also, malignant tumorigenesis (115).

Several reasons explain the decline in NK% with ongoing pulmonary cellular damage. Some suggest that perforin (cytotoxic factor) stimulates the killing of NK cells, others suggest the DNA damage of these lymphocytes is due to apoptosis, or that it`s because of NK degranulation (116). With time, NK cells lose their cytotoxic ability at later stages of the disease and become dysfunctional; as the aging of the immune system causes inefficient cleaning of abnormal cells (117). Another reason might be the overproduction of TNF-α by cancer cells, which causes NK dysfunction, and disables them to infiltrate tumor lesions (118). Some studies show that it`s because of NK cells accumulation in peripheral circulation; due to impaired recruitment. While other studies explain it by increased consumption of these cells in critical conditions, rather than primary deficiency (117).

The contribution of NTCP in NKs impairment is not only in the terms of BAs transportation and uptake, but also as a functional receptor for the entry of hepatitis B virus (HBV) and its satellite hepatitis D virus (HDV) in to hepatocytes. Chronic HBV infection leads to changes in hepatic environment, which will further disturb the antiviral immunity and cytokine response of NKs (31). Suggesting that NTCP can help in understanding HBV life cycle, and serve as a therapeutic target for hepatic and pulmonary immune disorders.

4.1.9 Elevation in AECs apoptosis

The presence of BAs at high levels in lung tissue is harmful at different levels; including airway, and alveoli (49). AECs I are large squamous cells that represent 96% of the internal air surface, they play a critical role in blood gas exchange, maintain lung function, and enhance alveolar repair; all by producing pulmonary surfactant (119). AECs II are the only cells that synthesize surfactant proteins, which contribute to surfactant stability, alveolar integrity, respiratory function, and inflammation. Both AECs are highly affected during BAs aspiration (120).

BAs exert direct cellular toxicity, depending on their type and water solubility, the higher the solubility, the greater the cytotoxicity. BAs stimulate the formation of ROS, causing AECs injury, lesion formation, vascular dilation and remodeling, and angiogenesis (50). Moreover, BAs activate sPLA2 synthesis in alveolar macrophages, which express BA G-protein coupled receptor (TGR5) (51). Normally, sPLA2 hydrolyzes phospholipids in pulmonary surfactant, regulating the first step in the inflammatory cascade. However, BAs-mediated overproduction of sPLA2 activates uncontrolled local inflammatory damage, that affects surfactant function and structure (120). In addition, BAs stimulate the synthesis and release of several inflammatory cytokines, including IL-1β, IL-6, IL-8, MMP9, neutrophilic elastase, and growth factors, such as TNF-α, all of which exacerbates systemic inflammation, and stimulates AECs apoptosis (119).

Apoptosis of AECs can be either extrinsic or intrinsic. Extrinsic apoptosis is due to interaction with a death ligand, such as TNF-α, and P53, that stimulate caspase 3, 8, and 9. On the other hand, intrinsic apoptosis occurs through an intracellular stimulus; such as DNA damage, or ROS (50). Both pathways stimulate endoplasmic reticulum stress, and perfusion of mitochondrial membrane. Eventually, respiratory cellular death decreases surfactant proteins synthesis, alveolar air space, and total lung size; leading to water displacement, disturbed ventilation and gas exchange, pulmonary damage, and collapse (119). Explaining the sudden death in infants born to mothers with hepatic disorders, where BAs and sPLA2 levels were elevated in their BALF and serum samples (73).

Since surfactant plays a crucial role in immune defense, BA-mediated inflammation causes a harmful immune modulation in the lungs, where non-pathological pulmonary microbes shift into the pathological form, causing antibiotic resistance and exacerbated lung damage (120). Despite being fatal; apoptosis caused by BAs can be beneficial in triggering tumor cells by suppressing their division and migration in several types of cancers, including lung, colorectal, and HCC (119).

4.2 Conclusion

Our study findings show that LF can cause lung injury, as evidenced by the elevation of inflammatory markers in BALF and AECs apoptosis. A link exists between LF and oxidative stress, which can implicate respiration, and overall pulmonary function and homeostasis. In LF mice models, abnormally elevated BAs levels pass through the

circulation and reach the lungs, where they can induce oxidative damage, causing pulmonary injury, inflammation, and fibrosis. Furthermore, BAs can suppress the activity of lung NKs, leading to further progression in lung injury, through disturbing immune response and respiratory homeostasis. Indicating that BAs are a critical factor in the crosstalk between LF and Pulmonary damage, which can further develop into lung cancer. This suggests that BAs may be valuable for treating and diagnosing LF and pulmonary complications. Antagonizing BAs receptors and transporters, and targeting CYP7A1 expression in pulmonary cells could serve as a novel treatment strategy to simultaneously address lung injuries and liver fibrosis, paving the way for integrated therapeutic approaches in clinical practice. This could improve patient outcomes and expand the understanding of the interconnectedness of organ systems in disease pathogenesis. Further research should be conducted in this field.

4.3 Limitations

Additional experiments; such as histopathology, would have been helpful in providing more evidence, but couldn't be performed due to time and budget limitations.

4.4 Conflict of Interest

The authors declare that this research was conducted in without any commercial or financial relationships that could potentially create a conflict of interest.

4.5 Recommendations

- More research is necessary to examine how BAs can affect lungs health.
- Periodical laboratory testing of BAs with liver function tests in patients with LF, as an indicator for lung status.
- Further research should be performed to examine pulmonary NK cells phenotype in LF models.
- Given the absence of published studies explaining the effects of BAs in the content of LF on lungs NK cells activity, and the lack of comprehensive understanding in other related studies, it is strongly recommended that additional studies be conducted in this area.

List of Abbreviations

Abbreviation	Meaning
AAD	Allergic Airway Disease.
AECs	Alveolar/Airway Epithelia Cells
AMP	Adenosine Monophosphate.
ARDS	Acute Respiratory Distress Syndrome.
α-SMA	Alpha-Smooth Muscle Actin.
BAs	Bile Acids.
BAAT	BA Coenzyme-A N-Acetyl Transferase.
BACS	BAs Coenzyme-A Synthase.
BAEP	BAs Export Bump.
BALF	Bronchoalveolar Lavage Fluid.
BEAS-2B	Bronchial Epithelia Cell Line.
BLM	Bleomycin.
CA	Cholic Acid.
CBDL	Common Bile Duct Ligation.
CCl_4	Carbon Tetrachloride.
CDCA	Chenodeoxycholic Acid.
CDR	Carbohydrate Recognition Domain.
CF	Cystic Fibrosis.
COPD	Chronic Obstructive Pulmonary Disease.
CRP	C-Reactive Protein.
CVDs	Cardiovascular Diseases.
CXCL	Chemoattractants.
CYP7A1	Cholesterol 7α-Hydroxylase.
DAMPs	Damage Associated Patterns.
DC	Dendritic Cells.
DCA	Deoxycholic Acid.
ECM	Extracellular Matrix.
ELISA	Enzyme Linked Immunosorbent Assay.
ET-1	Endothelin-1.
FGF	Fibroblast Growth Factor.
FGs	Foamy Macrophages.
FXR	Farnesoid X Receptor.
GFAP	Glial Fibrillary Acidic Protein.
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Factor.
GOR	Gastro-Esophageal Reflux.

Abbreviation	Meaning
GPBAR1	G Protein-Coupled BA Receptor R1.
GPX	Glutathione Peroxidase.
GSH	Glutathione (Reduced).
GSSG	Glutathione (Oxidized).
H2O2	Hydrogen Peroxide.
HCC	Hepatocellular Carcinoma.
HDM	House Dust Mite.
HFD	High-Fat Diet.
HIP	Hypoxic Inducible Factor.
HPS	Hepatopulmonary Syndrome.
HSCs	Hepatic Stellate Cells.
IBABP	Ileal BAs Binding Protein.
IBD	Inflammatory Bowel Disease.
ICP	Intrahepatic Cholestasis of Pregnancy.
IFN-γ	Interferon Gama.
IL	Interleukin.
IP	Intraperitoneal.
IPVDs	Intrapulmonary Vascular Dilation.
IRDS	Infant Respiratory Diseases Syndrome.
LCA	Lithocholic Acid.
LF	Liver Fibrosis.
MAFLD	Metabolic Dysfunction Associated Liver Disorders.
MDA	Malonedialdehyde
MMPs	Matrix Metalloproteinase.
MRP2	Multidrug Resistant Protein 2.
NAFLD	Non-Alcoholic Fatty Liver Disease.
NASH	Non-Alcoholic Steatohepatitis.
NBAP	Neonatal BAs Pneumonitis.
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells.
NKs	Natural Killer Cells.
NO	Nitric Oxide.
NO2-OA	Nitro-Oleic Acid.
NTCP	Na (Sodium)-Dependent Taurocholate Cotransporting Polypeptide.
OATP	Organic Anion Transporting Polypeptide.
OSTα/OSTβ	Organic Solute Transporter Alpha and Beta.
PDGF	Platelet Derived Growth Factor.
PI	Propidium Iodide.

Abbreviation	Meaning
Abbreviation	Meaning
PPH	Portopulmonary Hepatitis.
RAR/RXR	Retinoic Acid Receptor, Alpha and X.
ROS	Reactive Oxidative Species.
SCFA	Short-Chain Fatty Acids.
SHP	Short Heterodimer Partner.
SOD	Superoxide Dismutase.
SP	Surfactant Protein.
sPLA2	Surfactant Phospholipase A2.
sRAGE	Soluble Receptor for Advanced Glycation End products.
SSC	Systemic Sclerosis.
TGFβ1	Tissue Growth Factor Beta 1.
TGR5	Takeda G protein-coupled Receptor 5.
TLR-4	Toll Like Receptor 4.
TNF-α	Tumor Necrotic Factor Alpha.

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Appendices

Appendix A

IRB Approval

An-Najah National
University
Faculty of Medicine &
Health Sciences
Institutional Review Board

جامعة النجاح الوطنية
كلية الطب وعلوم الصحة
لجنة اخلاقيات البحث العلمي

Ref: Mas. Feb. 2023/3

IRB Approval Letter

Title of Research:

The Effect of Bile Acid Signaling Pathway on Lung Immune Cells in Liver Fibrotic Mice Model

Submitted by:
Ala`a Samir Mohammad Alfuqaha

Supervisor:
Jhonny Amer , Ahmad Salhab

Approved:
1st Feb. 2023

Your Study Title "**The Effect of Bile Acid Signaling Pathway on Lung Immune Cells in Liver Fibrotic Mice Model .**" reviewed by An-Najah National University IRB committee and was approved on 1st Feb. 2023

Hasan Fitian, MD

IRB Committee Chairman

IRB
Institutional Review Board

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جـامعـــة النجاح الوطنية
كليـــة الدراســـات العليـــا

تأثير مسار إشارات حمض الصفراء على الخلايا المناعية للرئة في نموذج الفئران للتليف الكبدي

إعـداد
آلاء سمـير محمد الفقها

إشـراف
د. جوني يعقوب نصري عامر

قدمت هذه الرسالة استكمالاً لمتطلبات الحصول على درجة الماجستير في الكيمياء الحيوية السريرية،
من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.
2024

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الملخص

الخلفية: اضطرابات الكبد مسؤولة عن 2 مليون حالة وفاة كل عام. حجر الرحى في معظم أمراض الكبد هو تليف الكبد (LF)، وهو استجابة التئام الجروح، الناجمة عن مختلف العوامل المسببة للإصابة؛ كالكحول، بالإضافة إلى العديد من الاضطرابات الأيضية والالتهابية. تؤدي الإصابة الكبدية طويلة المدى إلى إطلاق العديد من الأجسام المبرمجة والسيتوكينات الالتهابية، التي تنشط الخلايا النجمية الكبدية (HSCs)، وتحفز إنتاج الكولاجين. طالما لم يكن في مرحلة تليف الكبد المتقدمة، يمكن أن يكون LF قابلاً للعكس، وإلا فإن مستويات عالية من الكولاجين سوف تترسب في المصفوفة خارج الخلية (ECM)، مما يؤدي إلى تكوين ندبة تليفية، وفشل كبدي، والذي يمكن أن يتطور إلى سرطان الخلايا الكبدية. (HCC)، ومضاعفات أخرى خارج الكبد. يعد حمض الصفراء (BA) أحد أدوات التشخيص لـ LF؛ يتأثر كل من صناعته واستقلابه في الاضطرابات الكبدية. يمكن للمستويات المرتفعة بشكل غير طبيعي من BAs أن تمارس أضرارًا تأكسدية على أعضاء مختلفة، بما في ذلك الرئتين؛ حيث يمكن أن تؤدي إصابة الرئة إلى تحفيز التليف الرئوي، وفي النهاية، سرطان الرئة.

الأهداف: تهدف دراستنا إلى تحديد تأثير مسار إشارات BAs على صحة الرئتين، في نماذج الفئران LF. سيؤدي سد هذه الفجوة إلى توفير فهم أفضل لمحور الكبد والرئة؛ إعطاء رؤى قيمة حول الأهداف التشخيصية الجديدة والاستراتيجيات العلاجية.

الطرق: تم إنشاء نموذج الفئران LF عن طريق الحقن داخل الصفاق مع رابع كلوريد الكربون (CCl_4) لمدة أسبوعين (حاد) و 6 أسابيع (مزمن) (العدد= 12). تم التضحية بالفئران ثم تم جمع عينات من مصل

الدم، وسائل القصبة الهوائية (BALF)، وأنسجة الرئتين لفحصها. تم استخدام ELISA للكشف عن مستويات sRAGE والسيتوكينات الالتهابية (IL-1β، IL-4، IL-6، TNF-α) في عينات BALF، بالإضافة إلى مستويات BAs في كل من المصل وBALF. تم استخدام PCR لتحديد علامات الأكسدة (GPx، GSH:GSSG، MDA، SOD)، والإصابة (SP-D1) والتليف (α-SMA، MMP-9، GFAP) في نسيج الرئة. علاوة على ذلك، تم عزل وتقييم NKs المقيمة في أنسجة الرئة من أجل تنشيطها وقدرتها على البقاء من خلال تقييم مستقبلات CD107a وNTCP عبر قياس التدفق الخلوي (Flow Cytometry)، وهي نفس التقنية المستخدمة لتقييم الموت المبرمج لخلايا الطلائية التنفسية (AECs)النوعين الأول والثاني.

النتائج: ارتبطت مستويات حمض الصفراء (BAs) في كل من المصل وBALF خطيا مع تطورات LF. وقد وجد أن السيتوكينات الالتهابية (IL1-β، IL-4، IL-6، TNF-α)، وsRAGE زادت بشكل ملحوظ في عينات BALF لكل من مجموعات الفئران LF الحادة والمزمنة مقارنة بالcontrol. علاوة على ذلك، كانت علامات الإجهاد التأكسدي والإصابة والتليف مرتفعة بشكل ملحوظ في الأنسجة الرئوية لكلا المجموعتين LF، مقارنة بالcontrol. أظهرت بياناتنا زيادة كبيرة في إطلاق NTCP من trNKs الرئوية، والتي أثرت جزئيًا على نشاط trNK، وتسببت في انخفاض كبير في إطلاق CD107a. علاوة على ذلك، تم زيادة الموت المبرمج لخلايا الطلائية التنفسية (AECs) النوعين الأول والثاني بشكل ملحوظ مع تفاقم LF.

الاستنتاج: تظهر دراستنا وجود صلة بين الإصابة بتليف الكبد وتلف الرئة، حيث يعد حمض الصفراء عاملاً رئيسياً في هذا التداخل بين الكبد والرئة. يمكن أن يؤدي الارتفاع الناجم عن LF في مستويات BAs إلى حدوث إصابة مؤكسدة في الرئتين، مما يؤدي إلى تثبيط نشاط trNKs في الرئتين، وتنشيط الموت المبرمج لخلايا الطلائية التنفسية (AECs) النوعين الأول والثاني. مما يسبب التليف الرئوي، ويؤثر

على وظيفة الجهاز التنفسي. كل هذا يشير إلى أن BAs قد تكون بمثابة نهج قيم في علاج وتشخيص LF والمضاعفات الرئوية.

الكلمات المفتاحية: تليف الكبد، الرئتين، الأحماض الصفراوية، BALF، نموذج LF للفئران.