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THE PLASMA KALLIKREIN-KININ SYSTEM AS A MODULATOR IN LIVER INJURY/REMODELLING

by
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ABSTRACT
OF THE THESIS OF

Ibrahim Adeola Ahmed for Master of Science
Major: Biochemistry

Title: Plasma Kallikrein – Kinin System as a Modulator of Liver Injury/Remodeling

Background: Liver injury is an apparent condition of the body system owing to the numerous functions of the liver. Most of the etiologies are due to the actions of many drugs, toxins, hepatotropic viruses, and idiosyncratic factors. Moreover, the occurrence and persistence of hepatic injury lead to its remodeling; hence, acute, and chronic liver disease results due to apoptosis, necrosis, and inflammation. Besides, the compromise in the liver architecture during other organs or tissue's dysfunction could become a challenge.

The plasma kallikrein-kinin system (PKKS) plays diverse roles in coagulation, vascular injury, tissue inflammation, and wound-healing. In liver injury, the protease-activated receptors 1 and 2 play significant roles in the whole process while plasma kallikrein (PK), the main effector of the system role's is unknown. Yet, reports have shown the increase in PK's synthesis in acute liver injury. In terms of PK's significance in other diseases, a cohort study of type 1 diabetic patients observed the significant increase in the activity of PK compared to control. However, PK's role in liver injury and other disease-state liver like type 1 diabetes is still unclear.

Aims: In this study, the objective is to characterize the role of the PKSS in both acute and chronic liver injury models of non-diabetic and diabetic mice; and assess how it modulates its downstream targets in liver injury.

Methods: Acute liver injury was induced in 10-12 weeks old C57BL/6J male mice after a single injection of 0.6 mL/kg carbon tetrachloride (CCl_4), intraperitoneally. Chronic liver injury was induced after the injection of 0.6 mL/kg CCl_4, twice a week, for 2.5, 4, 6 or 7 weeks. In some group of mice, 0.05 mL/g streptozotocin (STZ) was injected daily for three to five days, to induce diabetes. Following the confirmation of the diabetic state, two weeks post STZ-injections by measuring the plasma glucose levels, the mice were injected with CCl_4 or its vehicle, mineral oil, either once for the acute liver injury or for 2.5 weeks for the chronic liver injury. In the liver, necrosis was observed by hematoxylin and eosin staining. Fibrosis was examined by assessing collagen fibers using picro sirius red staining and immunohistochemistry was performed for plasma pre-kallikrein. Quantitative polymerase chain reaction was used to assess the expression of hepatic genes such as the plasma kallikrein-kinin system, inflammation, and fibrosis. By Spearman correlation analysis, the PKKS genes were correlated to necrotic area, inflammatory and fibrotic markers, and

fibrotic area. *In vitro* studies were conducted via hepG2 cells to investigate cell-death and PK-related signaling pathways.

Results: A single injection of CCl_4 induced necrosis was negatively and weakly associated to the PKKS genes except for the *Kng1* gene (*Klkb1*, *F2r*, *Kng1*; r = -0.25, -0.20, -0.50 respectively; $p > 0.05$ except for *Kng1* gene). However, they were positively associated with cell-death ligand gene, *Hmgb1* (*Klkb1*, *F2r*, *Kng1*; r = -0.75, -0.44, -0.79 respectively; $p < 0.05$ except for *Kng1* gene). Analysis by immunohistochemistry showed the PPK around the necrotic areas at day 2 and 3 of CCl_4 induction, and significantly increased the *F2R* and *IL1B* genes while it decreased the *CCN2* gene expression in PK-induced hepG2 cells. Likewise, PK increased the proliferation of CCl_4-induced hepG2 cells by 140%. Varying time-points of liver fibrosis showed an increase in the mRNA levels of *F2r* (thrombin receptor), *F2rl1*, *Bdkrb1* and *Bdkrb2*. Spearman correlation showed a strong positive correlation to profibrotic factors, *Lgal3* and *Ccn2* genes, and fibrotic area; $p < 0.05$. In diabetes mice, we observed the upregulation of hepatic PKKS genes in response to chronic administration of CCl_4.

Conclusion: The PKKS play diverse role in liver injury, fibrosis, and resolution. Although PAR-1 and PAR-2 are implicated in liver fibrosis progression, they interact with diverse agonists. PK, which is an agonist, could be a purposed therapeutic agent in the regeneration of injured hepatocytes. However, additional investigations are needed to decipher the mechanism of interaction of PK in liver injury or fibrosis. Likewise, more research is also needed to understand the role of the bradykinin receptors in liver fibrosis.

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ABBREVIATIONS

ACE	angiotensin I-converting enzyme
AGE	advanced glycated end products
AFLD	alcoholic fatty liver disease
ALF	acute liver failure
ALT	alanine aminotransferase
AM	aminopeptidase
ASMA	alpha-smooth muscle actin
AST	aspartate aminotransferase
ATP	adenosine triphosphate
ALD	alcohol-induced liver disease
ATF3	activating transcription factor
AA	arachidonic acid
B1R	bradykinin 1 receptor
B2R	bradykinin 2 receptor
BK	bradykinin
BMDM	bone marrow-derived macrophages
CCl_4	carbon tetrachloride
CCl2/ Ccl2	C-C- motif chemokine ligand 2

CCl3/Ccl3	C-C- motif chemokine ligand 2
CHO	Chinese hamster ovary
cDNA	complementary DNA
COPD	chronic obstructive pulmonary disease
CPM	carboxypeptidase M
CPN	carboxypeptidase N
CTGF	connective tissue growth factor
CVD	cardiovascular disease
CYP450	cytochrome P450
DAMPs	damage-associated molecular patterns
DMEM: Dulbecco's Modified Eagle Medium	
DNA	deoxyribonuclease acid
DILI	drug-induced liver injury
EC	endothelial cells
ECM	extracellular matrix
EGF	endothelial growth factor
ET-1	endothelin-1
eNOS	endothelial nitric oxide synthase
F2	thrombin gene
FXa	factor Xa

FXII	factor 12
F2R	coagulation factor II thrombin receptor
F2RL1	coagulation factor II (thrombin) receptor-like 1
F2RL2	coagulation factor II (thrombin) receptor-like 2
F2RL3	coagulation factor II (thrombin) receptor-like 3
FBS	fetal bovine serum
GPCR	G-protein coupled receptors
g	gram
H&E	hematoxylin and eosin
HCC	hepatocellular carcinoma
HepG2	hepatocellular carcinoma cell lines
HMGB1	high-mobility group protein box 1
HMF	hepatic myofibroblast
HMWK	high molecular-weight kininogen
HSCs	hepatic stellate cells
IGF	insulin growth factor
IHC	immunohistochemistry
i.p.	intraperitoneal
i.v.	intravenous
IACUC	institutional animal care and use committee

KCs	Kupffer cells
KKS	kallikrein-kinin system
IL-1β	interlukin-1 beta
IL-6	interlukin-6
IL-10	interlukin-10
LAMP2A	lysosome-associated membrane protein 2A
LMWK	low molecular-weight kininogen
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
MFB	myofibroblasts
mg/kg	milligram per kilogram
mL/kg	milliliter/kilogram
MMPs	matrix metalloproteinases
MO	mineral oil
MOMP	mitochondrial outer membrane permeability
mPES2	microsomal prostaglandin E synthase 2
MPO	myeloperoxidase
NAFLD	non-alcoholic fatty liver disease

NASH	nonalcoholic steatohepatitis
NLRP3	NLR family pyrin domain containing 3
NO	nitric oxide
Ns	not significant
PAMPs	pathogen-associated molecular patterns
PARs	protease-activated receptors
PDGF	platelet-derived tissue growth factor
PGE2	prostaglandin E2
PGI2	prostaglandin I2
PSR	Picro Sirius red
PK	plasma kallikrein
PLC	phospholipase C
PPARɣ	peroxisome proliferator-activated receptor gamma
PPK	plasma prekallikrein
PRCP	Prolylcarboxypeptidase
PRR	pattern recognition receptor
RIPK	receptor interacting protein kinase
RNA	ribonuclease acid
ROS	reactive oxygen species
RT-qPCR	real-time quantitative polymerase chain reaction

SEM	standard mean of error
SMAC	second mitochondria-derived activator of apoptosis
STAT1	signal transducer and activator of transcription factor
STZ	streptozotocin
TBS	tris buffered saline
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TGF-β	transforming growth factor beta
TIMPS	tissue inhibitor of metalloproteinase
TK	tissue kallikrein
TLRs	toll-like receptor
TNF-α	tumor necrosis factor alpha
TNF-R1	TNF receptor 1
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	Regulatory T cells
VEGF	vascular endothelial growth factor
μg	microgram
μL	microliter
μM	micromolar

CHAPTER I

INTRODUCTION

A. Liver and Liver Injury

1. General

Liver injury is an eminent condition of the body system owing to the numerous functions of the liver (1). One of which is the role played in metabolism, biotransformation, synthesis and screening of biomolecules, and substances passed through it. More likely, the connection of the organ to the hepatic-portal vessels and the gastrointestinal tracts (2, 3) accounts for the role in the metabolism of almost all foreign substances (1). Hence, the numerous functions of the liver and interconnections with other organs and tissues (**figure 1**) serve as the basis of its name, ‘’the central organ of metabolic processes of the body’’. Likewise, these attributes confer a strenuous task and thereby subject the liver to injuries and diseases, which could be by direct or indirect causative factors. Clinical manifestation of hepatic injury is characterized by elevated levels of aminotransferases, alkaline phosphatase, and bilirubin. However, these parameters are dependent on the etiological factors of the injury. The supposed grouping of liver injury based on the cause is enormous due to the mechanism by which each causative agent acts. Hence, liver injury can be classified based on etiology and mechanism of action (1, 2): it is on these that incidence, prevalence, diseases types and players are reported.

2. Classification:

The etiological classification of liver injury is summarized into: chemical-induced liver injury (1), hypoxic hepatitis in intensive care unit patients (4), viral hepatitis, and autoimmune hepatitis (5). However, as etiology tends to distinguish one type of injury from the other, mechanism of action considers the paths of these injuries. Based on the later, some injuries may fall under similar categories. Though, this classification does not consider liver injury resulting from complications of other organs or systems in the body yet, primary and secondary causative factors form the basis of categorizing liver injuries.

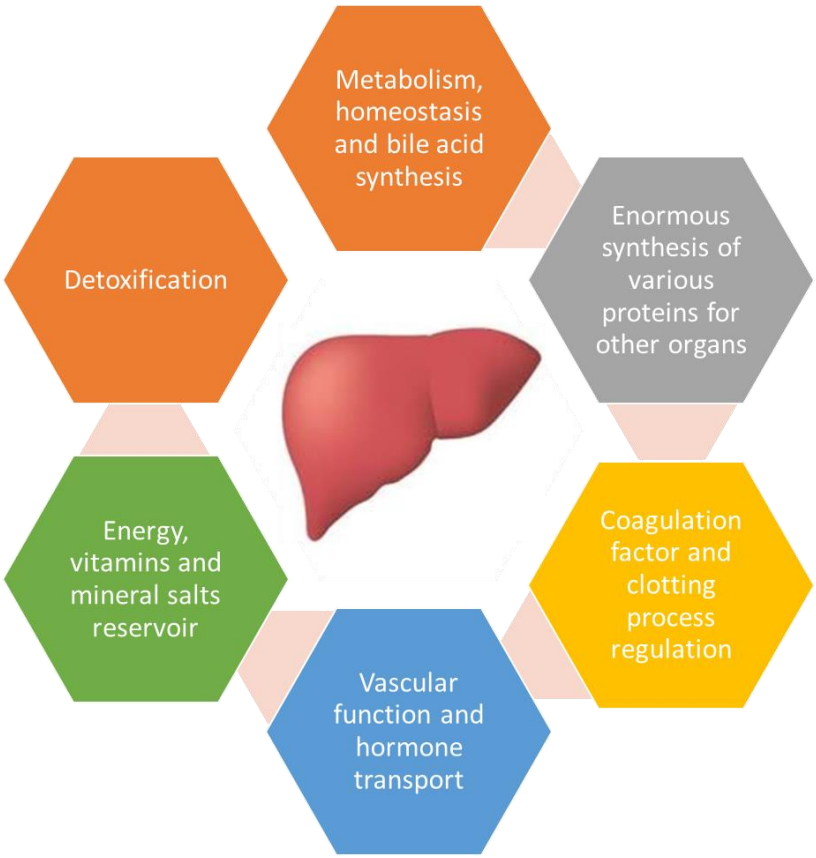


Figure 1. The Important functions of the Liver: The role of the liver owes it to its connection to numerous organs and its function in physiological and pathological conditions.

Mechanistic categorization of liver injuries tends to identify the differing routes in which causative factors of injury act. This allows investigators to specifically target each path of action and players for therapeutic interventions. Calcium homeostasis disorder, canaliculus disruption, mitochondrial injury, cytochrome P450 (CYP450) bioactivation, cell death components, and autoimmunity activation (1) are the diverse pathways implicated in liver injury.

As these pathways are involved in liver injury pathogenesis, their processes lead to liver injury progression. In drugs bioactivation leading to injury propagation, widespread cellular dysfunction is observed when reactive intermediates synthesized by CYP450 subtypes bind to cellular proteins (2). These scenarios are basically the profound characteristics of acute injury. Yet, chronic responses are initiated after acute liver diseases have set in (6) and is a result of complex interplays of different mechanisms. Hepatitis, acute liver failure (ALF), cholestasis, non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease (AFLD), steatosis, non-alcoholic steatohepatitis (NASH), inborn-errors of metabolism (Wilson's disease) and vascular injury are varying phenotypes of liver diseases observed due to complex interplays of biomolecules in the liver.

3. Pathophysiology

Liver diseases and a resulting effect to other organ dysfunction or disease have been found to exist (**figure 2**). Modification of the liver's architecture, regulation and physiology may occur due to drug interaction or intrinsic factors like hypoxia induced liver injury (7)- as the

liver produces the majority of biomolecules needed by other organs or tissues. These changes have been observed to cause coagulation abnormalities, acute kidney injury, impaired oxygenation, hepatic encephalopathy, modified immune response and altered myocardial contractility (7). Many drugs have been implicated in the idiosyncratic events of manipulating liver functions. Troglitazone, a type 2 diabetic mellitus (T2DM) drug, led to hepatic injuries (8). Drugs prescribed for diabetic patients could have hepatotoxic effects leading to increased risk of cardiovascular events (9); as an injured liver could compromise the integral secretion of plasma proteins like plasma kallikrein, needed to play roles in hemostasis. An implication is a profound effect in diabetes and cardiovascular injuries.

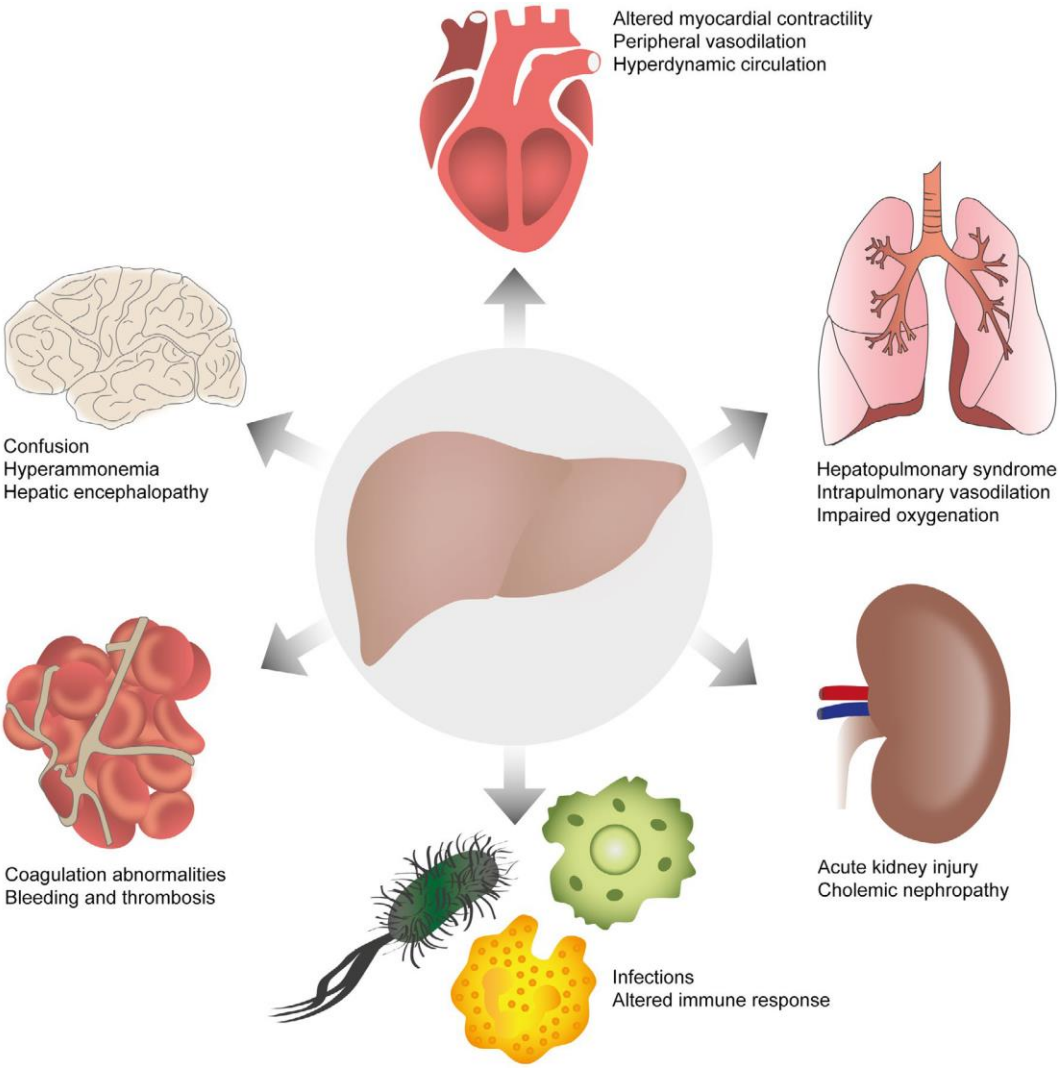


Figure 2. Schematic diagram of organ injury and failure due to liver interaction with intrinsic and extrinsic factors. Diagram was adapted from Horvatis et al., 2019 (7).

On the global scale, significant increase in morbidity and mortality are associated with 20% cases of liver injury caused by idiosyncratic drug interactions (1). Most of the epidemiological studies on liver injury or diseases were done on drug-induced liver injury (DILI) and ALF. Statistics on ALF in the United States account for about 1600 to 2000 people being affected per year. Most of these were attributed to DILI especially by

acetaminophen (8, 10). Nevertheless, a true estimate of incidence of liver injury is difficult to obtain due to asymptomatic and idiosyncratic interactions pertaining to different individuals involved (10, 11). Despite these challenges, retrospective studies in Sweden, United Kingdom, Germany, and United States identified DILI in patients of liver disease, parenchymal liver dysfunction, and medical inpatients to be 6.6%, 8.1% and 1.4% frequency among these patients respectively (12-14). An overall 4.0% incidence rate was reported in a study conducted at an Indiana community hospital involving new-onset jaundice (15).

A large variability in retrospective studies indicated the need for prospective studies. 13.9 ± 2.4 cases per 100,000 inhabitants incidence rate was observed in a three-year prospective population based studies in France (16). When applied to the United States population, ~44, 000 annual cases and at least 2700 deaths is observed with DILI. (17, 18). A current prospective cohort study conducted in Italy on DILI of 185 patients highlighted an incidence rate of 2% chronic liver diseases (19). Patients with bile duct loss were more likely to develop chronic liver injury than were those without such injury (94 vs. 47%) (20).

a. <u>Acute and chronic liver injury: propagators and cell death contribution</u>

The liver's physiological function as a detoxifier protects the body system from compounds passed through it for screening however, bioactivation by metabolizing enzymes could occur. These active substances contribute to toxicity within the liver landscape and eventually, injury. Hepatic injury of the liver occurs through the parenchymal hepatocyte and is further aggravated by the contribution of hepatic non-

parenchymal cell subtypes- the Kupffer cells, hepatic stellate cells (HSC), cholangiocytes, sinusoidal endothelial, natural killer cells and natural killer T cells (21, 22). As proposed in **figure 3**, the functions of hepatic cell subtypes, vulnerabilities and release of molecules that impact wound healing and remodeling contributes to liver injury progression and subsequently fibrosis (23).

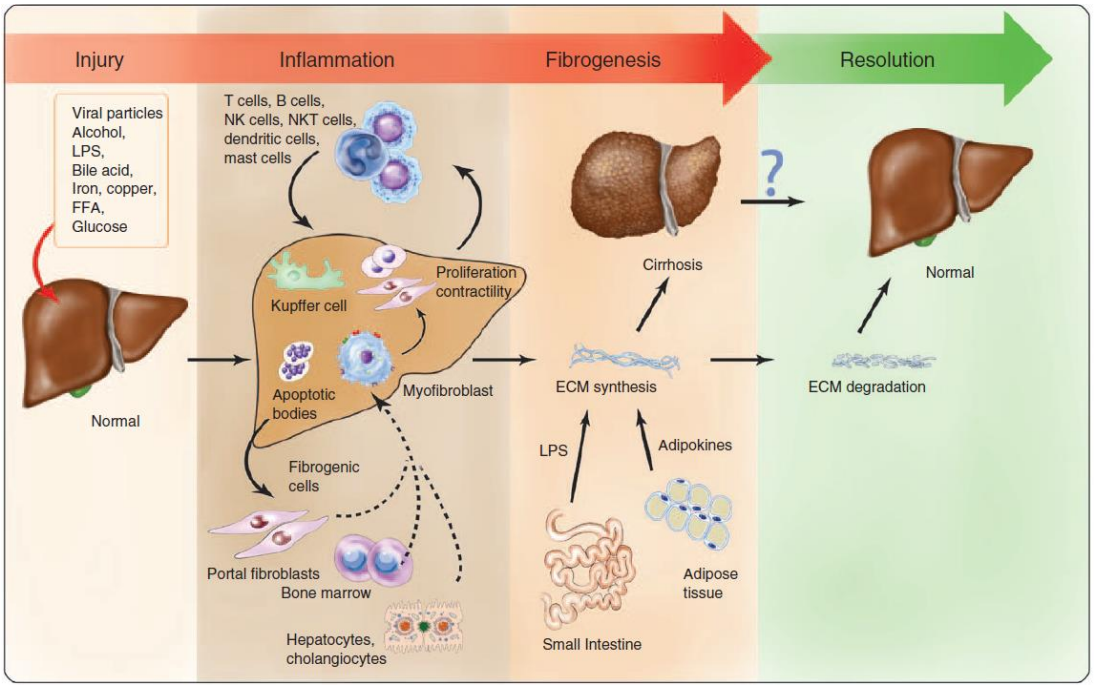


Figure 3. Illustration of liver injury and progression as propagated by many subsets of external players, hepatic cells, and released molecules. Scheme adapted from Michal Cohen-Naftaly and Scott L. Friedman 2011 (23).

Owing to these and the regenerative properties of the liver, the types of liver injury and the stages of liver diseases exist. Hepatocytes synthesize plasma proteins and coagulation factors like plasma kallikrein, thrombin, factor XII, which are upregulated in liver injury. Bile acids under toxic conditions of bile ducts blockage lead to severe

cholestasis through inflammatory-mediated repression of hepatobiliary transporters and canalicular contractility (7, 24). Steatosis results due to large lipid droplets accumulation in parenchymal cells- further insults by adipokines and advance glycated end products (AGE) ignites inflammatory mediators driven by Kupffer cells, which eventually leads to steatohepatitis (25, 26). Collagen fibers, fibronectins and elastin, the main features of liver fibrosis and cirrhosis are due to the activation of HSCs; the latter is stimulated by inflammatory and profibrogenic cytokines (23, 27, 28).

The complexity existing in liver injury due to the many players, mechanisms and contributing innate and recruited cells create numerous disease phenotypes. These consequences, from hepatitis, steatosis, and cirrhosis to autoimmune disorders are risk factors for portal hypertension, ascites, encephalopathy, cardiovascular instability, acute kidney disease, cerebral oedema infection susceptibility, and hepatocellular carcinoma (HCC) (23, 29). In a condition like type 1 diabetes, liver microangiopathy, hepatosclerosis, and glycogenopathy occur owing to morphological and physiological change within the confinement of the liver due to the preceding condition. This is a chronic form of liver injury arising from close ties with the originally affected system i.e. kidney, blood vessels and heart (30-33).

In many major mechanisms of liver injury, external substances engage possible bio-activators like CYP450 isoforms and reactive mitochondrial enzymes (monooxyenases), these later induce death molecules (FAS, TNF) by intracellular dysfunction caused by loss of ionic gradient, ATP decrease, cell swelling and rupture (1-3, 8, 34, 35). The distinction between acute and chronic hepatic injury is described in terms of cell-death and liver injury

recurrence- the presence of more necrotic cells and increased severity of liver damage occur in the former. In both, necrosis, necroptosis, apoptosis and autophagy occur (36) though, necrosis and apoptosis are the commonest form of cell-death observed (34). Inflammation is a common process observed in both acute and chronic liver injury however, the recruitment of immune cells through fibrotic factors and players have been implicated in the latter. As both injuries lead to hepatocellular damage, the biomarkers released to the blood stream during injury are used to assess liver function, however, these biomarkers are dependent on the injury subtypes. Alanine transaminases, aspartate transaminases, alkaline phosphatase, total bilirubin, creatinine are some of the biomarkers employed to assess hepatocyte. Though they indicate damage to liver cells, clinical presentations and histological examination are used to assess the disease phenotypes.

Difficulty exists in distinctly identifying the type of cell-death or intracellular death signals associated with acute and chronic liver injury due to interacting pathways. Necrosis is known to be a pronounced form of cell-death in acute and severe response while apoptosis dictate early, chronic and mild status (37). As injury is initiated in the liver, hepatocytes undergo both controlled and uncontrolled cell-death initiating inflammatory and immune response bodies like Kupffer cells, macrophages, neutrophils, lymphocytes, natural killer cells and the dendrites (35, 36, 38). Neutrophil recruitment engages many surface receptors, Lfa-1 and Mac-1, thereby stimulating ROS production. However, this could have been a defensive mechanism thereby aggravating the insults on hepatocytes-mitochondrial dysfunction and necrosis ensues (39, 40). In numerous studies, hepatic

apoptosis is considered a distinct contributor to chronic liver injury, and thereby dictate liver disease progression (41).

Mechanism of cell death by necrosis leads to hepatocyte swelling, rupturing of plasma membrane and the disruption of numerous pathways (35). Necrotic cells release nucleotides, DNA, uric acid, high mobility group box 1 (HMGB1) protein which are intracellular molecules. HMGB1, identified as a damage-associated molecular patterns (DAMP) is the most studied, could also be released by apoptotic cells (42). These molecules, recognize pattern recognition receptors (PRR) and elicit inflammatory responses (21). In addition, hepatocyte death occurs in an ATP-independent manner involving mitochondrial dysfunction (mediated by the Bcl-2 family of proteins) (35), activation of diverse proteases and phospholipases (43), and receptor interacting protein kinases (RIPK) (44). The latter, RIPK, is being shown to coordinate the necroptosis model of cell death. Besides, cell death by necroptosis is a regulated mode of necrosis with structural similarity though, its mode of initiation occurs through a kinase cascade (45, 46).

In contrast to necrosis, hepatocyte death by apoptosis is energy-dependent and regulated requiring the formation of apoptotic bodies hence, uptake by Kupffer cells and HSCs are inevitable (43). Apoptosis could be triggered by cytochrome c, death receptor ligands, growth factors, DNA damage and nucleotides (35, 43, 47). In the liver, the death receptor-signaling molecule is the major mediator, acting via Fas- Fas Ligand, tumor necrosis factor receptor 1 (TNF-R1), tumor necrosis factor alpha (TNF-α), death receptor-5 (DR-5), and tumor necrosis factor apoptosis inducing ligand (TRAIL) in disease states (21, 22). In a patient of acetaminophen-induced liver injury and fulminant liver failure, TNF- α,

TNF-R1, and Fas were elevated in the serum (38). Meanwhile, subsequent interplays between inflammatory bodies and death receptor ligands lead to the release of caspases which further contributes to a spike in apoptotic processes. The initiation of caspases 8 and 10 starts the proteolytic cascade processes leading to the involvement of many caspases like 3, 6, and 7 which are effectors and degraders of cellular proteins. All these processes contribute to cell death. Nevertheless, the involvement of caspases aggravates, and connects other processes like mitochondrial dysfunction involving the proapoptotic Bcl-2 proteins Bax and Bak; cytochrome c; and second mitochondria-derived activator of apoptosis (SMAC) to form the apoptosome thereby continuing the apoptotic cascades which leads to cell death eventually (21, 34, 35, 38). Likewise, mitochondrial dysfunction and mitochondrial outer membrane permeability (MOMP) combine with other organelle dysfunction (endoplasmic reticulum, lysosome) to form the intrinsic apoptotic pathway regulated by the Bcl family of proteins (21, 48).

Other defined hepatocyte cell death like autophagy is controlled and dependent on the lysosome for execution. The formation of an autophagosome coordinated by specific autophagy-related genes, chaperone heat shock cognate 70 (hsc70) and lysosome-associated membrane protein 2A (LAMP2A) ensures fusion and degradation in the lysosome (35). However, the major role of autophagy in the liver is still under investigation.

Investigation showing the contribution of cell death to liver injury is overwhelming and cannot be over-emphasized. However, hepatocyte cell-death in liver injury models has been defined to be the crux of the different pathways observed in liver diseases. The release

of the different DAMP molecules and reactive oxygen species (ROS) stimulates the inflammatory cascades and immune cells which further activates HSCs, the major contributor to fibrosis (under chronic injury). Still, the pathways to cell death occurrence, injury propagation, inflammation induction, immune cell infiltration and fibrosis occurrence are still being researched due to the involvement of many players.

b. Inflammation and fibrosis

An essential mechanism to be considered in several acute and chronic liver injury scenarios is inflammation (49-51). This event has been proposed to occur through necrotic and apoptotic release of DAMPs and ROS (49, 50). The consequence is the cellular production, activation and release of pro-cytokines - majorly interleukin (IL)-1β, and pro-caspases (49, 50). Numerous studies have proposed the activation of two intertwined pathways leading to the NLRP3 inflammasome build-up and subsequent diverse inflammatory signals' initiation by the action of Kupffer cells (51-53). ALI, ASH, NASH, DILI models have been studied to prove the existence and mechanism of inflammasome modulatory action in liver injury to fibrosis formation (51, 54-56). As inflammation is induced in both pathogen presence and tissue damage, clearance and repair mechanisms are reactions initiated for beneficial purposes (52). This observation in liver injury is as a result of the presence of numerous pro-and anti-inflammatory signals induced to attract immune cells *via* chemotaxis. Concurrent insults lead to a wound healing mechanism that gives birth to fibrous tissues formation in the liver as a result of HSCs activation. As schematically drawn below (**figure 4**), toll-like receptors (TLRs) activation by DAMPs

contributes to the first insult initiation process of pro-inflammatory cytokines. Through other DAMPs like ATP and ROS, subsequent activation of NRLP3 inflammasome formation, pro-caspase 1 and IL-1β occur. The invasion of neutrophils and activation of Kupffer cells eliminate the cell-deaths and debris or increase inflammation if insults re-occur (40).

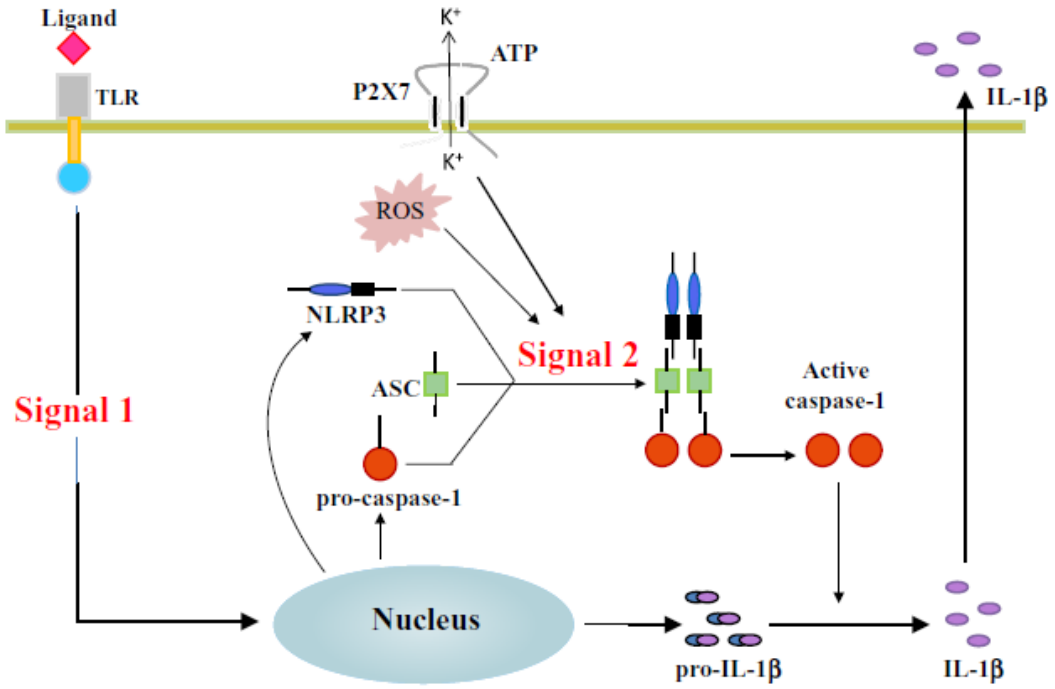


Figure 4. The organization of the inflammasome by Toll-like receptor (TLR) activation by DAMPs (Ligand 1) and subsequent ROS and ATP interaction with pro-caspase 1. Adapted from Hoque et al., 2013 (49).

Inflammatory and immune cells are important to the establishment of fibrosis in chronic liver disease. Inter-connections among these cells have been implicated in the generation of fibrous molecules and tissues. As resident macrophages are activated, they

stimulate the release of fibrogenic cytokines which contribute to the activation of HSCs into myofibroblasts-like cells (57). Regardless of etiology, extracellular matrix (ECM) degradation and generation is a feature of chronic liver injury- a wound-healing response orchestrated by the liver (58, 59). HSCs are the major contributor to liver fibrosis and subsequent cirrhosis through the contribution and expression of alpha- smooth muscle actin (ASMA), collagen type 1 and IV (Col 1α1 and Col IVα1), fibronectin, matrix metalloproteins (MMPs), Tissue inhibitors of metalloproteinases (TIMPs), several growth factors, inflammatory cytokines and chemokines and polymorphic leukocytes (28, 57, 60-64). Hepatocytes have also been implicated in liver fibrogenesis by releasing connective tissue growth factor (CTGF), a stimulator of fibrogenesis in a Transforming growth factor (TGF) -β-dependent mechanism (28, 65). The contribution of liver sinusoidal endothelial cells (LSECs) in liver injury and fibrosis is also under investigation. With evidence of infiltration of phagocytes and leukocytes through vascular tissues embedded in the liver, leakage of molecules like NO, bradykinin, adhesion molecules and myeloid cells is a possibility (liver microangiopathy), which could be likened to the phenomenon in atherosclerosis and other vascular diseases. Through sinusoid capillarization, angiogenesis, angiocrine signals, and vasoconstriction, LSEC contribute to chronic liver injury by stimulating the release of TGF-β and HSC activation (66). The role of Kupffer cell in chronic liver disease is another contribution to consider in liver fibrosis. They release numerous molecules, which influence neighboring hepatic cells contribution to liver fibrogenesis. Kupffer cells stimulate the release of pro-inflammatory IL-1β through the inhibition of peroxisome proliferated activated receptor-alpha (PPAR-α) in hepatic steatosis (25).

A well-delineated pathway to fibrogenesis and hepatic fibrosis establishment commences with a concurrent insult on the hepatocytes, the parenchymal cells able to regenerate and replace dead cells, which results in the ECM degradation and generation. To understand this mechanism of fibrogenesis, many complementary experimental and genetic animal models were studied (23, 60). Most of these models establish cell-deaths, oxidative stress and inflammation as mediators of fibrogenesis (60). However, the activation of HSCs from quiescent HSCs remains the major contributor to generation of collagen in liver fibrosis (57, 67). Activated HSCs is dictated by a myofibroblast (MFB) phenotype from mesenchymal cells and increased expression of ASMA (57, 68). The inducers of quiescent HSCs to a MFB type remain an on-going research. TGF-β, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), ROS, endothelin-1 (ET-1), nitric oxide (NO), fibronectin, insulin growth factor (IGF), apoptotic bodies, chemokines are possible stimulators of HSCs (23, 28, 57, 65, 67). Most of these are released by LSECs, phagocytic Kupffer cells and destroyed hepatocytes, which subsequently recruit inflammatory cells and activate stellate cells (23, 57, 65, 69, 70). The MFBs nature of HSCs alters the ECM of the liver architecture, impairing levels of collagens (1, III, and IV), MMPs, fibronectin, laminin, elastin, undulin, elastin, hyaluronan, proteoglycans, and TIMPs (23, 57, 67). In addition, activated HSCs express and stimulate the release of proinflammatory, profibrogenic and pro-mitogenic cytokines (71). Investigation by Kisserlva et al., around stellated cells activation observed their regression once the insult such as CCl_4 intoxication is stopped by undergoing cell-death by apoptosis and senescence (72). Also, reversion to the quiescent

state was being shown to occur (72). Further investigation showed the decline in profibrogenic cytokines like TGF-β with concurrent decline in ASMA and collagen I (72, 73).

Considering all these, liver fibrosis is a complex interplay among impaired physiological and regulatory processes occurring in other to maintain a normal functioning liver even at both transcriptional (70, 74) and epigenetic levels (71). Death-cell processes (37), inflammatory and fibrotic signals (50, 55, 75, 76), cell structure (77), coagulation responses (78), metabolic activities (79, 80), and immune cells mechanisms (55) are among investigated pathways in liver fibrosis.

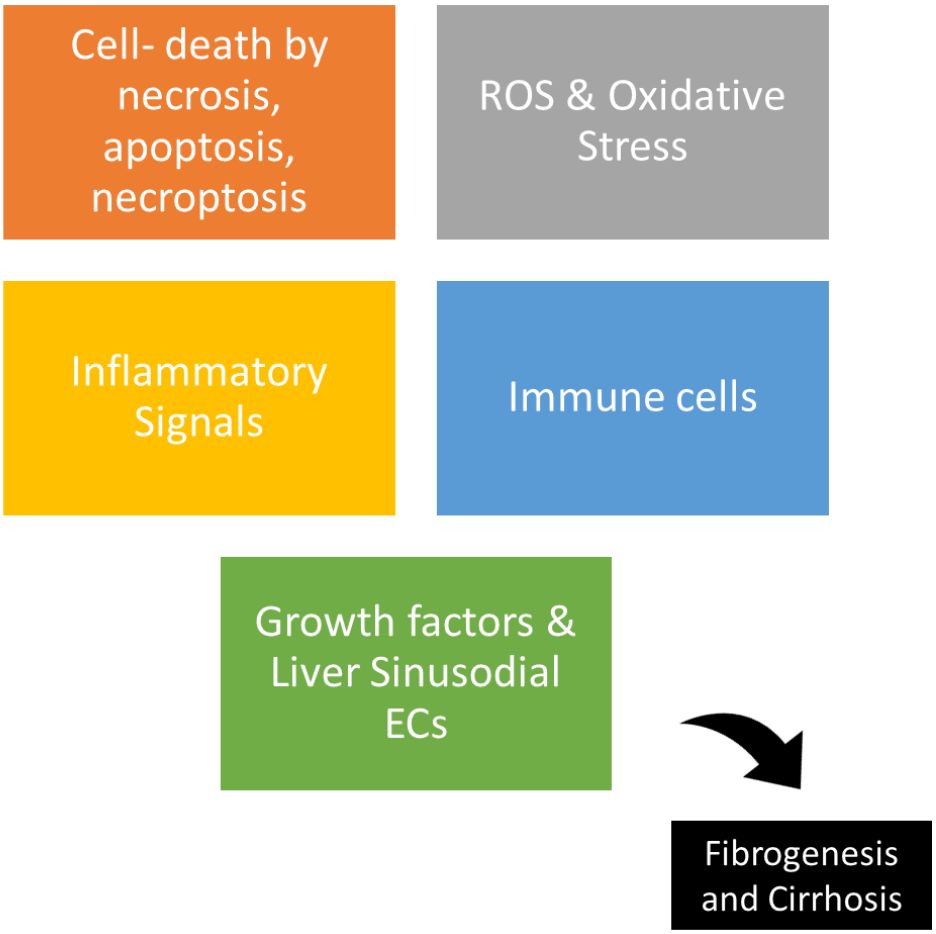


Figure 5. Mechanistic contributors of fibrogenesis and cirrhosis progression in the liver milieu.

Analysis on different players in liver fibrosis revealed the activities of members of the coagulation pathways of the cardiovascular system. Previous studies of chronic inflammation and tissue remodeling have highlighted the role of the kallikrein-kinin system (KKS) (81). Protease-activated receptors (PARs) 1 and 2 (82-87), and bradykinin (BK) receptor 2 (B2R) are upregulated with a possible involvement of the latter in resolution of liver fibrosis (81). However, some studies implicated B2R in liver injury while BK receptor

1 (B1R) was shown to resolve liver injury (88, 89). The cleaved fragment of high molecular-weight kininogen (HMWK), bradykinin (81), is a possible agonist of B2R in liver fibrosis though investigations are incomplete. Possible agonists of PARs 1 and 2 studied over the past years in other systems are thrombin, tissue factor, trypsin, mast tryptase, coagulation factors Xa and VIIa and plasma pre-kallikrein (PPK) (90, 91). Most investigations suggest the roles of other mentioned ligands for PARs except PPK due to their upregulation in chronic liver injury. Although PPK is observed to be upregulated in acute liver injury (92) and downregulated in liver fibrosis, its role in both cases is still to be determined.

B. The plasma kallikrein-kinin system

The KKS is thought to play diverse roles in bodily functions. Supported by numerous investigations and evidences, this system modulates physiological and pathophysiological actions pertaining to neutrophil aggregation, coagulation, vasodilation, inflammation, complement activation and vascular tone (93-96). Comprising pre-kallikrein, kininogen (high- and low- molecular weight), kinins, kininases and their receptors, the actions of this system modulate cardio-vasculature basically through the potent effector BK (96). Plasma kallikrein (PK), the active form of PPK and high molecular weight kininogen (HMWK) make up the plasma KKS while tissue KKS comprises the action of tissue kallikrein (TK) on low-molecular weight kininogen (LMWK). Both PK and TK are the main initiator of the KKS through the kininogens (96, 97) though, BK and its related-peptides (Lys-BK, des-Arg^9-BK and des-Arg^9-Lys-BK) are the bioactive kinin molecules

cleaved from kininogen by the kallikreins (94). Recent researches also show the kallikreins acting through the PARs (98) and B2R (99). The kinins are formed by both the actions of kallikreins (PK and TK) and kininases I (aminopeptidase M (AM), carboxypeptidases N and M(CP)) (93) while Kininase II (angiotensin I-converting enzyme, ACE) is involved in the hydrolysis of these peptides (95).

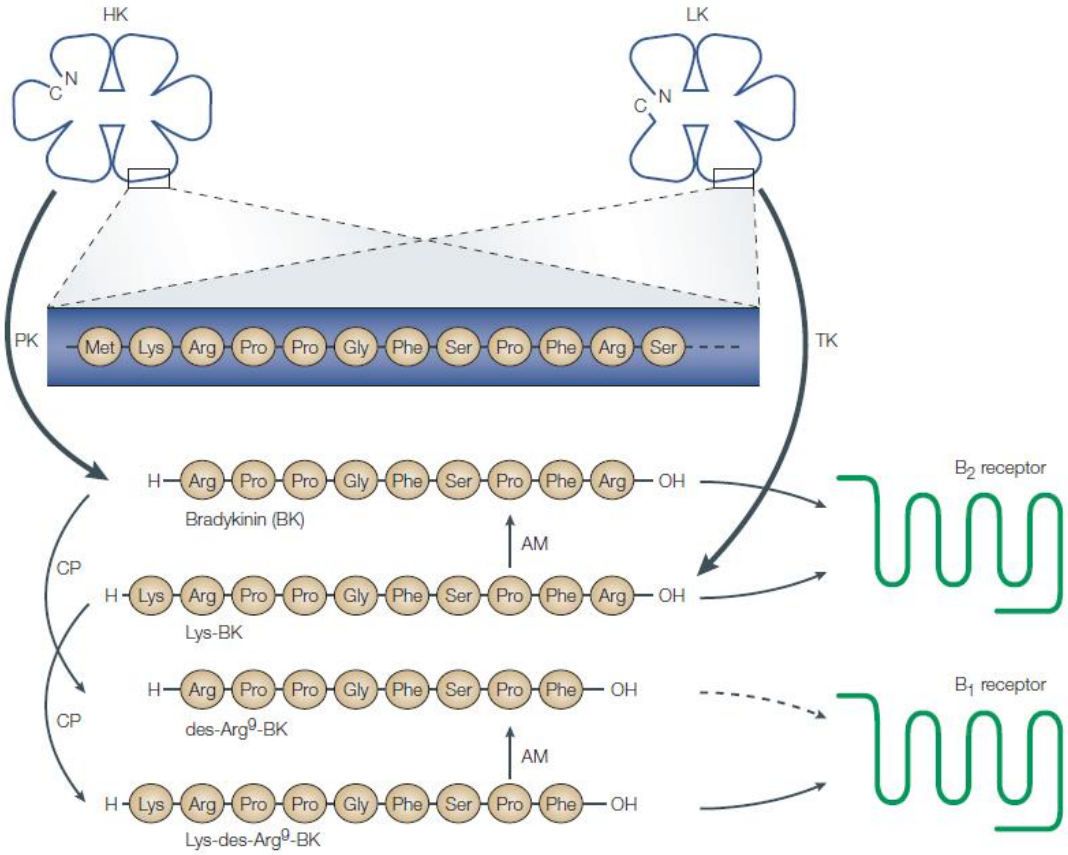


Figure 6. Schematic presentation of the kallikrein-kinin system (KKS). The diagram depicts the kallikreins, players in the activation and deactivation of the physiological kinins, and the B1 and B2 receptors. HK, High molecular weight kininogen; LK, Low molecular weight kininogen; AM, aminopeptidase M; CP, carboxypeptidase N and M. Adapted from Marceau and Regoli, 2004 (100).

Bradykinin release on endothelial cells facilitates vasodilation by regulating blood flow and pressure (101), angiogenesis (97), proinflammation (102) although, it plays a role in vascular or endothelial permeability (95), hypertension pathogenesis (96), mitogenesis (103) and hereditary angioedema (104). Nonetheless, the process of vasodilation by BK leads to the synthesis and action of other vasodilators like NO, prostaglandin (PG) E_2 (PGE_2) and PGI_2 (100). In normal circumstances, BK mediates its action through one of its receptors, while activating the other induced receptor during abnormal situations. As an antithrombin agent, BK simulates B2R leading to inhibition of platelet aggregation (105) - a role also played by des-Arg^9-BK, NO and PGI_2 (105, 106).

1. The bradykinin receptors 1 and 2 (B1R and B2R) and liver injury

To date, two receptors of BK have been discovered and explored, the B1R and B2R (107, 108). Encoded by separate genes, these receptors have been largely explored due to the beneficial and detrimental roles played in normal functions and diseases of the body system. These receptors are G-protein coupled receptors (GPCRs) mediating the actions of kinins through the activation of phospholipase C (PLC) and the mobilization of intracellular calcium (Ca^{2+}) (100) although PK could also act through B2R.

BK and other kinins moderate their effects of vascular tone, mitogenesis, inflammation, neovascularization and coagulation through the bradykinin receptors 1 and 2 (B1R and B2R) (95-97, 103). Indeed Lys-BK and des-Arg^9-Lys-BK, which are liberated from the actions of TK and CP on LMWK and Lys-BK respectively, interact with B1R and

B2R. In additional, B1R has been shown to have strong affinity for the arginine CP (des-Arg^9-BK is liberated from BK by CP as well) generated kinins (94). Under normal condition, B2R is constitutively expressed in many tissues and involved in vasodilation while the B1 receptor is induced in pro-inflammatory conditions of cytokines, endotoxins and tissue injury actions (103). Also, the B2 receptor has been shown to play protective roles on cardiac pathologies. In a pathological state of liver function i.e. liver fibrosis, bradykinin was shown to play a protective role of hindering liver damage by acting through the B2 receptor (81). Whereas, in a model of liver injury by trichloroethylene sensitization, both B1R and B2R contributed to increased liver injury (89). This studies show differing implications of bradykinin receptors in liver injury or fibrosis in mice based on the causative factor, the injury model or disease phenotype. PK is the only proven protease involved in the cleavage of kininogen to BK yet, these studies did not shown any relationship or interaction in the liver disease models investigated.

2. The protease-activated receptors

Although bradykinin and its related-peptides are the major substrates for the B1R and B2R, PK has been shown to directly activate transfected human B2 receptor in the absence of cell-bound kininogen carried-out in a Chinese hamster ovary (CHO) cell line (99). This indicates that PK's role in body function is not limited to its proteolytic activities as a serine protease. Normally, PK's activity anecdotally probed in many systems showed the activation of thrombin and trypsin receptors PAR-1, 2 and 4. In a study by Abdallah et al. 2010, PK was observed to cleave and internalize PAR-1, 2 and 4 in HEK293 cells (98).

Like bradykinin receptors, PARs are GPCRs encoded by separate genes, the F2R, F2Rl1, F2Rl2 and F2Rl3 genes for PAR-1, PAR-2, PAR-3, and PAR-4 respectively (91). Mostly, proteases that cleave PARs do so at specific cleavage sites and sequences (**figure 6**), the hirudin site, which eventually induce conformational and affinity changes that activates signaling mechanisms through intracellular G proteins (91, 109). PARs cleavage by numerous types of proteases, allow for biased signaling due to the various conformations initiated and the recruitment of preferred specific G proteins (110, 111). Moreover, as PARs have activators, they have deactivators. PARs could be cleaved at specific N-terminal regions that render them unresponsive. One study observed the shedding of a specific cleavage site initially responsive to thrombin however, this region became irresponsive to thrombin agonist when tested. Yet, they responded to ligands from adjacent PARs (112).

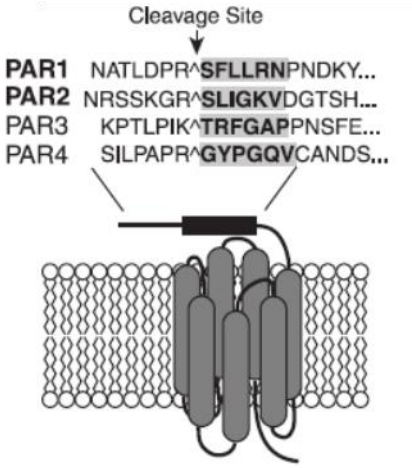


Figure 7: Representative cleavage sites of PARs ligands especially PK at hirudin site. Adapted from Abdallah et al., 2010, (98).

PARs are found expressed in lots of body cells, tissues. Physiologically, they play a major role in hemostasis, wound healing, pain, cellular proliferation and inflammation (110). Due to their functional roles, they have proportionately been implicated in varying diseases like diabetes (113), liver fibrosis, steatohepatitis (83), inflammation and obesity (114), asthma, cardiac injury (115, 116), cancer(90), arthritis (90), atopic dermatitis (117), and lung fibrosis (118).

3. PARs in liver injury/fibrosis

Both PAR-1 and 2 have been shown to have functional roles in liver fibrosis. Many experimentally induced liver fibrosis observed the reduction in liver fibrosis when mice knockouts of PAR-1 and PAR-2 were used. In a PAR-1 knockout model of C57BL/6J mice, liver injury was not affected in PAR-1 $^{-/-}$, PAR-1 $^{+/-}$ and PAR-1 $^{+/+}$ mice nonetheless, the extent of fibrosis was reduced by 56% in PAR-1 $^{-/-}$ compared to PAR-1 $^{+/+}$ mice (84). In addition, alpha-smooth muscle actin (ASMA) cells decreased by 80% suggesting a role played by PAR-1 in the pathogenesis of liver fibrosis (84). Similar role and function was observed in PAR-2 $^{-/-}$ mice after eight weeks of CCl_4 treatment (85). Although PAR-1's modulation and complicity in liver fibrosis was studied in the past, current studies also implicate PAR-2 in the progression of liver fibrosis to cirrhosis. Due to the induction of these receptors by many agonists, investigations are on-going to really ascertain the main culprits in liver injury. Earlier studies have associated tissue factor and thrombin to the activation of protease activated receptors in NAFLD (119-121). Most compelling evidences stems from thrombin-factor Xa induced-PAR-1 playing a role in tissue fibrogenesis while

mast cell tryptase and factor Xa acts through PAR-2 to cause fibroblast proliferation, differentiation and migration (85, 122-124). In additional, experimental and human studies of acute and chronic liver disease showed the upregulation of thrombin, factor Xa and tissue factor therefore implicating them as inducers of the PARs in liver fibrogenesis and fibrosis progression (125, 126). A research on the association of necroinflammation and extent of fibrosis with prothrombic conditions in 90 patients with chronic hepatitis B showed an independent association of thrombin with fibrosis (125, 127).

Another modulator of the PAR system is PK. However, investigation on PK's impact in liver injury has been short coming due to its translational decrease in liver fibrosis. However, a research on the function in liver injury showed the proteolytic cleavage of TGF-β in part by PK; TGF-β happens to be significant driver of liver fibrogenesis and the most potent pro-fibrotic player studied (92, 128). In this recent research, PK-cleaved TGF- β in HEK293 cells released a product that could be detected in liver disease patients and many mouse liver injury models (58, 92, 128). These studies revealed the function of PK via TGF-β in murine models of liver disease however, researches regarding PK actions through PARs, B2R and BK are yet to be shown.

C. Diabetes mellitus and the liver

Emerging evidence revealed the modulation of the function of liver by hyperglycemia. In a particular study, inflammatory cells, cytokines and hepatic transaminase levels were significantly increased (129). In addition, other investigation also

observed changes in the liver of diabetic rats based on oxidative damage, metabolic profiles, hydroxyl radicals and mitochondrial alterations (130-134). Many researches have studied the risk of nonalcoholic fatty liver disease (NAFLD) in diabetes mellitus; with fewer studies on their relationships (135-137). Patients with diabetes have shown manifestation of NAFLD however, the mechanism of liver damage due to hyperglycemia in these patients remains unclear (32, 129).

Furthermore, numerous phenotypes of diabetic-induced liver damage have been revealed over the past few years. These associated diseases like hepatic diabetic microangiopathy (31, 32, 138), glycogenic hepatopathy (30, 139, 140), Mauriac syndrome (141, 142) are due to hepatic complications of chronic exposure of the liver to high glucose. Type 2 diabetes has been associated with an increased risk of liver cirrhosis in various large cohort study (143-145). Targher et al., 2010 revealed the association between NAFLD and type 1 diabetes mellitus among subjects having a higher prevalence of cardiovascular disease (146). In most of the investigated patients with TIDM, hepatic fibrosis was detected after liver biopsy was done (147). Moreover, numerous clinical and experimental research showed that liver fibrosis could result due to long term diabetes. All these suggest a complication of bodily organ functions due to hyperglycemia's induction of a compromise in liver functions; with CVD being a major risk because of its direct interaction to the liver.

Although, mechanisms driving the complications of diabetes-induced hepatic injury are yet to be established, some factors of oxidation, inflammation, fibrosis, coagulation and cell-death which have been studied in liver injury and fibrosis could be enquired. A study on experimental type 1 diabetes using streptozotocin-induced hyperglycemia focused on

the effects of regulatory T cells (Tregs) in the liver based on previous researches that had established Tregs' loss in hepatic inflammation (129). Some other studies had looked at roles of antioxidants (148); signal transducer and activator of transcription factor (STAT1 and activating transcription factor (ATF3) (149); NF-kβ (150); microsomal prostaglandin E synthase 2 (mPES2) (151); and hematopoietic tissue factor induced-PAR-2 signaling (152) in diabetes mellitus effects on liver.

Furthermore, studies involving T2DM in ten subjects, showed that NO and BK had lower concentration with a decreased PK activity however, a higher DesArg-BK concentration was detected in plasma (153). More recent studies associated PK activity to time duration of T1DM, one of the study observed a reduced PK activity in diabetic nephropathic patients (154); while others targeted PK using inhibitors as a possible treatment for diabetic macular edema (155). while also pharmaceutically and therapeutically targeting different members of the PK-kinin system (156). However, less studies have been conducted on experimental models of DM and liver injury with basis on the plasma kallikrein-kinin system.

CHAPTER II

AIMS OF THE STUDY

The aim of this study is to understand the roles of the plasma kallikrein-kinin system in liver injury knowing fully well that this system has been investigated and observed to play diverse functions in coagulation, inflammation and injury. Emphasis will be placed upon PK and its action through its receptors. Based on past reports, the KKS has contributed to chronic liver injuries and vascular dysfunction through their receptors. On-going investigations involving T1DM have shown the increased activity of PK in patients without complications however, as discussed previously, this activity decreased with an underlying complication like diabetic nephropathy.

Based on this, we will establish our objectives on the following questions:

1. Does plasma kallikrein and bradykinin interact with their receptors in liver injury to affect or alter inflammatory and fibrotic processes knowing that these receptors have been implicated in liver fibrosis?
2. Is there a possibility of PK or BK being used or targeted as a supposed therapeutic agent in liver injury or fibrosis?
3. How does this system contribute to other disease-state like T1DM in the liver?

To carry out these hypotheses, the following aims will be examined:

1. Determine the correlation of liver injury, inflammation and fibrotic factors to expressed genes of the plasma kallikrein-kinin system in 10-12 weeks old C57BL/6J mice using the carbon tetrachloride (CCl_4) model of fibrosis
2. Assess whether liver injury induced by carbon tetrachloride (CCl_4) will modulate the expression of components of the kallikrein-kinin system in type 1 diabetic mice.
3. Investigate the PK/BK-related mechanistic pathway in Human hepatocellular carcinoma HepG2.

CHAPTER III

MATERIALS AND METHODS

A. *In Vivo* Experiments:

10-12 weeks old male C57BL/6J mice were used for the experiments. Mice were obtained from the Animal Facility of the American University of Beirut. Protocols were approved by the Institutional Animal care and Use Committee (IACUC)

1. Acute liver injury in mice:

Acute liver injury was induced by a single-intraperitoneal (i.p.) injection of diluted (1/10) 0.6 mL/kg carbon tetrachloride (CCl_4) (Sigma, 270652) in mineral oil (MO) (Sigma, 8042-47-5). The mice were sacrificed by cervical dislocation and liver tissues were harvested after one, two and three days (**figure 8**).

2. Liver fibrosis:

Different time-points (2.5 weeks, 4 weeks, 6 weeks and 7 weeks) of chronic liver injuries were induced by i.p. injection of diluted (1/10) of 0.6 mL/kg CCl_4 in MO, twice a week. After the last injection of CCl_4, mice were sacrificed after one and three days (**figure 9**).

3. Type 1 diabetes mellitus:

Type 1 Diabetes Mellitus (T1DM) was induced by i.p. injection of 0.05 mg/g of streptozotocin (STZ; Sigma, S0130-G) dissolved in freshly prepared cold 0.1 M sodium citrate (Na Citrate) buffer (pH 4.5), for three consecutive days. Diabetes was confirmed by fasting blood glucose level, through the tail vein using the Performa glucometer (ACCU-CHECK Performa, 04680430003) and Performa test strips (ACCU-CHECK Performa, 06454011). When necessary, a fourth or fifth day injection was given. All mice were allowed to rest for 10-14 days and hyperglycemia was monitored with blood glucose test, to ascertain the stability of high glucose level.

4. Liver injury on mild hyperglycemic mice:

After the establishment of mild hyperglycemia which was determined by statistical analysis, all mice, including the controls, were divided into four groups: vehicle, CCl_4, Diabetes, Diabetes + CCl_4. Each of the 4 groups was divided into two sub-groups. The first sub-group, vehicle or CCl_4 injections were given once to induce acute liver injury (one-time CCl_4 injection). The chronic injury was established in the second sub-group by 2.5 weeks of CCl_4 injection. The acute CCl_4-treated group was sacrificed at 48 hours while the chronic CCl_4-treated group was sacrificed at 24 hours and 72 hours post last CCl_4 injection.

Treatments	**Acute Injury on Diabetes (n=6 each)**	**Chronic Injury on Diabetes**
Mineral Oil	Vehicle	Vehicle
CCl_4	CCl_4	CCl_4
Mineral Oil	Diabetes	Diabetes
CCl_4	Diabetes + CCl_4	Diabetes + CCl_4

5. Histological and immunohistochemical experiments:

Upon sacrifice, mice were dissected and 4 parts were removed from four liver lobes and placed in 10% formaldehyde. Tissues were embedded in paraffin and cut at 4-µm thickness on microscopic slides at the Histology Laboratory, Department of Anatomy, Cell Biology and Physiological Science, American University of Beirut.

a. Hematoxylin and Eosin (H&E) and Picro Sirius Red (PSR):

The H&E staining was performed to detect liver injury. The PSR staining was performed for the detection of collagen fibers. Both staining were conducted on 4-µm thick formalin-fixed paraffin-embedded tissue. Images were taken at 40x magnification. Morphometric pixel analysis was performed on 10 non-overlapping randomly chosen fields per mouse, using ImageJ software (NIH, USA).

b. Immunohistochemistry (IHC):

IHC was carried out for PK on paraffin-embedded mouse liver tissue sections using a rabbit polyclonal antibody anti-PK (Invitrogen). Before this, blocking for endogenous hydrogen peroxidase was done using 3% hydrogen peroxide in tris buffered saline (TBS). Subsequent blocking was performed by utilizing goat serum. After the application of the primary antibody, Biotinylated goat anti-rabbit secondary antibodies were added for an hour while staining using DAB and counterstaining with hematoxylin (Leica Biosystem) were carried out as described previously (157). Images were taken at 40x magnification. PK positive area from all mice (ten fields per mouse) were quantified with ImageJ by utilizing the image, adjust and color threshold tabs. No staining was observed when omitting the primary antibody.

6. Gene expression profiles:

a. RNA extraction:

For each mouse, 2 samples from the median and left lobes were snap frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted using Qiazol Lysis reagent (Qiagen, Hilden, Germany, 79306) and homogenized using a Tissue Lyser (QIAGEN II) and transferred to RNAeasy mini columns. After extraction of total RNA, the pellet was re-suspended in 60 µL RNase/DNase free water. RNA quantification was measured using a Nanodrop Spectrophotometer (Thermo Fisher Scientific).

b. Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR):

The High Capacity Reverse Transcriptase kit (Thermo Fisher Scientifics, 004007363) for RNA reverse transcription to cDNA was performed according to the manufacturer's instruction. 2 µg of the total extracted RNA was reverse transcribed into cDNA in a final volume of 20 µL. The reaction mix done in a BioRad T100 Thermo Cycler machine (Bio-Rad laboratories, California, USA), according to the manufacturer's instructions. The conditions for the experiment was set at: 25°C for 10 minutes, 37°C for 2 hours, 85°C for 5 minutes and finally at 4°C.

Using the iTaq™ Universal SYBR Green Supermix (Bio-Rad Laboratories, 1725121), polymerase chain reactions (PCR) were done on the obtained samples in a CFX384 system (Bio-Rad Laboratories, California, USA):

The primers were previously described (157) and the oligonucleotide sequences were synthesized and purchased from Macrogen Inc. Before usage, they were centrifuged at 1,000 rpm for 1 minute, reconstituted, and a working solution of 50 µM was prepared.

For each reaction mix, 5 µL of SYBR Green Supermix, 2.3 µL of DNase/RNase free water and 0.1 µL each of forward/reverse primers were added into each tube of 2.5 µL cDNA sample. Results were calculated using the ΔΔCT method and normalized to the housekeeping gene 18S.

B. *In Vitro* Experiments:

1. Hepatocellular Carcinoma cell line, culture, and treatment

a. Cell culture:

Hepatocellular carcinoma cell line, HepG2, was received from ATCC, Virginia, USA. All necessary procedures from storage to culturing were according to the guidelines of ATCC. The cells were cultured in low glucose Dulbecco's media (DMEM, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (complete media), and incubated at 37C for two days. The media was changed at day 3 while the cells were split into three flasks at day 5 after a confluence of about 90% was reached.

b. Treatment:

Cells were plated in either 96 well- or 12- well plates depending on the experiment to be performed. CCl_4, Staurosporine (STS, Calbiochem), and PK were used as treatment conditions. When necessary, PAR-1, PAR-2, and B2R antagonists were used 30 minutes before treatment conditions were applied. The experiments were divided into the following parts:

i. MTT assay:

MTT assay was performed on HepG2 cells after treating cells with CCl_4 or STS or PK. For these experimental studies, HepG2 cells were plated in 96-well plate to perform cytotoxicity test and proliferation rate for different concentrations of CCl_4, and STS treatments. In a quintuplet for each concentration and conditions, 100,000 cells/ well were

plated for cytotoxicity assay using MTT as described previously while 10,000 cells/well were plated for the proliferation assay. Briefly, the cells were plated in low DMEM media supplemented with 10% FBS and 1% P/S and treated for 24 hours. Various treatment concentrations of CCl_4 in 0.5% DMSO (1 mM, 2 mM, 4 mM and 8 mM) and STS (50 nM, 100 nM and 1 μM were used. Media was aspirated and treated with 10 μL MTT solution and 90 μL low DMEM media, supplemented with 10% FBS and 1% P/S, and treated for 120 minutes. The plate was kept under the dark in the cell culture incubator at 37 °C and 5% CO_2. 150 uL of stop solution (MTT assay) was added in each well. The absorbance was checked at 595 nm wavelength. The percentage cytotoxicity and proliferation rate were calculated. The 1 mM CCl_4 in 0.5% DMSO, and 1 μM for STS, were used for the subsequent experiments.

% Cytotoxicity = 100 – [(Sample- Background/ Control Sample) * 100]

% Proliferation = (Sample- Background) * 100

ii. Cell death and PK study:

400,000 cells/well were plated in 12-wells plates for RNA extraction. After 24 hours, the media was changed and replaced with low glucose complete DMEM media but with a 5% FBS. The conditions: vehicle (media only), 1 mM CCl_4 in 0.5% DMSO, and 1 μM STS were added in triplicates and the experiment was stopped after24 hours. For the PK study, the cells were treated with 2.5 ng/mL PK.

Receptors inhibition: Receptor antagonists of PAR-1 (SCH530348), PAR-2 (GB83) and B2R (HOE-140) were purchased from Axon Medchem BV (Cedarlane, Canada), and where used at 0.5 μM, 2 μM and 1 μM, respectively. The inhibitors were diluted in low glucose complete DMEM media but with a 5% FBS from the initial stock of 2 μM and 1.82 μM respectively and added to the cells prior to 30 minutes stimulation with PK. The cells were harvested after 24 hours.

c. Total RNA extraction and RT-qPCR:

Treatments (Paragraph II) were stopped by washing the plates with Phosphate Buffer Saline (PBS; without Ca^{2+}/Mg^{2+}; Sigma-Aldrich) and aspirated after one minute. According to the manufacturer's protocol, 500 μL of TRIzol™ Reagent (Ambion; Life Technologies) was added in each well for 30 minutes and scraped thoroughly. The cell lysates were transferred into Eppendorf tubes and were vortexed for about 30 seconds. 100 μl of chloroform were pipetted into each tube, and vortexed to ensure homogeneity. The tubes which were incubated for 5 minutes at room temperature, were centrifuged for 15 minutes at 12,000 × g at 4°C. The aqueous phases (RNA) were collected and transferred into new Eppendorf tubes. 200 μL of isopropanol was added for precipitation, vortexed again for 15 seconds, and left at room temperature for 10 minutes. Samples were centrifuged for 15 minutes at 12,000 × g at 4°C, and the appeared white gel-like pellets (RNA) were submersed in 400 μL of 75% ethanol while the supernatants were discarded. This was followed by centrifugation for 5 minutes at 7,500 × g at 4°C. The latter step was repeated with 75% ethanol and 7,500 × g at 4°C centrifugation after removing the supernatant. To

guarantee the elimination of the ethanol in the samples, the tubes were left to air-dry under the hood at room temperature for 15 minutes. Finally, 20 μL of DEPC- treated DNase/RNase-free water was added to the RNA samples, and were placed at 60°C for 5 minutes. Furthermore, to evaluate the purity and concentration of the precipitated RNA, the samples were placed on ice and the absorbance of 1 μL of each sample and DNase/RNase-free water (used as blank) were measured using the DeNovix DS-11FX Spectrophotometer. Lastly, the RNA samples were stored at -80°C. The protocol for real time quantitative polymerase has been described above (*In vivo*, 6b) likewise, the results were calculated using the ΔΔCT method but normalized to the housekeeping gene *Gapdh*.

C. Statistical analysis:

Statistical analysis was done using GraphPad Prism 8, (version 8.4.3 for windows, GraphPad software, La Jolla, CA 92037, USA). Multiple comparisons between groups were done using One-Way Analysis of Variances (ANOVA) or (Mann-Whitney U test), $p<0.05$ was considered statistically significant. Also, correlation analysis where carried out by Spearman correlation of non-parametric test.

CHAPTER IV

RESULTS

A. PK and PAR-1 plays a role in inflammation and activation of neutrophil in acute liver injury:

1. Experimental design:

We studied the role of the PKKS in acute liver injury by the treatment of C57BL/6J mice with one single injection of 0.6 mL/Kg of CCl_4 or vehicle (MO). Mice were sacrificed after one, two or three days (**figure 8**).

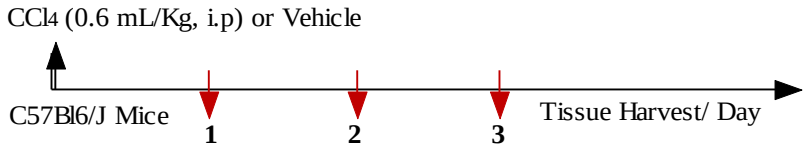


Figure 8. Schematic representation of CCl_4-induced acute liver injury. C57BL/6J male mice (10-12 weeks old) were treated with vehicle (mineral oil) or 0.6 mL/Kg CCl_4 i.p. once. The mice were separated into groups to be sacrificed at three time-points, day 1, 2 and 3 (n=7 for vehicle and n=7 for CCl_4 per group).

2. Acute liver injury assessment:

Acute liver injury was assessed to determine the induction of hepatic injury by H&E staining of liver sections. The necrotic area is presented by the delineated area of liver section as shown in the images in **figure 9a**, while the quantified sections in **figure 9b**. Quantification was done using the ImageJ software. Briefly, Images opened on the ImageJ

software were marked and drawn by freehand selection around the bothers of the necrotic areas (necrosis was determined by lighter color and hepatocyte absent areas). The selected parts were measured and the area fraction calculated to the total area of each image. There was a 45.1% increase in cell death by necrosis after one day CCl_4 treatment compared to vehicle however, a gradual decrease in necrotic area of 39.3% and 22.9% was observed from day one to two, and two to three days respectively (**figure 9b**).

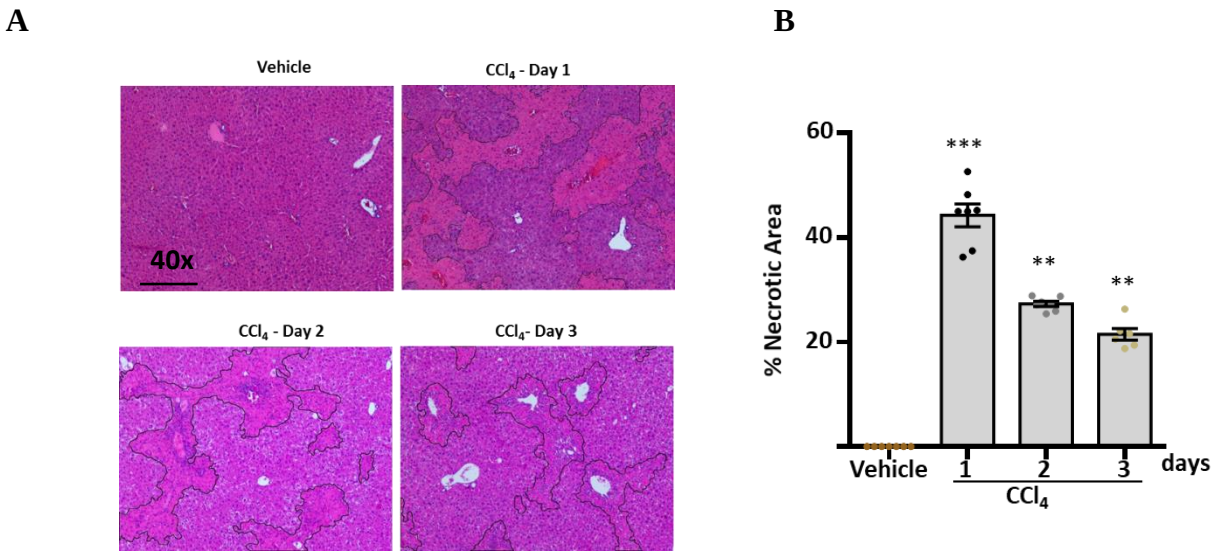


Figure 9. Carbon tetrachloride (CCl_4)-induced acute liver injury in mice shows gradual decrease in injury from day 1 to 3. (A) Schematic representation of CCl_4-induced acute liver injury. C57BL/6J male mice (10-12 weeks old) were treated once with vehicle (MO) or 0.6 mL/Kg CCl_4 i.p and sacrificed at day 1, 2, and 3. (B) Representative hematoxylin and eosin (H&E, original magnification x40) staining of liver sections. Necrotic areas are delineated by marked areas. (C) Quantification of necrotic areas using ImageJ software. Data are shown as mean ± SEM (n=7 per group); Statistical significance was determined by Mann-Whitney test with **p<0.005, ***p<0.0005, (CCl_4 versus Vehicle).

3. Time-points assessment of the PKKS genes and thrombin:

CCl_4 treatment of mice resulted in the 42.5% decrease in mRNA expression of the *Klkb1,* gene at day 1 which later increased at day 2 and 3 of analysis (**figure 10**). A statistical increase was observed in gene expression at day 3 when compared to vehicle. Since PK activation have been shown to modulate the action of kininogen and PAR-1, we assessed the gene expression of their genes. **Figure 10** shows similar pattern of expression as seen with the PK gene (*Klkb1*). As observed, there was a 51.92% and 16.67% decrease in day 1 mRNA levels of Kininogen and PAR-1 genes respectively. *F2rl1, Bdkrb1* and *Bdkrb2* genes were not induced.

Since PAR-1 has numerous ligands of which kallikrein is a part, we also carried out RT-qPCR experiment on the thrombin gene. We noticed a similar pattern of gene expression to PAR-1 however, mRNA levels at day 1 increased instead of a decrease, as observed with PAR-1 (**figure 10**).

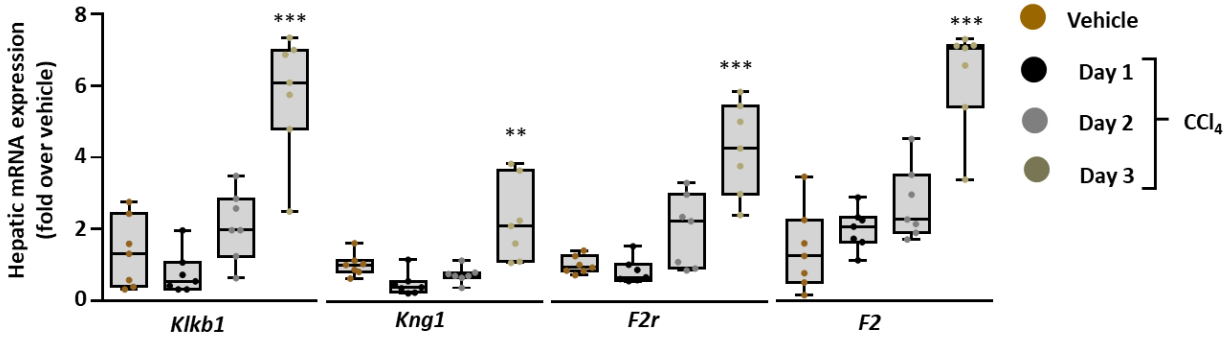
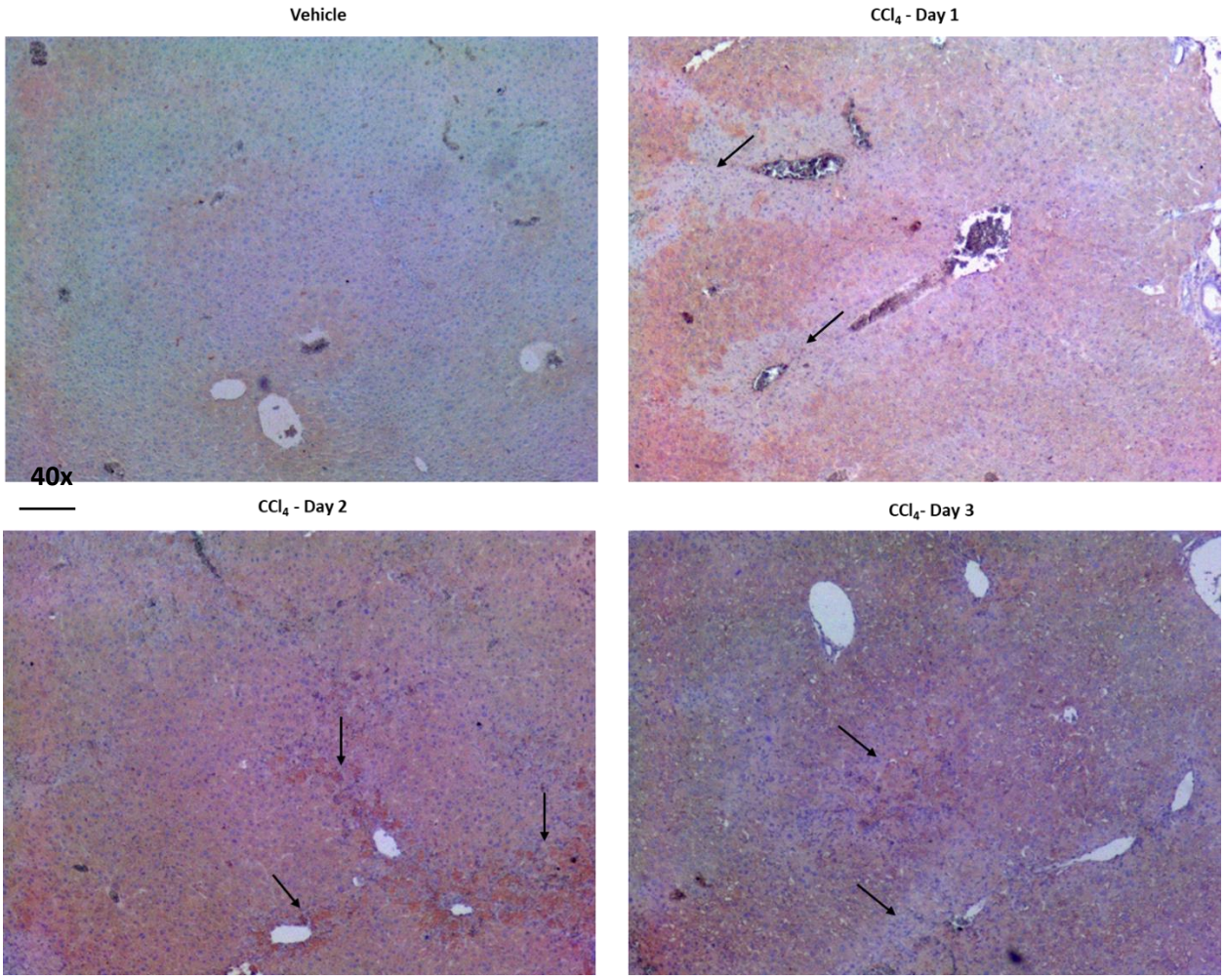


Figure 10: **Pronounced modification of hepatic PKKS genes in acutely injured mice**. RT-qPCR expression analysis showed a modification to the *Klkb1, Kng1, F2r* and *F2* genes over the time-points assessed. Data are shown as minimum to maximum values of boxplots (n = 7); Statistical significance was determined by one way ANOVA * $p<0.05$; **$p<0.005$; ***$p<0.0005$ (CCl_4 versus Vehicle).

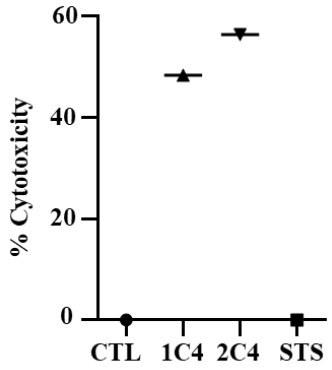
4. Klkb1 gene is translated to the pre-kallikrein protein and PK increases the proliferation of hepG2 cells:

We next assessed the translatory possibility of the prekallikrein gene to the protein. By immunohistochemical analysis, the pre-kallikrein protein was present in a similar pattern of expression to the *Klkb1* gene in the time-points analyzed. It decreased at day 1 (**figure 11 A; black arrows, PPK is absent at necrotic areas**) and was observed to increase at day 2 around the injured areas in clusters whereas a decrease in these clusters seems to occur at day 3 (**figure 11A; black arrows, PPK is seen as dark brown clusters at day 2 and day 3**). We proceeded to see the effect of inducing hepG2 cells with PK. First, we conducted *in vitro* cell effects of toxic compounds like CCl_4 and STS, which have been studied to cause cell-death by necrosis and apoptosis, respectively. We pre-incubated hepG2 cells with 1 mM, 2 mM of CCl_4 and 1 μM of STS for 24 hours and by the MTT assay, to conduct the cytotoxicity test. About 48.4% and 56.4% toxicity were observed with 1 mM and 2 mM CCl_4-treated cells respectively however, STS-treated cells showed no toxicity after MTT assay (**figure 11B**). Secondly, we corroborated the results of the acute liver injury finding on PK protein assembling at necrotic areas. In this regard, HepG2 cells were pre-incubated for 24 hours with CCl_4 and STS. The media was changed and the cells were further treated with PK for 24 hours. MTT assay for proliferation was conducted afterwards. PK's effects on HepG2 cells was elicited by a 23.9% increase in proliferation rate compared to the control of 13%. Although, PK increased the proliferation rate of 1 mM CCl_4-treated cells to 16.8% compared to 6.9% for control, it had no effect on 2 mM CCl_4-treated and STS-treated HepG2 cells (**figure 11C**) in the experiment conducted.

A


Vehicle
CCl_4 - Day 1
40x
CCl_4 - Day 2
CCl_4- Day 3

B


% Cytotoxicity
60
40
20
0
CTL
1C4
2C4
STS

C

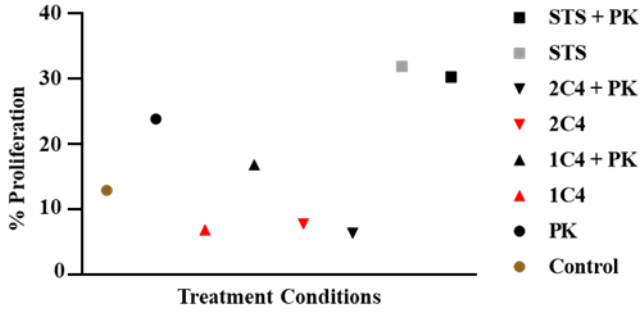

% Proliferation
40
30
20
10
0
Treatment Conditions
STS + PK
STS
2C4 + PK
2C4
1C4 + PK
1C4
PK
Control

Figure 11: **PK is involved in hepatocyte regeneration?** (A) Representative images of immunostaining of PPK (black arrows; dark brown clusters) at day 1, 2 and 3 of liver injury. (B) % of cytotoxicity level of pre-incubated HepG2 cells with 1 mM, 2 mM CCl_4, and 1 μM STS, assessed by MTT assay. (C) % of proliferation of HepG2 cells as determined by MTT assay was performed by incubating 1 mM, 2 mM CCl_4, and 1 μM STS treated cells with PK for 24 hours. 1C4 (1 mM CCl_4), 2C4 (2 mM CCl_4).

5. mRNA expression of a cell-death marker in the liver:

In tissue injury, some genes have been shown to propagate cell death by necrosis. An example is Hmgb1, a marker of cell-death. Moreover, it has been shown to have pro-inflammatory effects in many studies (158-162). We assessed the mRNA level of the *Hmgb1* gene and found a close pattern to the induced PKKS genes (**figure 12a**). Likewise, a Spearman correlation studies with the quantified necrotic area was performed however, a weak correlation of $r = 0.15$ and a p value of 0.45 (**figure 12b, table 1**) were obtained. This result indicates a probable or possible diverse role of what in acute liver injury rather than just necrotic functions. The Hmgb1 molecule has been showed to play inflammatory functions in different studies. Due to this previous studies, we will be considering it has both a DAMP capable of inducing inflammatory processes.

A **B**

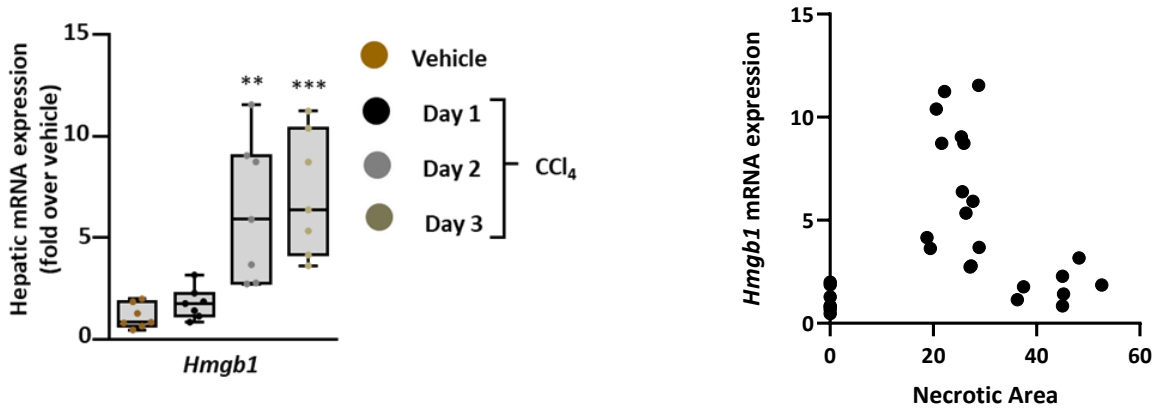


Figure 12. *Hmgb1* gene plays other role than necrosis. (**A**) Time-points mRNA expression of *Hmgb1*. (**B**) Absence of association between necrotic areas and *Hmgb1* gene (r= 0.15, p = 0.45). Data are shown as minimum to maximum values of boxplots (n = 7); Statistical significance was determined by one way ANOVA where **p<0.005, ***p <0.0005 (CCl_4 versus Vehicle); correlative plots by Spearman Correlation

6. Correlation studies between necrotic area, Hmgb1, and PKKS and thrombin genes:

We carried out Spearman correlation analysis to observe the association between the PKKS genes and necrotic areas. Based on the correlation coefficient and *p* values, there was no or weak negative associations between necrotic areas and the *F2*, *Klkb1* and *F2r* genes however, a negative association (r = -0.51, p = 0.006) exists with the *Kng1* gene (**table 1**). We next proceeded to conducting a correlation analysis with the cell-death ligand gene, *Hmgb1* instead and observed or found a positive association between *Klkb1*, *Kng1*, and *F2r* genes (**figure 13**)

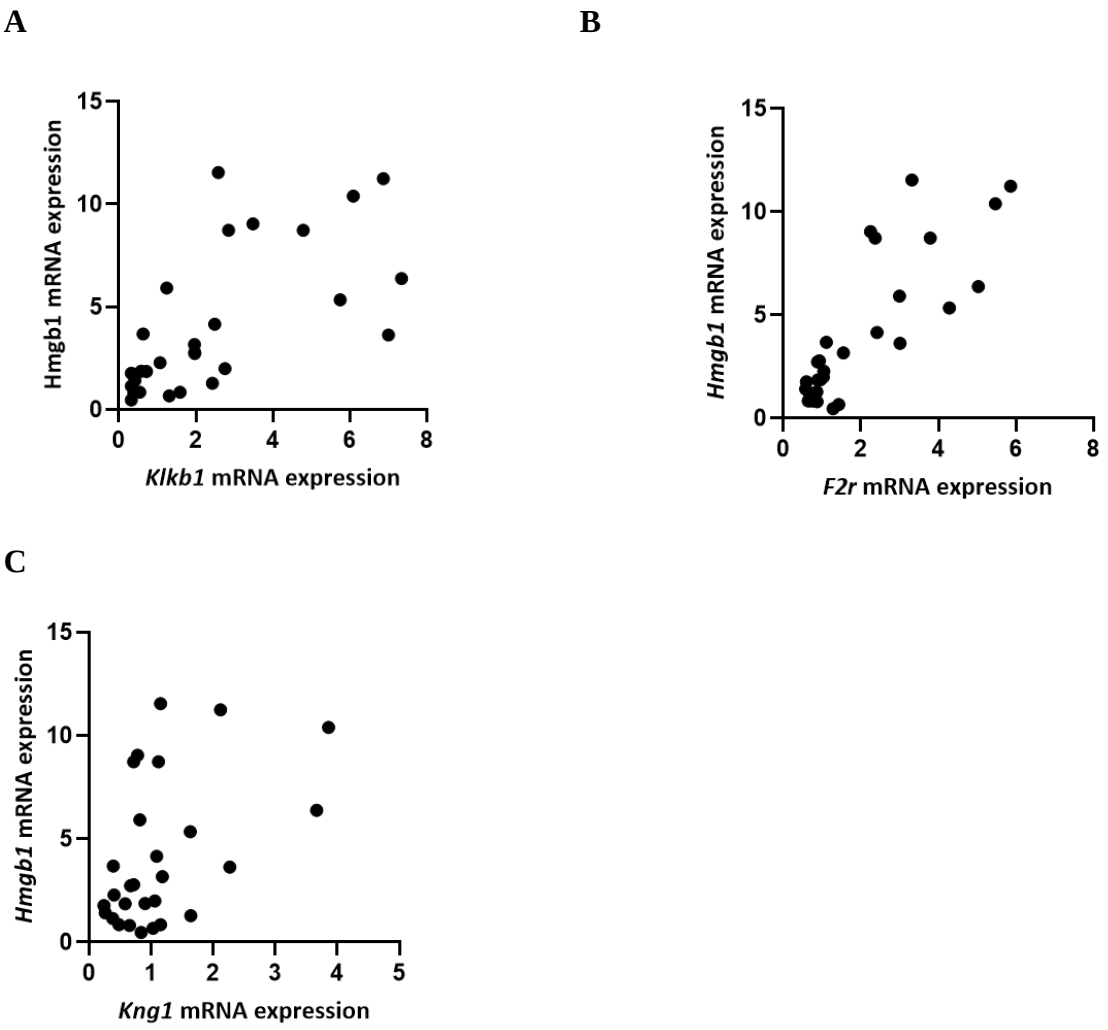


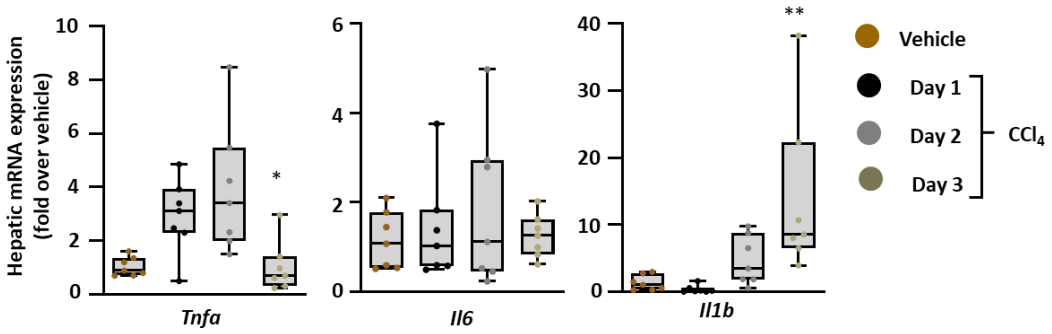
Figure 13. Association between necrotic areas and induced genes of the PKKS. Correlative studies by Spearman shows the negative associations between necrotic areas and (A) *Klkb1* gene, r= 0.75, $p < 0.0005$ (B) *F2r* gene, r= 0.79, $p < 0.0005$. (C) *Kng1*, r = 0.44, $p < 0.0005$. Correlative plots by Spearman Correlation.

7. Modifications to hepatic gene expression levels of inflammatory markers could involve the PKKS genes:

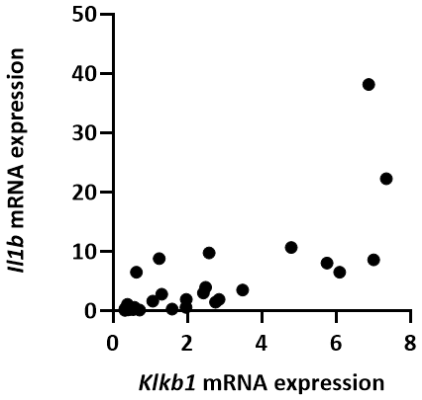
The effectors of the PKKS, plasma kallikrein and BK through their receptors have been implicated in vascular and tissue inflammation. We assessed the expression of some

pro-inflammatory genes like *Tnfa*, *Il6*, and *Il1b*. We noticed a modification to the mRNA levels of the inflammatory players assessed except *Il6* at the observed time-points (**figure 14**). Spearman correlation studies with the PKKS genes and thrombin gave a strong relationship with the *Il1b* gene (**table 1**).

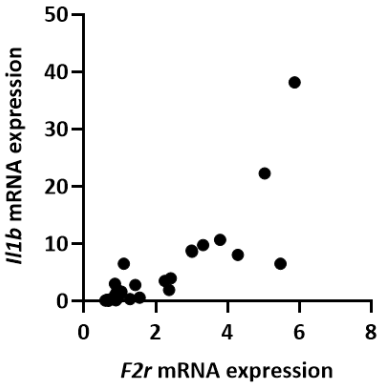
A



B



C



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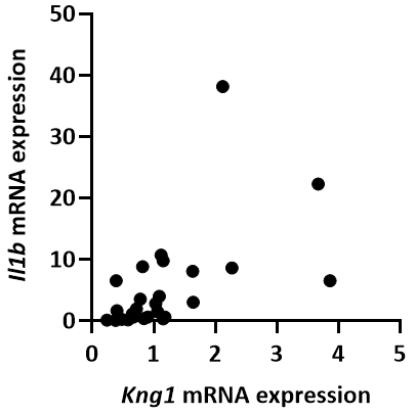


Figure 14. *Tnfa* and *Il1b* genes induction implicates inflammatory response to liver injury with a probable involvement of the PKKS. (A) *Tnfa* and *Il1b* genes. Correlation studies between (B) the *Klklb1* gene and *Tnfa* and *Il1b* genes (r= -0.25, p = 0.20; r= 0.77, p < 0.0001) (C) the *F2r* gene and *Tnfa* and *Il1b* genes (r= -0.19, p = 0.34; r= 0.87, p < 0.0001) (D) the *Kng1* gene and *Tnfa* and *Il1b* genes (r= -0.47, p = 0.012; r= 0.66, p = 0.0001); Statistical significance was determined by one way ANOVA where *p<0.05, **p <0.005 (CCl_4 versus Vehicle); correlative plots by Spearman Correlation.

8. Chemotaxis response and the PKKS genes:

Inflammatory response of tissues induces chemotaxis which aids immune cells infiltration. Some subsets of immune cells have been detected in acute injury site. We investigated two C-C motif chemotactic markers, CCL2 and CCL3, which aid in the recruitment of monocytes, neutrophils and resident macrophages (Kupffer cells). By RT-qPCR, their gene expression were modified (**figure 15**) with only a strong positive coefficient correlation to the PKKS genes for *Ccl3* gene gave; *Klkb1, Kng1* and *F2r* have an r of 0.67, 0.57 and 0.77 (p < 0.005) respectively (**table 1**). It is not surprising as PK has been shown to activate neutrophil and also modulate the inflammatory properties of

BMDM as studied in our laboratory. This indicates the possible involvement of the latter in immune cells' presence or functions during acute hepatic injury

A

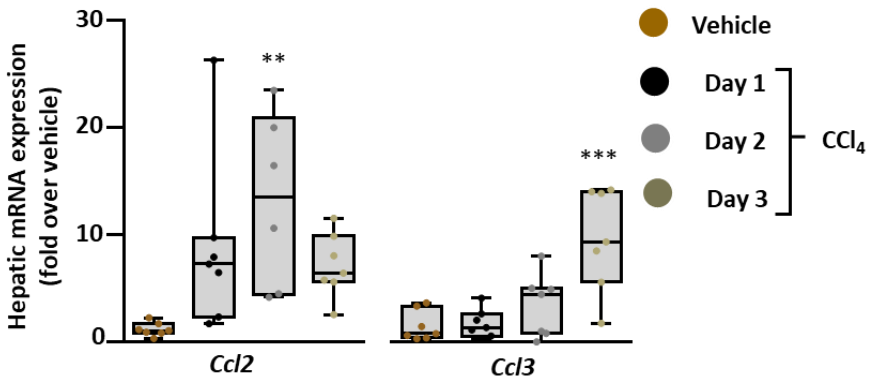


Figure 15. ***Ccl3* gene showed strong association to PKKS genes**. Data are shown as minimum to maximum values of boxplots (n = 7); Statistical significance was determined by one way ANOVA; **p<0.005, ***p <0.0005 (CCl_4 versus Vehicle).

9. Neutrophils are activated:

We looked at the possibility of the PKKS genes playing a role in immune cells recruitment? *Ccl3* gene has been investigated to be involved in the recruitment of macrophages and neutrophils. *Adgre (gene of F4/80)*, *Ly6c*, and *Ly6g* genes, which are markers of immune cells filtration were assessed. Results of gene expression indicate the presence of monocytes/macrophages and neutrophils populations. It has been previously demonstrated in Dr. Jaffa's laboratory a modulation of macrophage phenotype by PK; likewise, PK has been shown to activate neutrophil extracted from the bone marrow. So, the expression of myeloperoxidase gene, *Mpo* which is a marker of neutrophil activation, was observed with a gradual increase in expression of the mRNA level at the time-points

assessed (**figure 16B**). Correlation analysis with the PKKS genes shows a strong positive correlation especially, with the Klkb1 gene, $r = 0.67$, $p = 0.000086$ (**figure 16C**).

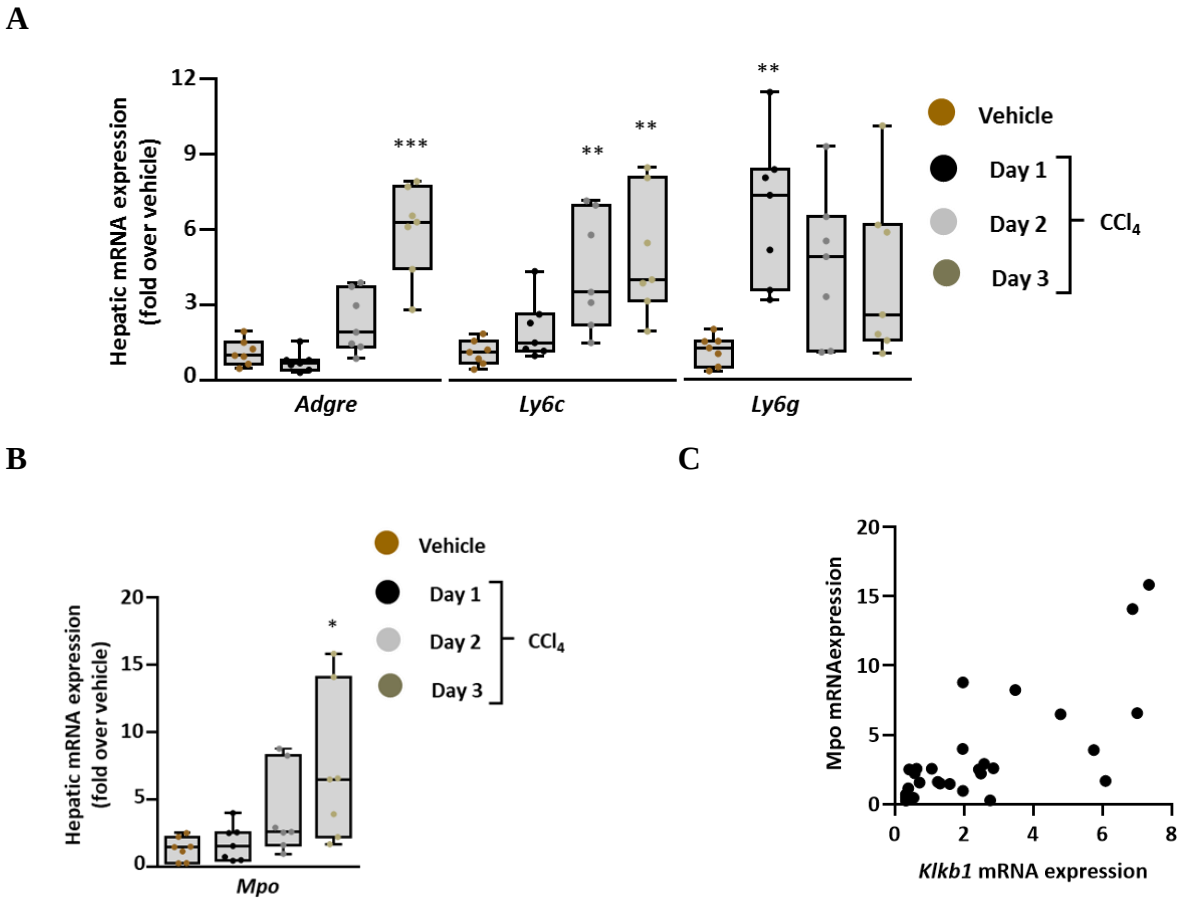


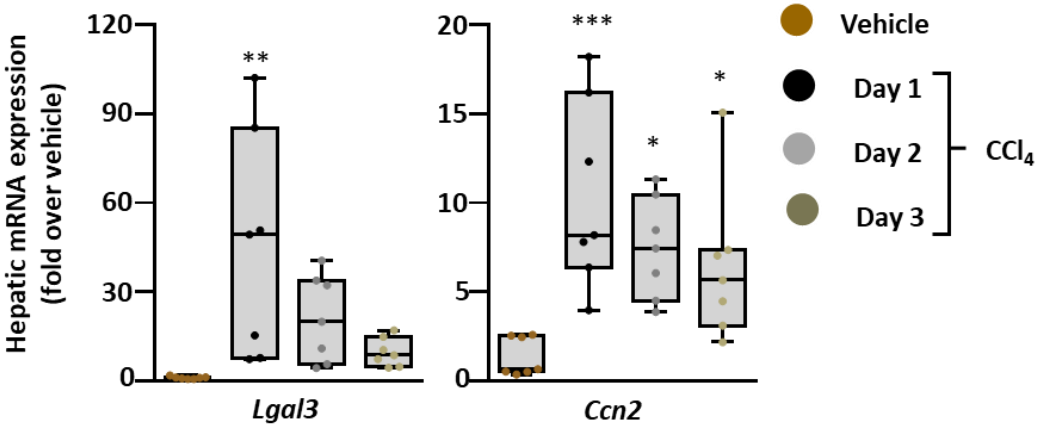
Figure 16: PK might be involved in acute liver injury recruitment of neutrophil. mRNA expression levels of (A) *Adgre*, *Ly6c*, and *Ly6g* genes. (B) *Mpo* genes (C) correlation with *Klkb1* gene ($r = 0.61$ $p > 0.0005$). Data are shown as minimum to maximum values of boxplots (n = 7); Statistical significance was determined by one way ANOVA; *p<0.05, **p<0.005, ***p <0.0005 (CCl_4 versus Vehicle); correlative plots by Spearman Correlation

10. Pro-fibrotic factors induction:

Gene expression analysis on pro-fibrotic factors, galectin-3 and Ctgf (*Lgal3* and *Ccn2*) and their correlation analysis indicate their stimulation with no correlation to the

PKKS genes (**figure 17A & table 1**). However, these genes share a strong positive correlation with the necrotic area of liver injury, p< 0.0005 (**table 1 & figure 17B**)

A



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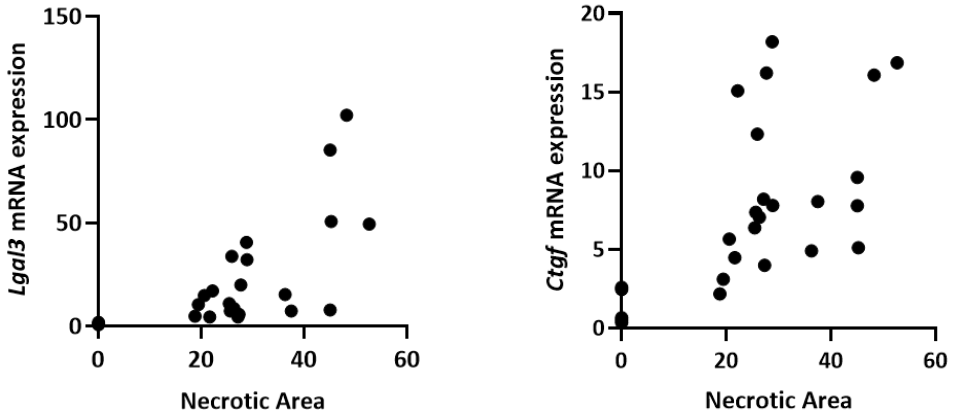


Figure 17: **Early induction of pro-fibrotic factors in acute liver injury**. (A) mRNA expression of pro-fibrotic factors *Lgal3* and *Ccn2*. (B) Spearman correlation plot between pro-fibrotic factors *Lgal3* and *Ccn2* and necrotic area (r = 0.8 and 0.77 respectively, p < 0.0005). Data are shown as Minimum to Maximum values of boxplots (n = 7); Statistical significance was determined by one way ANOVA; * p<0.05; **p<0.005; ***p <0.0005 (CCl_4 versus Vehicle); correlative plots by Spearman Correlation

Table 1: Correlation matrix of analyzed genes and necrotic area in acute liver injury

Parameters	*Klkb1*	*Kng1*	*F2r*	*F2*	*Il1b*	*Tnfa*	*Hmgb1*	*Ccl2*	*Ccl3*	*Adgre*	*Ly6c*	*Ly6g*	*Mpo*	*Lgal3*	*Ccn2*	Necrotic Area
Klkb1	1.00	0.78	0.77	0.75	0.77	-0.24	0.75	0.31	0.67	0.81	0.69	0.13	0.67	0.06	0.12	-0.25
Kng1	0.78	1.00	0.71	0.56	0.66	-0.47	0.44	0.00	0.57	0.70	0.45	-0.02	0.46	-0.19	-0.13	-0.50
F2r	0.77	0.71	1.00	0.71	0.87	-0.19	0.79	0.47	0.77	0.85	0.72	0.21	0.61	0.20	0.23	-0.20
F2	0.75	0.56	0.71	1.00	0.62	-0.08	0.76	0.54	0.57	0.74	0.74	0.38	0.70	0.35	0.30	0.03
Il1b	0.77	0.66	0.87	0.62	1.00	-0.20	0.75	0.40	0.77	0.84	0.65	0.18	0.69	0.10	0.13	-0.25
Tnfa	-0.24	-0.47	-0.19	-0.08	-0.20	1.00	0.21	0.55	-0.09	-0.14	0.22	0.50	0.07	0.53	0.62	0.55
Hmgb1	0.75	0.44	0.79	0.76	0.75	0.21	1.00	0.71	0.65	0.73	0.82	0.40	0.70	0.44	0.52	0.15
Ccl2	0.31	0.00	0.47	0.54	0.40	0.55	0.71	1.00	0.50	0.44	0.79	0.69	0.53	0.84	0.76	0.58
Ccl3	0.67	0.57	0.77	0.57	0.77	-0.09	0.65	0.50	1.00	0.65	0.67	0.40	0.53	0.39	0.35	0.07
Adgre	0.81	0.70	0.85	0.74	0.84	-0.14	0.73	0.44	0.65	1.00	0.81	0.18	0.78	0.14	0.11	-0.26
Ly6c	0.69	0.45	0.72	0.74	0.65	0.22	0.82	0.79	0.67	0.81	1.00	0.57	0.72	0.63	0.53	0.23
Ly6g	0.13	-0.02	0.21	0.38	0.18	0.50	0.40	0.69	0.40	0.18	0.57	1.00	0.46	0.85	0.78	0.67
Mpo	0.67	0.46	0.61	0.70	0.69	0.07	0.70	0.53	0.53	0.78	0.72	0.46	1.00	0.39	0.30	0.11
Lgal3	0.06	-0.19	0.20	0.35	0.10	0.53	0.44	0.84	0.39	0.14	0.63	0.85	0.39	1.00	0.80	0.80
Ccn2	0.12	-0.13	0.23	0.30	0.13	0.62	0.52	0.76	0.35	0.11	0.53	0.78	0.30	0.80	1.00	0.77
Necrotic Area	-0.25	-0.50	-0.20	0.03	-0.25	0.55	0.15	0.58	0.07	-0.26	0.23	0.67	0.11	0.80	0.77	1.00

11. Induction of PK in HepG2 cells:

In the mice model, we observed the upregulation of the kallikrein gene, and by correlation analysis, an association exists with *F2r* and *Il1b* genes, we preincubated HepG2 cells with 2.5 ng/mL of PK. After 24 hours, we observed an increase in mRNA levels of F2R, F2RL1, and IL1B (**figure 18**). In similar manner, we assessed the pro-fibrotic factors genes, LGAL3 and CCN2; the induction of PK in hepG2 cells significantly upregulated the mRNA level of LGAL3 gene however, the CCN2 gene decreased (**figure 18**).

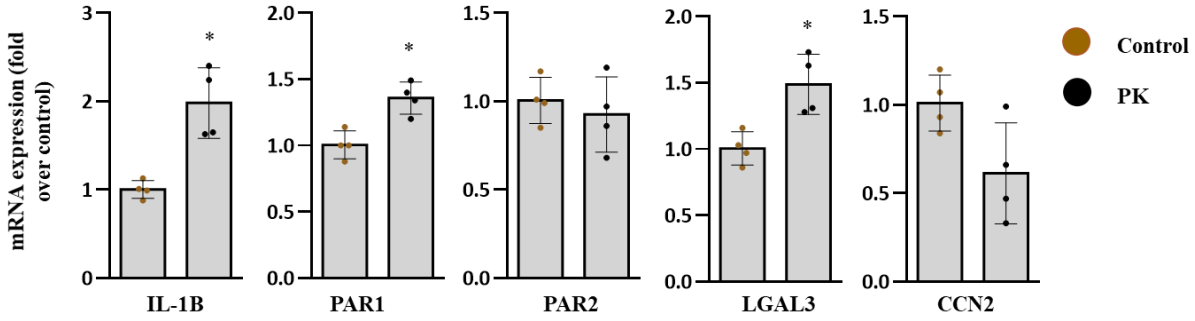


Figure 18: Plasma kallikrein increased the mRNA levels of F2R, F2RL1, IL1B and LGAL3 genes while the CCN2 gene decreased in expression. Data are shown as mean± SEM (n= 4 separate experiments); (PK versus control corresponding to untreated cells).* $p<0.05$

B. Fibrogenesis as a way of initiating processes of resolution in chronic liver injury

1. Experimental Design:

We investigated the effect of early liver fibrogenesis on the PKSS in order to understand the role of PK in chronic liver injury. C57BL/6J mice were subjected to 0.6mL/Kg of vehicle (MO) or CCl_4 for 2.5 weeks. These mice were treated with same volume of vehicle or CCl_4 twice per week and sacrificed after the last injection on day 1 and 3. Liver tissues were extracted and processed for histological examination and mRNA extraction.

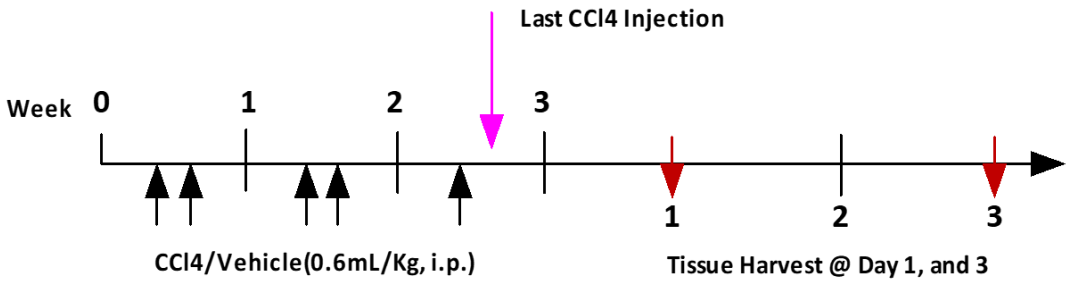


Figure 19: Schematic representation of CCl_4-induced chronic liver injury. C57BL/6J male mice (10-12 weeks) were treated with MO or 0.6 mL/Kg CCl_4 i.p. for three weeks. The mice were separated into groups to be sacrificed at the time-points, day 1 (n = 5 for vehicle and n = 6 for CCl_4) and day 3 (n = 5 for CCl_4) after the last injection of CCl_4.

2. Chronic liver injury assessment:

Chronic liver injury was assessed at day 1 to determine the induction of hepatic injury by H&E staining. The necrotic area is shown by the delineated area (.**figure 20)** Quantification was done using ImageJ software and shows a 28.2% increase in cell death by necrosis when compared to vehicle after one day CCl_4 treatment.

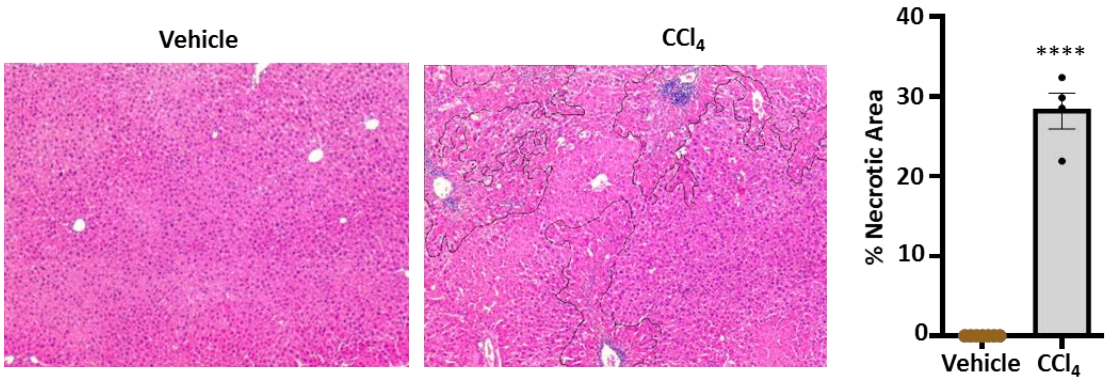
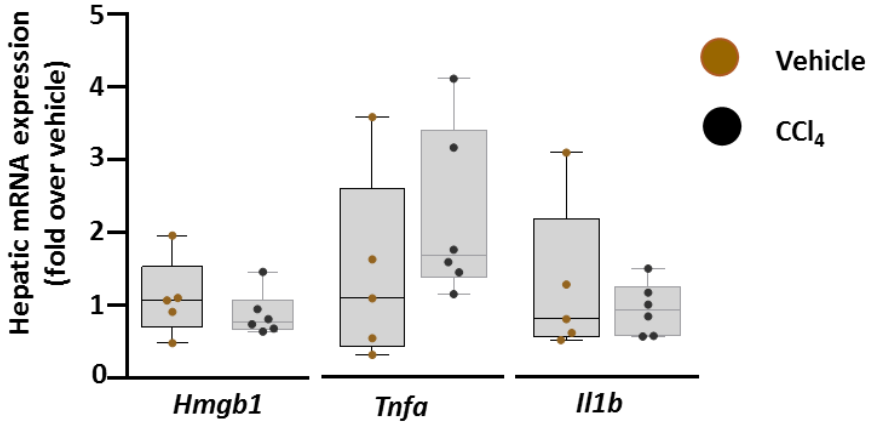


Figure 20: CCl_4-induced chronic liver injury in mice at day 1. *Left*, representative section of liver tissue stained by hematoxylin and eosin (H&E, original magnification x40) after i.p. injection of CCl_4 or vehicle (MO). Necrotic areas are delineated by marked areas. *Right*, quantification of necrotic areas using ImageJ by morphometric analysis. Data are shown as mean± SEM (n=5-6 mice per group); ***P<0.0005, (CCl_4 versus Vehicle).

3. Gene expression of cell-death, inflammatory, chemoattractant and neutrophil activation markers:

Since injury by necrotic cell death was observed by H&E staining, we carried out RT-qPCR on the *Hmgb1* gene to observe its expression at day 1. **Figure 21A** shows the unchanged expression of the *Hmgb1* gene in this timepoint of liver fibrosis model exploited. We assessed the mRNA levels of *Tnfa* and *Il1b* genes and we noticed the increase in *Tnfa* expression levels however, *Il1b* gene remained unchanged. Likewise, by gene expression analysis, we assessed the possible changes in *Ccl2* and *Ccl3* genes as seen earlier in the inflammatory genes of the acute injury time-points observed. There were statistically significant increase of *Ccl3* gene expression.. While *Ccl2* has been studied to be involved in monocyte recruitment, the *Ccl3* gene is involved in both resident macrophage and neutrophil recruitments. Likewise, since we noticed the upregulation of the *Mpo* gene (marker of neutrophil activation) in acute liver injury, we also assessed the gene in this time-point. **Figure 21B** showed an unchanged mRNA levels in the *Mpo* gene after CCl_4 treatment compared to vehicle.

A



B

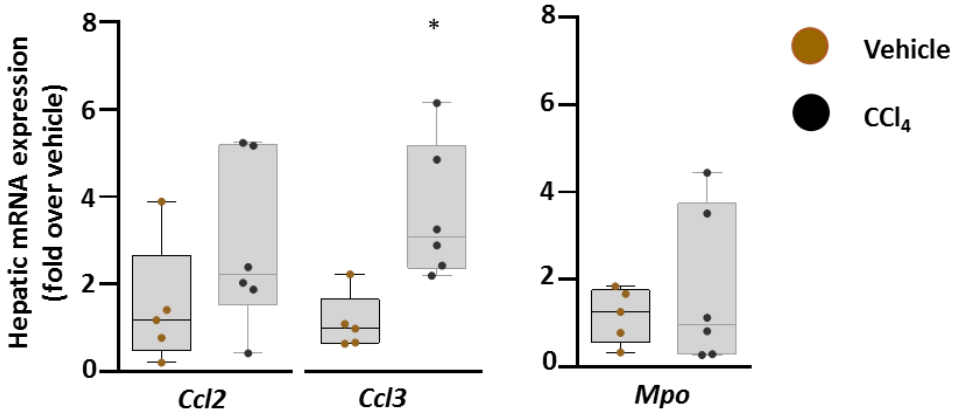
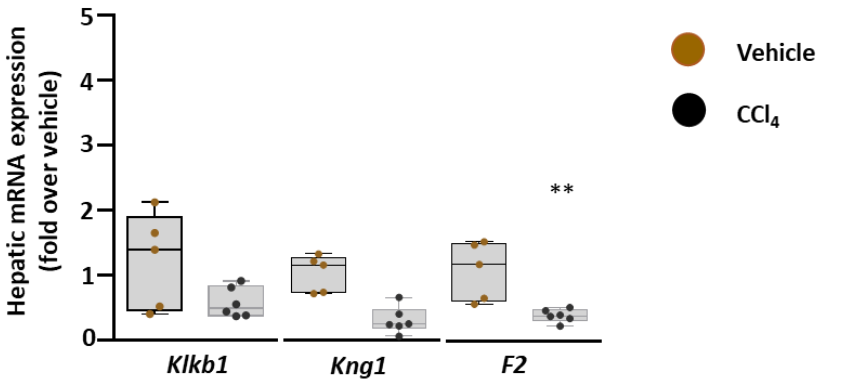


Figure 21. Day 1 hepatic mRNA expression of (A) cell-death marker, *Hmgb1* gene, inflammatory markers, *Tnfa* and *Il1b* genes. (B) Chemokines *Ccl2* and *Ccl3* genes and neutrophil activation marker *Mpo* gene. Data are shown as minimum to maximum values of boxplots (n = 5-6 mice per group);*p<0.05, **p<0.005, ***p <0.0005 (CCl_4 versus Vehicle).

4. Gene expression analysis of the PKSS and thrombin genes:

As observed in the chronic injury time point, there was a shift from the gene expression of both *Hmgb1* gene and other inflammatory markers in acute liver injury. This could probably be a short-time adaptation of the liver to subsequent insults or injury Based on the role of the PKKS genes in inflammatory processes, we revealed a decrease in gene expression of the PKKS and *F2* genes except the *F2rl1* and *Bdkrb1* genes (**figure 22B**). Most notable are the decrease in *F2r* gene, and its ligands *Klkb1* and *F2* (**figure 22**).

A



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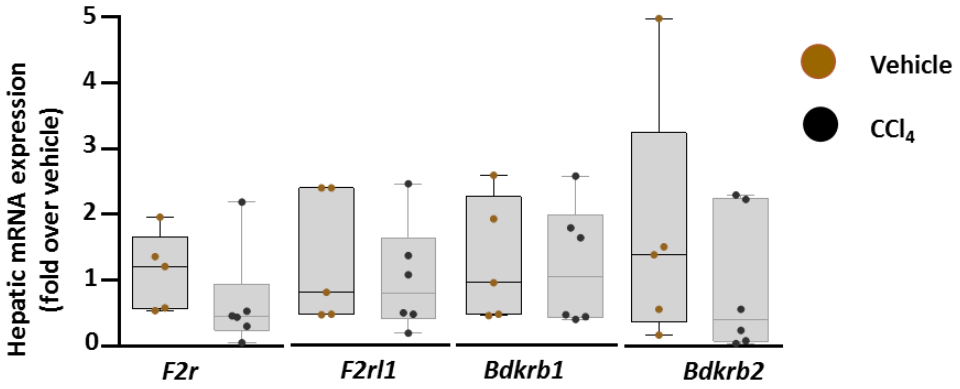


Figure 22. Inducers of inflammation regulated. Hepatic mRNA expression of the PKKS members, changes in gene expression among PKKS members showed a downregulated change in hepatic PAR-1 gene expression and its ligands (a) *Klkb1*, *Kng1*, *F2* genes. (b) *F2r*, *F2rl1*, *Bdkrb1*, *Bdkrb2* genes Data are shown as minimum to maximum values of boxplots (n=5-6 mice per group); * $p<0.05$; **$p<0.005$ (CCl_4 versus Vehicle).

5. Assessment of liver fibrosis:

Our study has revealed some findings on the PKKS genes and inflammation, and since fibrosis has been shown to occur in this timepoint of chronic liver injury, we assessed fibrosis by looking at the deposition of collagen (an ECM component) through PSR staining. Images of stained sections are shown in (**figure 23, left)** while quantified parts

done by area fraction analysis using ImageJ software is shown in **figure 23, right**. There was a significant increase in collagen deposition in the CCl_4 group over vehicle as shown by the PSR staining.

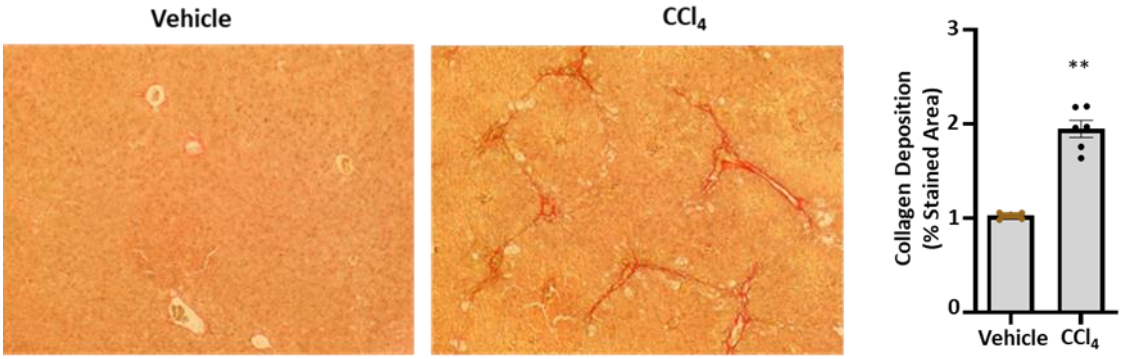


Figure 23. Fibrogenesis is induced as collagen deposition is observed in the liver. *Left*, Representative images of collagen fibers stained with Picro Sirius Red for control as vehicle, and treated mice after the last injection of CCl_4 (stained red). *Right*, quantification of histological staining of SR. Mice were treated as described in the legend for **figure 8**. Data are expressed as mean ± SEM, (n=5 mice per group); $^{**}p < 0.005$ (Mann-Whitney test).

6. Gene expression analysis of pro-fibrotic and fibrotic factors:

By gene expression, we noticed the increase in mRNA expression of pro-fibrotic factors and fibrotic players in liver fibrosis. **Figure 24** indicates higher expressions for the *Ccn2, Lgal3, Mmp2, Mmp9* and *Timp1* genes after chronic administration of CCl_4 compared to the vehicle. In all, *Lgal3, Mmp2* and *Timp1* genes were statistically upregulated compared to other genes.

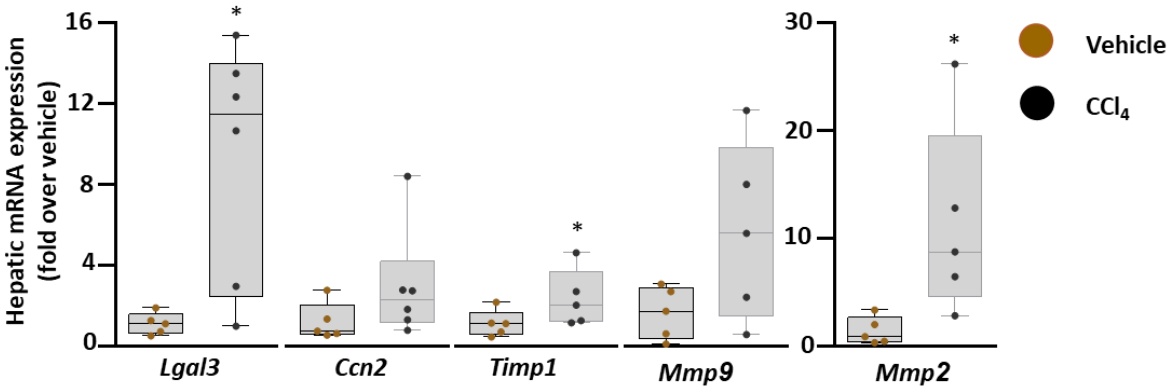


Figure 24: Hepatic fibrogenesis activities regulate inflammatory processes by increased gene expression of fibrotic markers. Upregulation of hepatic mRNA expression of pro-fibrotic markers *Lgal3*, *Ccn2*, matrix metalloproteases *Mmp2* and *Mmp9* and the regulator *Timp1*. Data are shown as minimum to maximum values of boxplots (n = 5-6 mice per group); * $p<0.05$; (CCl_4 versus Vehicle).

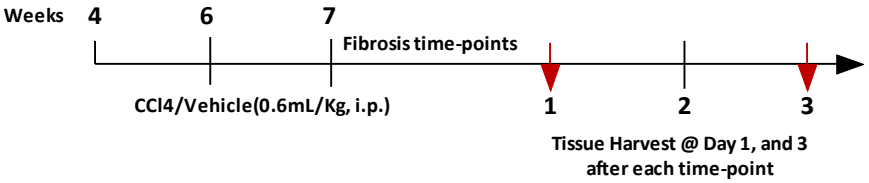
C. Varying gene expressions of PKKS members in different timepoints of liver fibrosis model suggest rhythmic mode of gene regulation:

1. Experimental Design:

To corroborate the obtained results on the effect of liver fibrogenesis on the PKKS in chronic liver injury, we studied different timepoints of CCl_4-induced liver fibrosis (four, six and seven week). This relates to early, progressive, and late liver fibrosis respectively. The C57BL/6J mice were subjected to 0.6mL/Kg of vehicle (MO) or CCl_4 for 4, 6 or 7 weeks. These mice were treated with same volume of vehicle or CCl_4 twice per week and sacrificed after the last injection on day 1 and 3. Lastly, the liver tissues were extracted and processed for histological examination and mRNA extraction. Fibrosis was established in all time-points however, there was a gradual increase in fibrosis from approximately 3 folds

in the 4 weeks' time-point to 3.4 and 4.2 folds in the 6 and 7 weeks' time-points, respectively.

A



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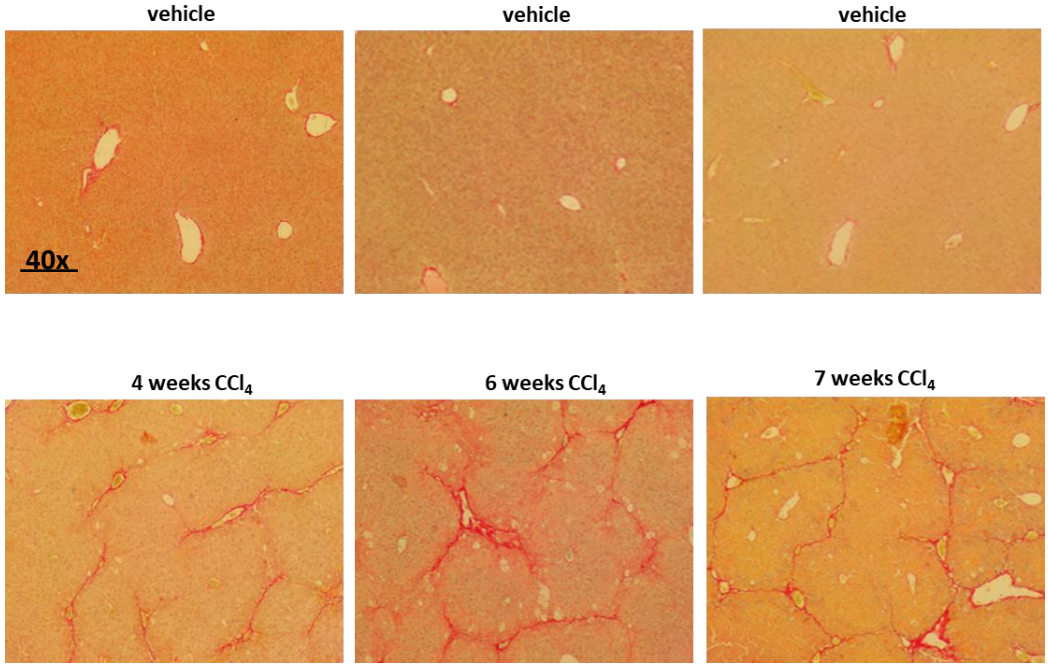

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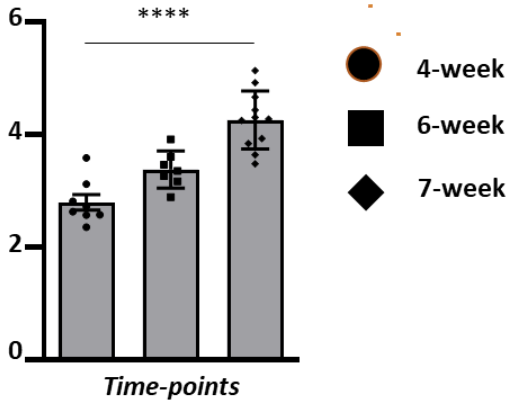


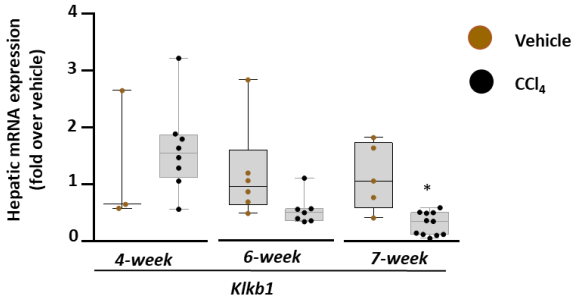
Figure 25: Increase in fibrosis across the time-points of liver fibrosis induction by CCl_4. (A) Schematic representation of CCl_4-induced liver fibrosis, C57BL/6J male mice (10-12 weeks) were treated with MO or 0.6 mL/Kg CCl_4 i.p. for four, six and seven weeks. (B) Varying Representative images of collagen fibers stained with Picro Sirius Red for 4, 6, and 7 weeks after the last injection of CCl_4 (stained red). (C) Quantification of histological staining of SR by Image J software. The quantified areas in each sample were normalized to their average time-points controls before statistical analysis plotted. Data are expressed as mean ± SEM, (n=7-10 mice per group); $^{**}p < 0.0005$ (one-way ANOVA).

2. Gene expression analysis of the PKKS:

As assessed by RT-qPCR, there was a decrease in the mRNA expression of the *Klkb1* genes (**figure 26A**) across all time-points except the 4-week timepoint. Gene expression analysis in other members of the PKKS and *F2* gene indicates a differing mode of regulation across all time-points (**figure 26**). The *Kng1* gene (**figure 26B**) of the CCl_4 groups displayed a significant upregulation of its mRNA across all timepoints except in the 6-week timepoints where it was decreased. Similarly, the *F2* mRNA expression (**figure 26C**) increased across all timepoints except in the 7-weeks. The *F2r* gene (**figure 26D**) also increased significantly at the early and late stages of liver fibrosis. Whereas, the *F2rl1* gene

(**figure 26E**) that was unstimulated and unchanged in acute and 3-week liver injury, increased across all time-points and showed a more pronounced role in the progressive and late liver fibrosis. Likewise, both the bradykinin receptors (**figure 26 F&G**) displayed an increase over all time-points while the *Bdkrb2* gene (**figure 26G**) increased tremendously.

A



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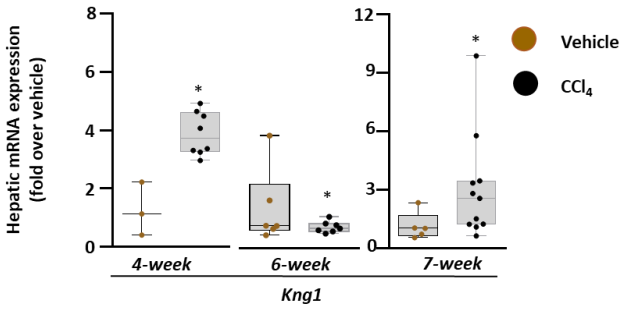

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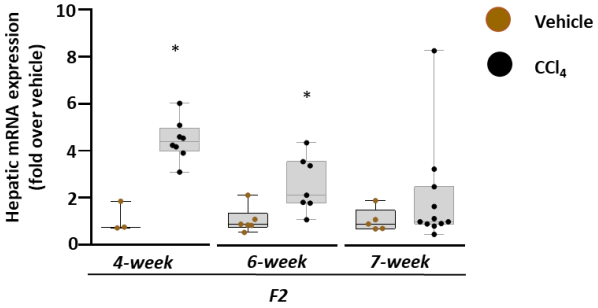

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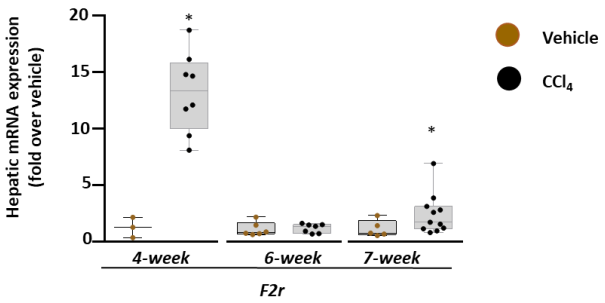


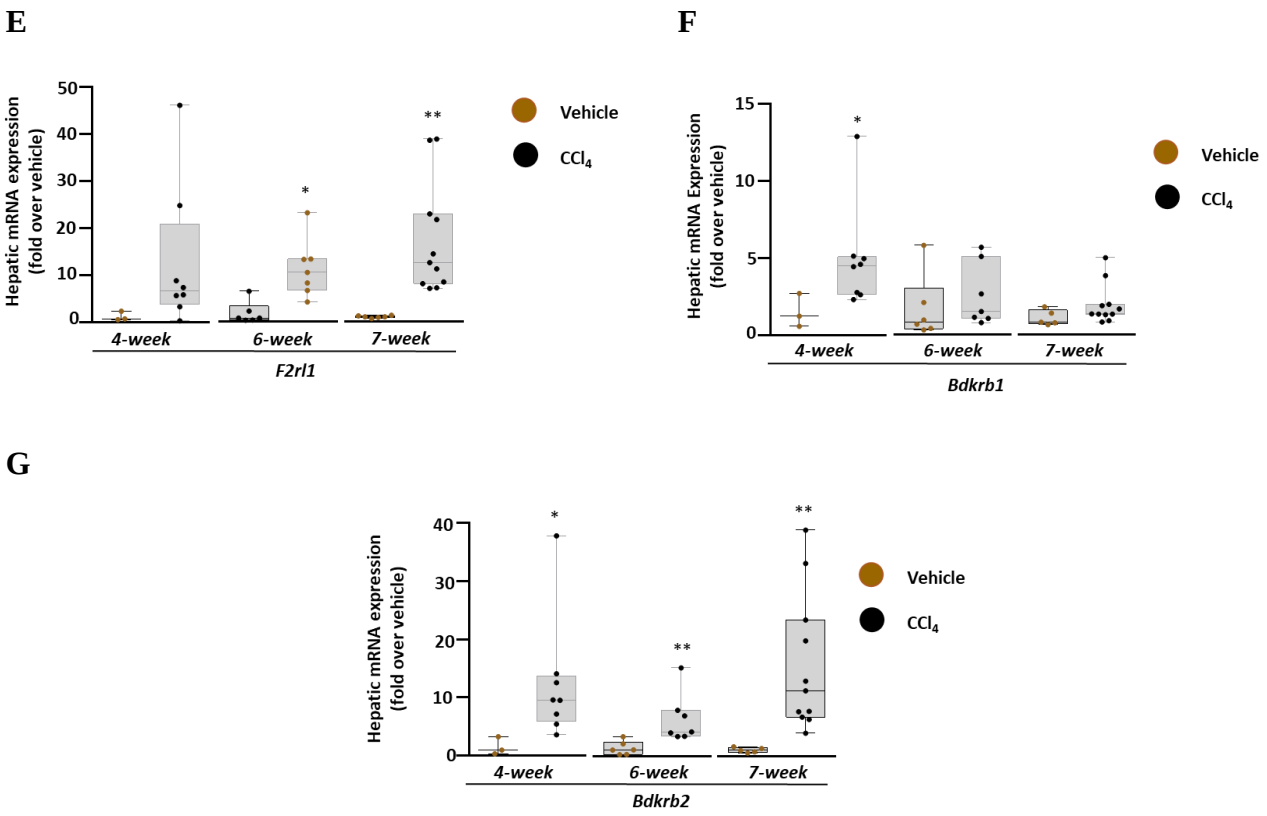


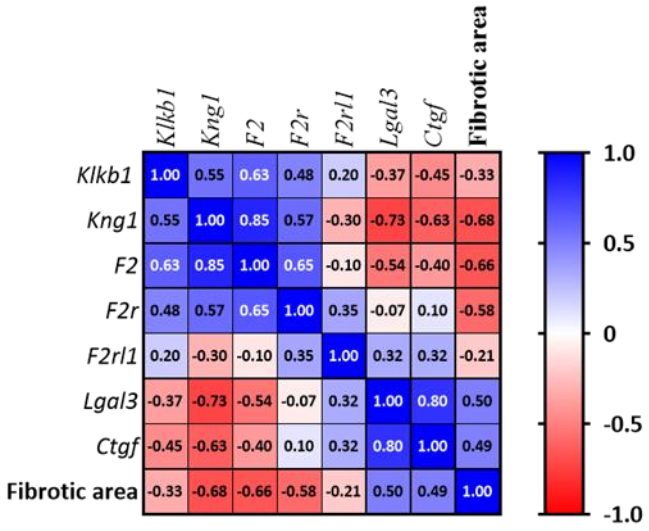
Figure 26: Differential gene expression of members of the PKKS genes indicate distinct players at different time-points of chronic injury model. Hepatic mRNA expression of (A) *Klkb1* gene. (B) *F2* gene (C) *Kng1* gene. (D) *F2r* gene, in a 4-week CCl_4 exposure. (E) The *F2rl1* gene, (F) *Bdkrb1* gene. (G) *Bdkrb2* gene, Data are shown as minimum to maximum values of boxplots (n = 3-10 mice per group); Statistical significance was determined by Mann-Whitney test where *p<0.05; **p<0.005; ***p<0.0005 ****p<0.0005 (CCl_4 versus Vehicle).

3. Correlation analysis by Spearman of fibrotic area and markers, Lgal3 and Ccn2 genes to PKSS genes:

Spearman correlation was used to analyze the association between the PKKS genes and pro-fibrotic factors, and fibrotic areas. Due to the enormous charts produced, we generated a heat map for each time-points of fibrosis and PKKS genes. In addition, a plot to assess the varying changes of each correlative data to a fibrotic parameter over time was

produced. Varying expression of gene was observed across fibrotic time-points of 2.5 (**figure 27A**), 4 (**figure 27B**), 6 (**figure 27C**) and 7 weeks (**figure 27D**). When *Klkb1* (**figure 27E**) gene was correlated to fibrotic area and pro-fibrotic genes of galectin 3 and CTGF (*Lgal3* and *Ccn2*) across all time-points, an insignificant weak negative relationship was observed except for the fibrotic area at 6 weeks ($r = 0.71$, $p = 0.05$; **figure 27 E**). Yet, the receptors of the kallikrein gene, *F2r* and *F2rl1* genes were associated to some extent to the pro-fibrotic factors and fibrotic areas. Distinguishingly, the *F2r* (**figure 27F**) gene was more correlated at 4 weeks and 7 weeks' time-points while the *F2rl1* (**figure 27G**) genes increased in correlation from the 4th week till the 7th week time-points of liver fibrosis. Furthermore, the *Kng1* (**figure 27H**) gene showed an oscillating significant correlative structure early in liver fibrosis (2.5 weeks' time-point) and was impacted by the strong association of its receptors, *Bdkrb1* (**figure 27I**) and *Bdkrb2* (**figure 27J**), to the pro-fibrotic factors and fibrotic area in the progressive and late liver fibrosis time-points (**figure 27 F-H**).

A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Klkb1 1.00 0.55 0.63 0.48 0.20 -0.37 -0.45 -0.33
Kng1 0.55 1.00 0.85 0.57 -0.30 -0.73 -0.63 -0.68
F2 0.63 0.85 1.00 0.65 -0.10 -0.54 -0.40 -0.66
F2r 0.48 0.57 0.65 1.00 0.35 -0.07 0.10 -0.58
F2rl1 0.20 -0.30 -0.10 0.35 1.00 0.32 0.32 -0.21
Lgal3 -0.37 -0.73 -0.54 -0.07 0.32 1.00 0.80 0.50
Ctgf -0.45 -0.63 -0.40 0.10 0.32 0.80 1.00 0.49
Fibrotic area -0.33 -0.68 -0.66 -0.58 -0.21 0.50 0.49 1.00
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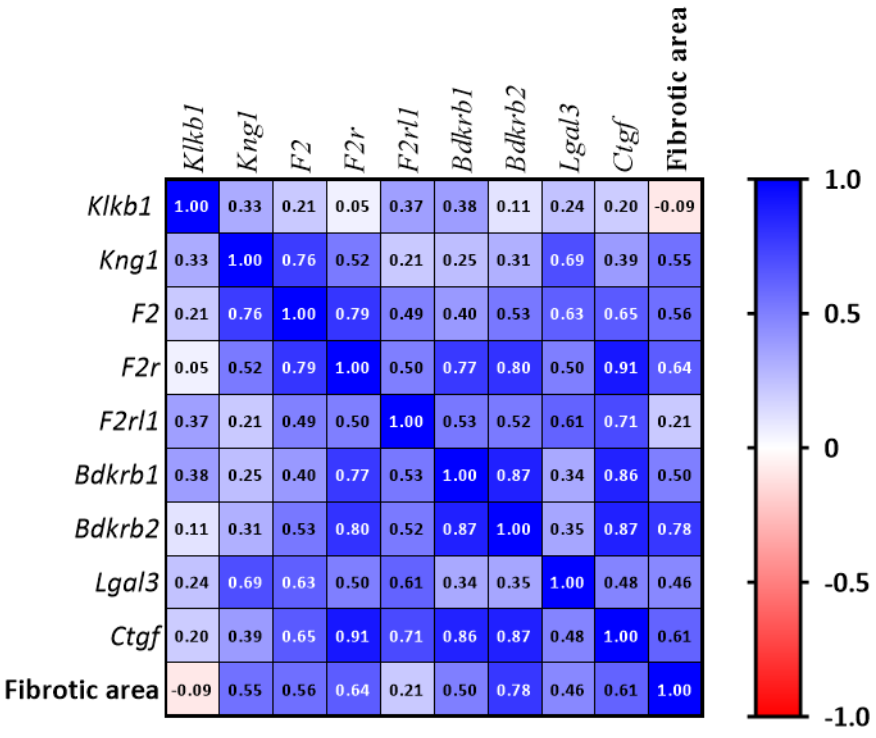
Klkb1 Kng1 F2 F2r F2rl1 Bdkrb1 Bdkrb2 Lgal3 Ctgf Fibrotic area
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Kng1 0.33 1.00 0.76 0.52 0.21 0.25 0.31 0.69 0.39 0.55
F2 0.21 0.76 1.00 0.79 0.49 0.40 0.53 0.63 0.65 0.56
F2r 0.05 0.52 0.79 1.00 0.50 0.77 0.80 0.50 0.91 0.64
F2rl1 0.37 0.21 0.49 0.50 1.00 0.53 0.52 0.61 0.71 0.21
Bdkrb1 0.38 0.25 0.40 0.77 0.53 1.00 0.87 0.34 0.86 0.50
Bdkrb2 0.11 0.31 0.53 0.80 0.52 0.87 1.00 0.35 0.87 0.78
Lgal3 0.24 0.69 0.63 0.50 0.61 0.34 0.35 1.00 0.48 0.46
Ctgf 0.20 0.39 0.65 0.91 0.71 0.86 0.87 0.48 1.00 0.61
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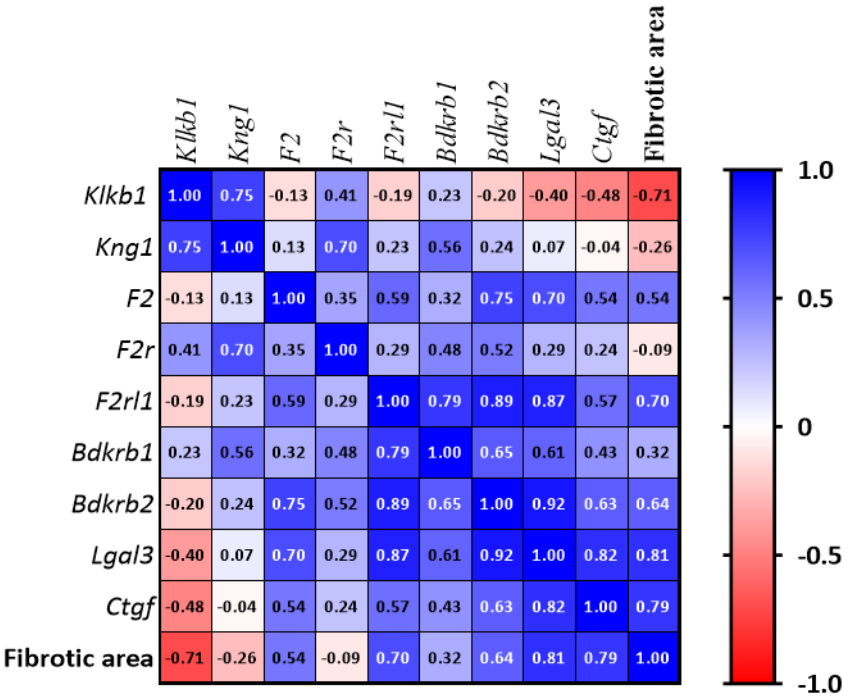
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Kng1 0.75 1.00 0.13 0.70 0.23 0.56 0.24 0.07 -0.04 -0.26
F2 -0.13 0.13 1.00 0.35 0.59 0.32 0.75 0.70 0.54 0.54
F2r 0.41 0.70 0.35 1.00 0.29 0.48 0.52 0.29 0.24 -0.09
F2rl1 -0.19 0.23 0.59 0.29 1.00 0.79 0.89 0.87 0.57 0.70
Bdkrb1 0.23 0.56 0.32 0.48 0.79 1.00 0.65 0.61 0.43 0.32
Bdkrb2 -0.20 0.24 0.75 0.52 0.89 0.65 1.00 0.92 0.63 0.64
Lgal3 -0.40 0.07 0.70 0.29 0.87 0.61 0.92 1.00 0.82 0.81
Ctgf -0.48 -0.04 0.54 0.24 0.57 0.43 0.63 0.82 1.00 0.79
Fibrotic area -0.71 -0.26 0.54 -0.09 0.70 0.32 0.64 0.81 0.79 1.00
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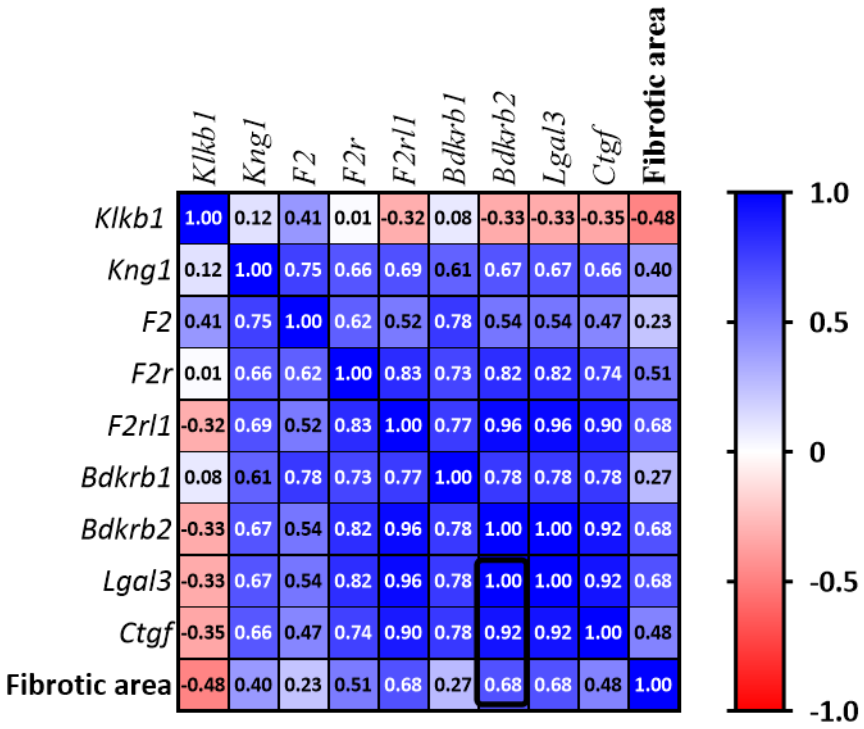
Klkb1 Kng1 F2 F2r F2rl1 Bdkrb1 Bdkrb2 Lgal3 Ctgf Fibrotic area
Klkb1 1.00 0.12 0.41 0.01 -0.32 0.08 -0.33 -0.33 -0.35 -0.48
Kng1 0.12 1.00 0.75 0.66 0.69 0.61 0.67 0.67 0.66 0.40
F2 0.41 0.75 1.00 0.62 0.52 0.78 0.54 0.54 0.47 0.23
F2r 0.01 0.66 0.62 1.00 0.83 0.73 0.82 0.82 0.74 0.51
F2rl1 -0.32 0.69 0.52 0.83 1.00 0.77 0.96 0.96 0.90 0.68
Bdkrb1 0.08 0.61 0.78 0.73 0.77 1.00 0.78 0.78 0.78 0.27
Bdkrb2 -0.33 0.67 0.54 0.82 0.96 0.78 1.00 1.00 0.92 0.68
Lgal3 -0.33 0.67 0.54 0.82 0.96 0.78 1.00 1.00 0.92 0.68
Ctgf -0.35 0.66 0.47 0.74 0.90 0.78 0.92 0.92 1.00 0.48
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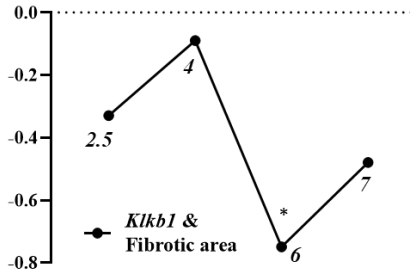
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Klkb1 &
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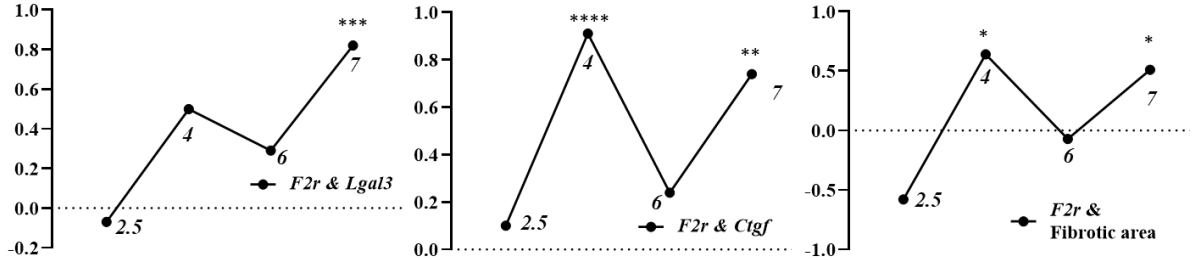
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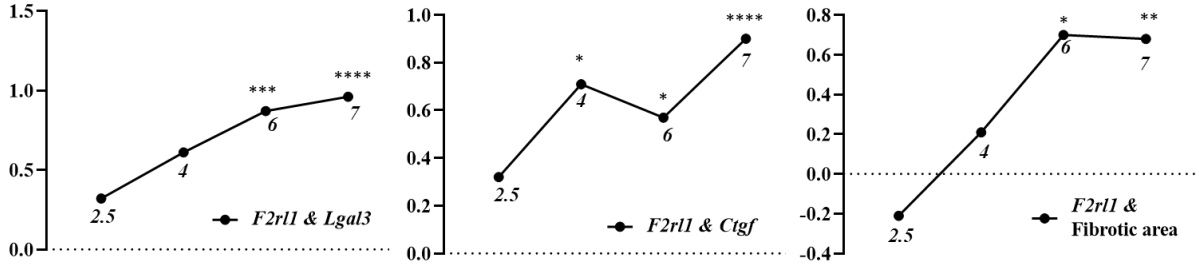
Fibrotic area

G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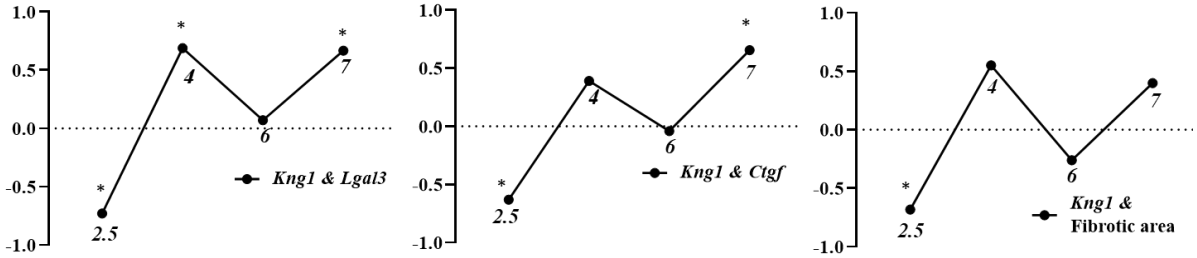
H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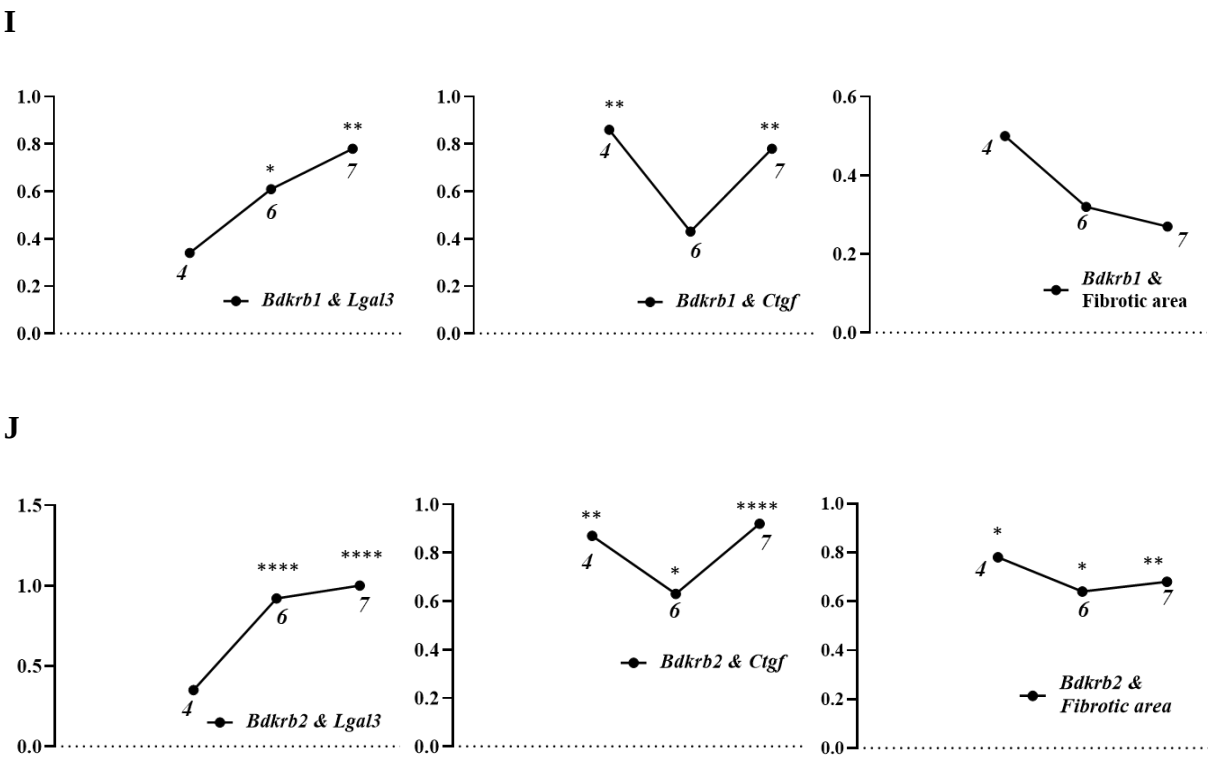


Figure 27: Spearman Correlation analysis showed association between receptors' genes of PKSS and pro-fibrotic factors, and fibrotic areas. (A-D) Heatmap showing correlation coefficients of the PKSS, *Lgal3*, *Ctgf* genes and fibrotic areas. Plot analysis of the association between (E) *Klkb1* and fibrotic area (F) *F2r* gene, *Lgal3*, *Ctgf* genes and fibrotic areas (G) *F2rl1* gene, *Lgal3*, *Ctgf* genes and fibrotic areas (H) *Kng1* gene, *Lgal3*, *Ctgf* genes and fibrotic areas (I) *Bdkrb1* gene, *Lgal3*, *Ctgf* genes and fibrotic areas (J) *Bdkrb2* gene, *Lgal3*, *Ctgf* genes and fibrotic areas. 2.5, 4, 6 and 7 corresponds to 2.5 weeks, 4 weeks, 6 weeks and 7 weeks respectively. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.00005$.

D. Liver Injury and Hyperglycemia induction by streptozotocin (STZ) injection

1. Model for Hyperglycemia

One of the risks of cardiovascular disease is diabetes. A previous study done by Jaffa et al., 2015 showed an increase in plasma kallikrein activity among cohorts of T1DM patients (163). As said earlier, diabetic liver disease in form of hepatosclerosis and NAFLD has been defined in some diabetic patients. In order to examine the involvement of the KSS system, we induced liver injury in diabetic mice. Analyses were performed by histological examination and RT-qPCR technique on the modifications to the mRNA expressions of the PKKS genes as a result of injury on hyperglycemia.

2. Experimental Design:

We employed a model of type 1 diabetes by inducing mild hyperglycemia through STZ injection in C57BL6/J mice. 10 weeks old C57BL6/J mice were injected i.p. with 0.05mg/g of STZ in 0.1M sodium citrate or vehicle on a daily basis for three to five days, until hyperglycemia was established in the STZ-treated mice. By ACCU-CHECK Performa glucometer and strip, we measured the glucose levels and statistical differences between the two groups were determined by Matt-Whitney U test. **Figure 28** shows the glucose level between the two groups.

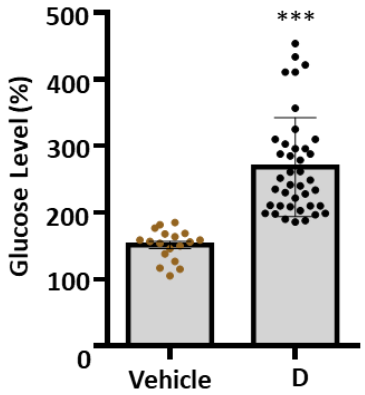


Figure 28: STZ-Injection induces hyperglycemia in C57BL6/J Mice. STZ-induced diabetes mellitus in 10 weeks old C57BL/6J male mice. Mice were treated with vehicle or STZ, i.p., for three to five days were necessary. The graph shows measured glucose level. Data are shown as mean ± SEM values of boxplots (n = 11 for vehicle, and n = 39 for Diabetes group); Statistical significance was determined by Mann-Whitney test where ***p<0.0005 (CCl_4 versus Vehicle); Vehicle (Na Citrate buffer), D (Diabetes).

3. Liver Injury Induction in Diabetic Mice:

a. Experimental Design:

After hyperglycemia was induced, the mice were allowed to rest for fourteen days while glucose level was measured to ascertain hyperglycemia stability. Mice were distributed into acute injury and chronic injury groups.

b. Acute Liver Injury in Diabetic Mice:

The diabetic C57BL/6J male mice were treated with either MO as vehicle or 0.6 mL/Kg CCl_4 i.p once and were sacrificed after forty-eight hours. We assessed acute liver injury using H&E staining. The area of necrosis is outlined by the delineated area. Images

of and quantification of liver sections are shown in **figure 29**. Quantification was done using ImageJ software. H&E staining a 30% increase necrosis in the CCl_4 group compared to vehicle. In the diabetic group, no significant necrosis was detected yet, diabetes seems to aggravate injury as portrayed in the diabetic + CCl_4 group which is impacted by a 36% increase in necrosis compared to the vehicle and diabetic group, and a 16% necrotic increase compared to the CCl_4 group (**figure 29, right**).

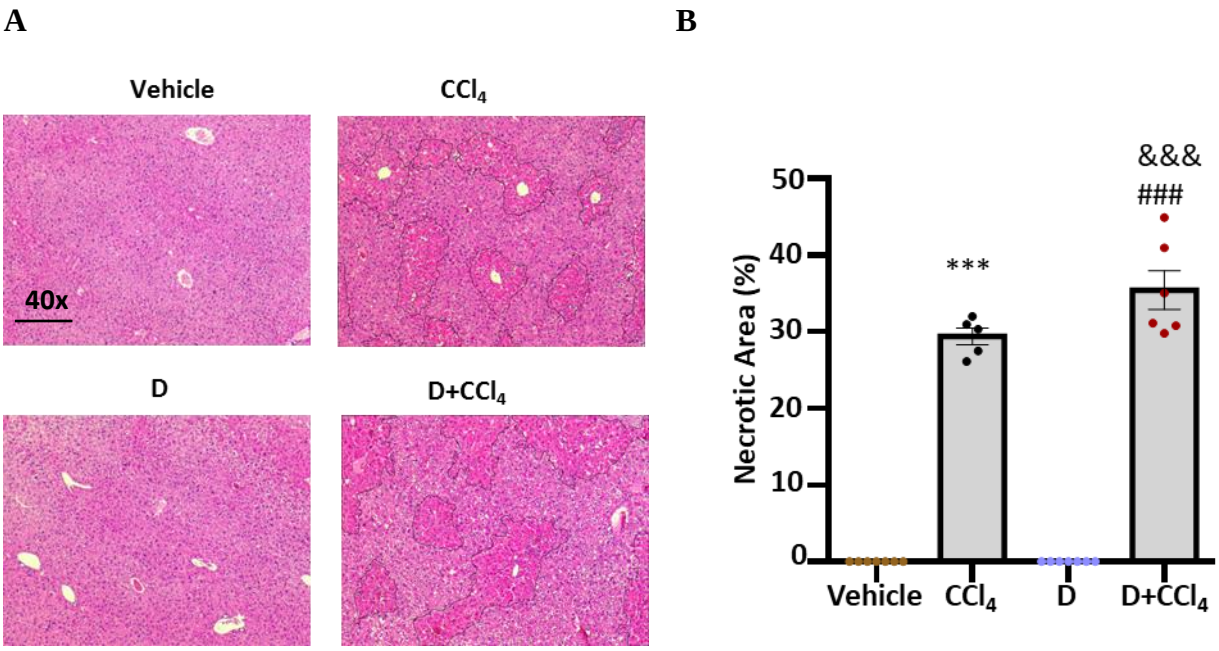


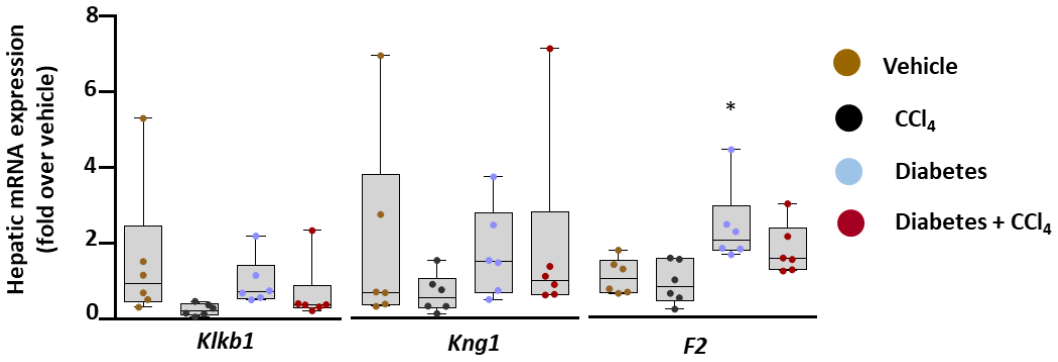
Figure 29. Representative images of necrotic areas of acute injury on diabetic mice. CCl_4-induced acute liver injury in diabetic mice after 2 weeks of hyperglycemia stability. Diabetic C57BL/6J male mice were treated with MO or 0.6 mL/Kg CCl_4 i.p. once. *Left*, Representative staining of liver tissue sections stained with H & E (original magnification x40) in Vehicle (MO), CCl_4-treated (once), diabetic or diabetic+CCl4-treated mice. Necrotic areas are marked. *Right*, Quantification of necrosis area by Image J software. Data are shown as mean ± SEM and minimum to maximum values by boxplots (n = 6 per group); Statistical significance was determined by Mann-Whitney and one-way ANOVA test with multiple comparisons where $*p<0.05$ (CCl_4 versus vehicle; D versus vehicle); $***p<0.0005$ (CCl_4 versus vehicle); $^{\&\&\&}p<0.0005$ (D+ CCl_4 versus CCl_4); $^{\#\#\#}p<0.05$

(D+CCl4 versus Vehicle or D); Vehicle (Na Citrate/Mineral Oil), D (Diabetes), D+CCl_4 (Diabetes + CCl_4)

c. Expression of the PKKS and thrombin genes:

We noticed the increased necrosis by single injection of CCl_4 as observed by H&E staining. Studies on murine models of diabetes and the complications have witnessed the modification to plasma kallikrein (156, 164-166) as well as inflammatory processes (166-168). We proceeded to observe the change in gene expression of the PKSS in both diabetic and injured diabetic mice. Our analyzed results showed no significant alteration across the diabetic and the injury-induced diabetic mice (**figure 30**). Although *F2* mRNA level increased by 100% in the diabetic group, the induction of injury by CCl_4 had no effect (**figure 30A**). Similarly, the mRNA level of *Bdkrb1* genes decreased after CCl_4 injection on normal mice however no effect was obtained on diabetic mice (**figure 30B**).

A



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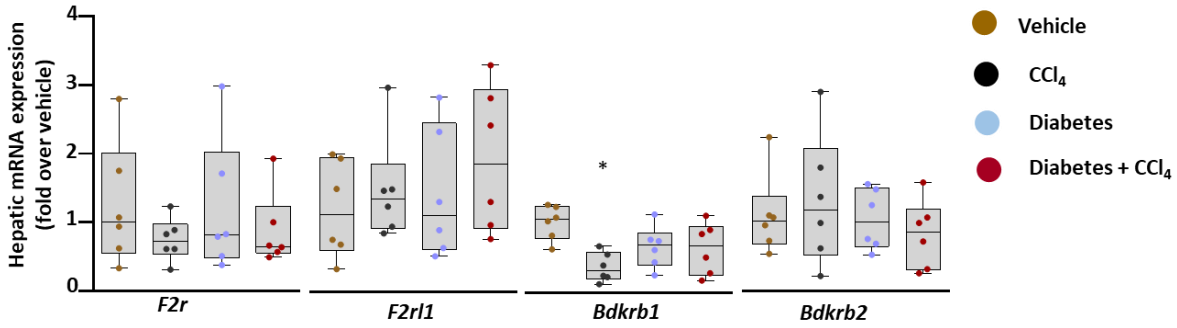


Figure 30: The PKKS is not modulated by hyperglycemia. (A) Hepatic mRNA expression of the *Klkb1*, *Kng1* and *F2* genes. (B) Hepatic mRNA expression of the *F2r*, *F2rl1*, *Bdkrb1* and *Bdkrb2* genes. Data are shown as mean ± SEM and minimum to maximum values by boxplots (n = 6 per group); Statistical significance was determined by Mann-Whitney and one-way ANOVA test with multiple comparisons where *p<0.05 (CCl_4 versus vehicle; D versus vehicle); Vehicle (Mineral Oil), D (Diabetes), D+CCl4 (Diabetes + CCl_4).

4. Chronic Liver Injury in diabetic mice

a. Fibrosis is induced in chronic injured diabetic mice

We proceeded to observe the modification to the PKSS in diabetic mice by the administration of vehicle or CCl_4 for 2.5 weeks. By this, we hope to observe the effect of a chronic liver injury in diabetic mice. After the last injection, the mice were sacrificed twenty four hours (day 1; n = 5 for vehicle and n = 6 for CCl_4) and seventy-two hours (day 3; n = 5 for CCl_4) after the last injection of CCl_4. Liver fibrosis was assessed by similar method described above; fibrosis was induced by collagen deposition across the group except control. Collagen deposition had a 32.35 % increment in the diabetic-chronic injury group compared to the CCl_4 group and a corresponding 153.56% and 196.67% increase

compared to the diabetic and vehicle group (**figure 31**). The effect of hyperglycemia increased collagen deposition compared to vehicle in this experimental model. Likewise, injury on diabetes potentiated the deposition of collagen and is statistically significant compared to the diabetes and CCl_4 groups (**figure 31, right**).

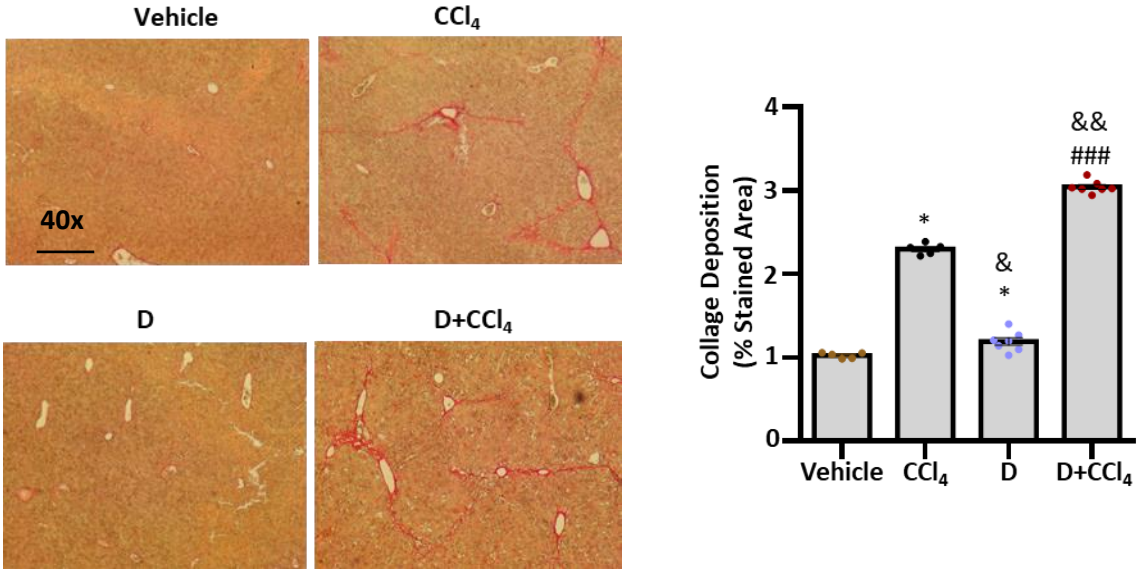
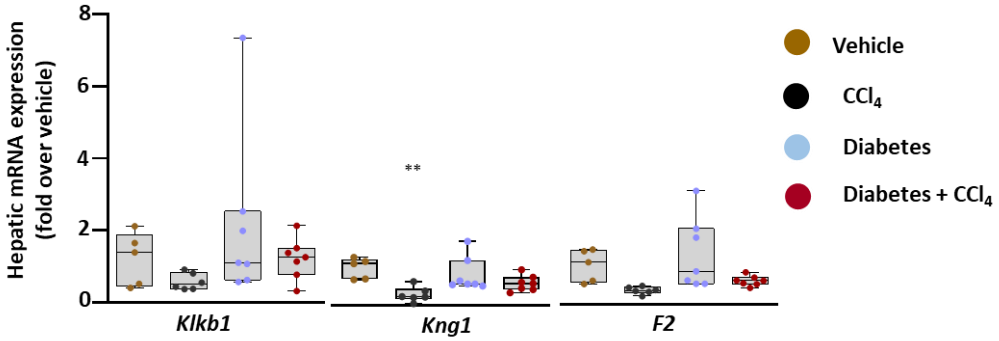


Figure 31. Fibrogenesis is induced as collagen deposition is observed in the liver. *Left*, CCl_4-induced chronic liver injury in diabetic mice after 14 days hyperglycemia stability. Diabetic C57BL/6J male mice (10-12 weeks) were treated with MO or 0.6 mL/Kg CCl_4 i.p. for 2.5 weeks**.** *Left*, representative staining of liver tissue sections stained with Picrosirius red (original magnification x80) in Vehicle, CCl_4-treated, diabetic or diabetic+CCl_4-treated mice. Diabetes was induced by a 3-day STZ injection and where necessary for 5 days, CCl_4 was injected in normal and diabetic mice after 14 days hyperglycemia stability for 2.5 weeks. *Right*, quantification of necrosis area. Data are expressed as mean ± SEM, (n=5 mice per group) by Mann-Whitney U test; *P < 0.05; (CCl_4 versus vehicle; D versus vehicle); $^{\&}p<0.05$, $^{\&\&}p<0.005$, (D versus CCl_4; D+ CCl_4 versus CCl_4); $^{\#\#\#}p<0.0005$ (D+CCl_4 versus D); Vehicle (Mineral Oil), D (Diabetes), D+CCl4 (Diabetes + CCl4).

b. Gene expression analysis on the PKKS genes and thrombin:

By qPCR, the increase in gene expression was observed in the *Klkb1*, *F2r* and *F2rl1* genes of diabetic- chronic injury group compared to the diabetic group. In all the treatment groups, hyperglycemia tends to cause a reduction in gene expression of the PKKS and *F2* genes except the *Bdkrb1* gene which had a slight increase in mRNA level compared to vehicle (**figure 32**).

A



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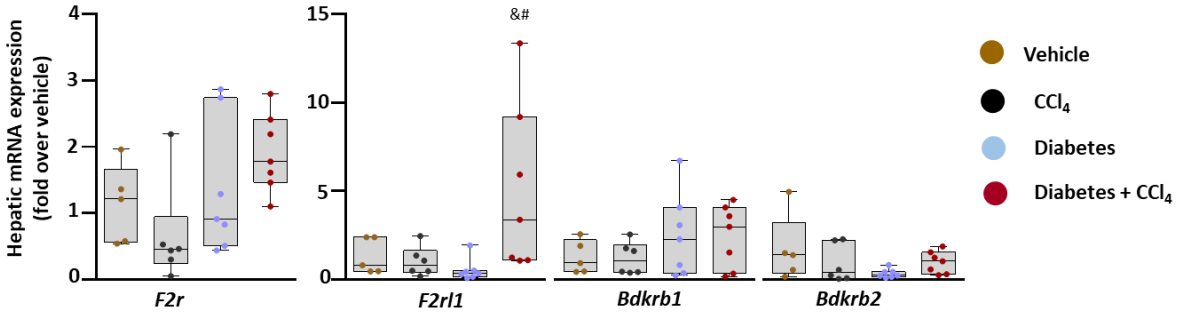


Figure 32. Effect of chronic injury and hyperglycemia on the genes of the PKKS. Chronic injury on hyperglycemia increases the mRNA expression of the *F2r*, *F2rl1* and *Bdkrb1* which are possible influencers of inflammatory and fibrotic responses. However, the effectors are not changed in this study. Hepatic mRNA expression of (**A**) *Klkb1*, *Kng1* and *F2*. (**B**) *F2r*, *F2rl1*, *Bdkrb1*, and *Bdkrb2* Data are shown as mean ± SEM and minimum to maximum values by boxplots (n = 5-7 per group); Statistical significance was determined by Mann-Whitney and one-way ANOVA test with multiple comparisons where

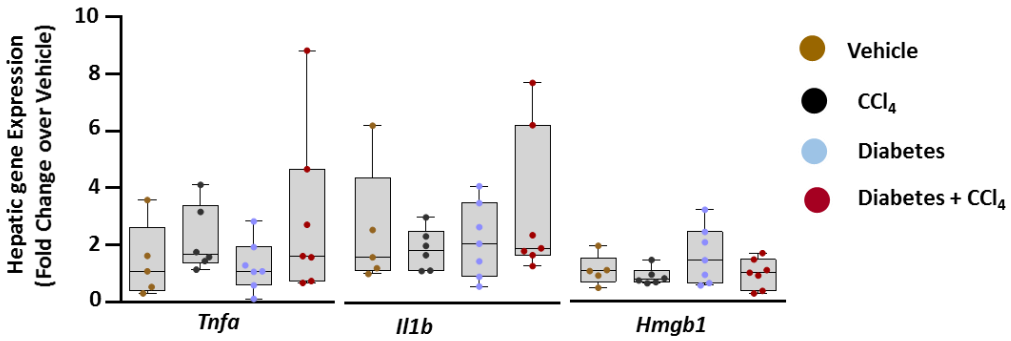
*p<0.05, **p<0.005, ***p<0.0005 (CCl_4 versus vehicle; D versus vehicle); $^{\&}p$<0.05, $^{\&\&}p$<0.005, $^{\&\&\&}p$ <0.0005 (D versus CCl_4; D+ CCl_4 versus CCl_4); $^{\#}p$<0.05, $^{\#\#}p$<0.005, $^{\#\#\#}p$<0.0005 (D+ CCl_4 versus D)

c. Inflammatory and fibrotic genes induction:

After observing a modifications to some PKKS genes in hyperglycemic and injured-diabetic mice groups, we conducted gene expression analysis to see if inflammatory or fibrotic markers are induced; as both hyperglycemia and chronic liver injury have been shown to modulate the effect of inflammatory players, fibrotic and ECM remodeling factors. Moreover, in our study of 2.5 weeks fibrogenesis induction, *Tnfa, Mpo, Ccl3, Lgal3, Ccn2, Mmp2, Mmp9,* and *Timp1* genes were affected as assessed by mRNA gene expression levels. In this study, mRNA levels of inflammatory markers *Tnfa*, *Il1b* and *Hmgb1* were not different across all groups (**figure 33A**). Diabetes does not potentiate an additional increased inflammatory response across all markers when compared to the control group. However, chemokines *Ccl2* and *Ccl3* genes had an increase in mRNA expression in all groups compared to control although, statistical significance was not reached (**figure 33B**). In addition, the mRNA levels of *Mpo* were decreased across all groups compared to the control (**figure 33B**). Pro-fibrotic factors, *Lgal3* and *Ccn2* had increased mRNA levels in all groups compared to the vehicle (**figure 33C**). ECM remodeling factor *Mmp2* had increased gene expression in the injured-diabetic groups compared to diabetic or CCl_4 groups. Injury on diabetes did not increase the mRNA levels of *Mmp9* although CCl_4 and hyperglycemia individually had an effect on the gene injury. *TIMP1* gene expression was not modulated by hyperglycemia alone but there was an

increase in mRNA levels in the CCl_4 and diabetes+CCl_4 group though statistical significance was not reached (**figure 33D**).

A



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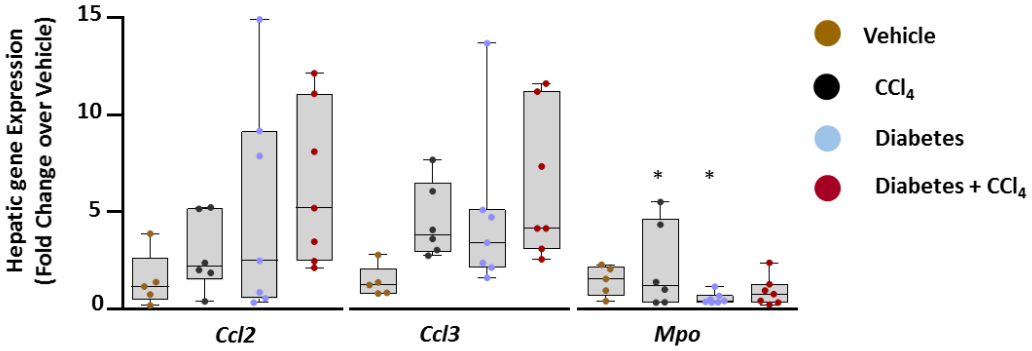

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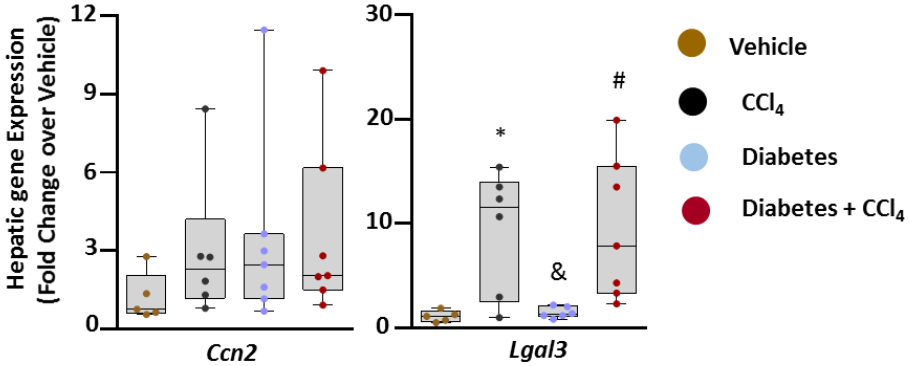

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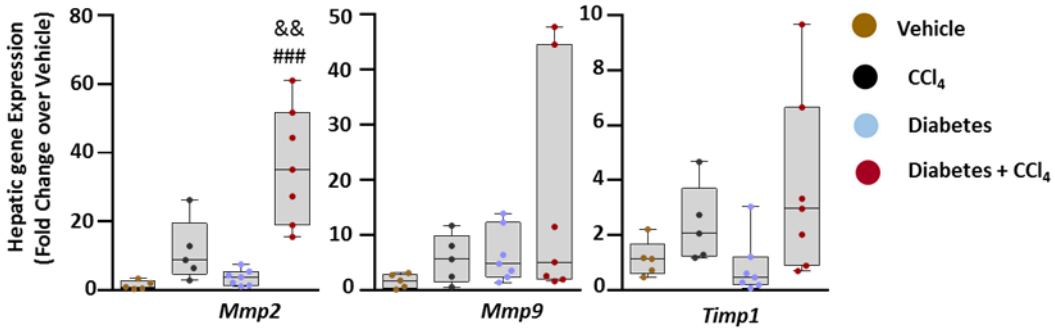

Figure 33: **Effect of hyperglycemia on the genes of inflammation, chemoattractant, neutrophil activation, fibrosis**. (**A, B**) Hepatic mRNA expression of inflammation *Tnfa* and *Il1b*, cell-death *Hmgb1*, chemokines *Ccl2* and *Ccl3*, and neutrophil activation *Mpo*, markers. (**C, D**) Stimulation of fibrotic markers *Ccn2* and *Lgal3*, and matrix metalloproteases and inhibitor *Mmp2*, *Mmp9* and *Timp1*. Data are shown as mean ± SEM and minimum to maximum values by boxplots (n = 5-7 per group); Statistical significance was determined by Mann-Whitney and one-way ANOVA test with multiple comparisons where $*p<0.05$, $**p<0.005$, $***p<0.0005$ (CCl_4 versus vehicle; D versus vehicle); $^{\&}p<0.05$, $^{\&\&}p<0.005$, $^{\&\&\&}p<0.0005$ (D versus CCl_4; D+ CCl_4 versus CCl_4); $^{\#}p<0.05$, $^{\#\#}p<0.005$, $^{\#\#\#}p<0.0005$ (D+ CCl_4 versus D)

CHAPTER V

DISCUSSION AND PERSPECTIVES

Many studies have investigated hepatic injury and fibrosis in the context of mechanisms driving the injury, extracellular matrix remodeling and regeneration pattern. Different players have been implicated of which the receptors of the PKKS are part of the main casts. Likewise, past and on-going researches have established paths and outlined the possible roles of sub-populations of cells, resident and recruited, driving the genesis, progression, and resolution of liver injury and fibrosis. Furthermore, among many, these researches implicated coagulation factors' players like PAR-1, PAR-2, BDKRB2, factor Xa and XII, thrombin; cell-death propagators (38), caspases, Bax, Bak, TNFR, TRAIL, HMGB1, FasL, JNK; inflammatory markers, NFk-β, IL6, TNF-α, IL1, IL10; and fibrotic factors- PDGF, VEGF, CTGF, TGF-β, GALECTIN-3, MMPs, ASMA. Yet, the connection among these players is incomplete as more players are being involved to play a role in the diseases. For example, whilst PAR-1 and PAR-2 have been established as a drivers of liver fibrosis, the ligands, thrombin and factor Xa contributes to the production of proliferative fibroblasts of HSCs and the progression of liver fibrosis (122). Moreover, these receptors seem to be targeted by many proteins including plasma kallikrein.

In this study, we investigated the involvement of the PKKS genes in liver injury and fibrosis by correlative analysis to cell-death, inflammatory and fibrotic makers. Specifically, we looked at the possibility of PK as a player in liver injury, and progression to fibrosis. Earlier studies by Borges et al., showed the increased clearance rate of PK in

murine models of acute phase inflammation (169) while it decreased in chronic liver injury (170). The investigation supposedly implicated galectin-3 as the receptor involved in endocytic mediation of this clearance. Using time-point liver injury induction, our study shows the increased expression of the *Klkb1*, *Kng1*, *F2r* and *F2* genes over time. By Spearman correlation, these genes showed a strong positive association with cell-death marker, *Hmgb1* gene; inflammatory marker, *Il1b* gene, *Ccl3*; immune cell recruitment markers, *Adgre*, *Ly6g* genes; and neutrophil activation marker, *Mpo* gene. The positive association between the *Hmgb1* gene and the induced PKKS genes could be influenced by the inflammatory roles of the former (159). When we looked at the role of PK in HepG2 cells, considering its cluster formation around injured areas as seen by IHC, the protein increased the proliferation of CCl_4-intoxicated cells. Although, doubling the concentration of CCl_4 seems to abrogate the effect of PK on cells' proliferation. However, it did decrease the expression of the *Ccn2* gene in HepG2 cells. Thus, PK showed a huge potential in increasing the proliferation of HepG2 cells and in decreasing the *Ccn2* gene. Though more investigation is needed in ascertaining PK's regeneration and anti-fibrotic tendencies, our correlative analysis to the *Ccn2* and *Lgal3* gene yielded a weak negative relationship status. Nevertheless, the induction of PK in HepG2 cells led to an increase in *F2r* and *Il1b* mRNA levels probably, signifying the action through PAR-1 and *Il1b*. However, we should consider that thrombin induces an increase in CTGF synthesis in fibroblast cells (76) thereby not ruling out the possible induction of CTGF by PK in fibroblast cells. Yet, more studies are needed to guide through these findings. Likewise, the regulation of these genes, *Klkb1*, *F2r*, *Kng1*, could be involved in the recruitment of the immune cells' macrophages and monocytes, and the activation of neutrophil, as they yielded positive association with

their markers. One should not also rule out the involvement of the LSEC in resolving or propagating liver injury or fibrosis based on the PKKS contribution on endothelial surfaces (171, 172).

In chronic liver injury time-point, which mimics liver fibrosis time-points of fibrogenesis, early, progressive and late settings, we correlated gene expression of PKKS genes to these time-points. We saw a change in the expression of the assessed genes compared to the acute liver injury scenario. Yet, as fibrotic area increased, profibrotic factors and MMPs increased in expression. The regulation of these molecules at the gene level could be a feedback mechanism owing to their subsequent roles in acute liver injury and injury progression. Likewise, the PKKS genes known to contribute to inflammatory processes decreased in mRNA level over this time-point, the regulation of the inflammatory markers assessed has been expected. Furthermore, PK continued to show a decreased expression over all time-points except 4 weeks, and had a no correlation with the profibrotic factors and area assessed. However, investigations surrounding PK in liver inflammation and fibrosis suggest the involvement in cleaving *Tgfb* (58, 173, 174). This observation and our study suggest a regulation over the *Klkb1* gene's expression. Nevertheless, the receptors of PK's action, *F2r* and *F2rl1* genes, were increased in all time-points except at 2.5 weeks. This was expected as they are upregulated along with possible ligands ,thrombin, factor Xa, and mast tryptase (83-85, 122, 123). Besides, the *F2r* gene is supposedly an inducer of the *Ccn2* gene which was increased in this time-point (84). However, the regulation of the *F2r* gene could have affected in part the *Ccn2* gene. Also, the *F2r* gene showed strong positive association at 4 and 7 weeks of fibrosis establishment

and the *F2rl1* gene was more associated to fibrosis at 4 weeks through 7 weeks. Since both genes are significantly associated to profibrotic factors *Ccn2* gene at 4 and 7 weeks, this supports previous experiments showing the PAR-1 as an inducer of CTGF. However, the total knockout of the *F2r* gene did not abrogate the expression of the Ccn2 gene (84). Which suggests another player like the *F2rl1* gene or others, *F2rl2* and *F2rl3*, could be involved. Also, it is established that a cross-talk exists between PAR-1 and PAR-2 especially with the TGF-β system (175). The *F2rl1* gene showed more association to the fibrotic area especially at 6 and 7 weeks' time-point. This is also prominent with the Lgal3 gene at 4, 6 and 7 weeks. Our study reveals an association between *F2rl1* and profibrotic factors, *Lgal3* and *Ccn2* genes, and fibrotic areas in early, progressive and late fibrosis. However, more investigation is needed to ascertain it as a possible indicator of fibrosis by correlating it with the well-studied fibrotic gene procollagen 1.

Furthermore, *Kng1* gene showed a strong negative association to *Lgal3* and *Ccn2* and fibrotic area corresponding to the relationship witnessed in acute liver injury. This observation was found opposite in other time-points of liver fibrosis except 6 weeks' time-point which showed no association. *Kng1* gene could be a supposed player in liver fibrosis as studies showed the inhibition of thrombin-induced platelet aggregation by bradykinin in humans (105). Likewise, the infusion of bradykinin corrected hepatocellular damage in a chronic liver injury in rats via hepatocytes (81). Although in an another experimental model of trichloroethylene induction of liver injury, bradykinin through the B2R activated the Kupffer cells and contributed to liver injury (89). This shows the varying interactions of implicated players of the PKSS with cell types within the liver architecture. We also saw

the association of the bradykinin receptors with liver fibrosis. Especially, the *Bdkrb2* gene showed strong positive associations through all time-points except in 2.5 weeks. Whether bradykinin through the B1R and B2R receptors contributes or plays a major role in liver injury required further investigation. Below is a scheme showing the PKKS in liver fibrosis production and possible reduction (figure 34).

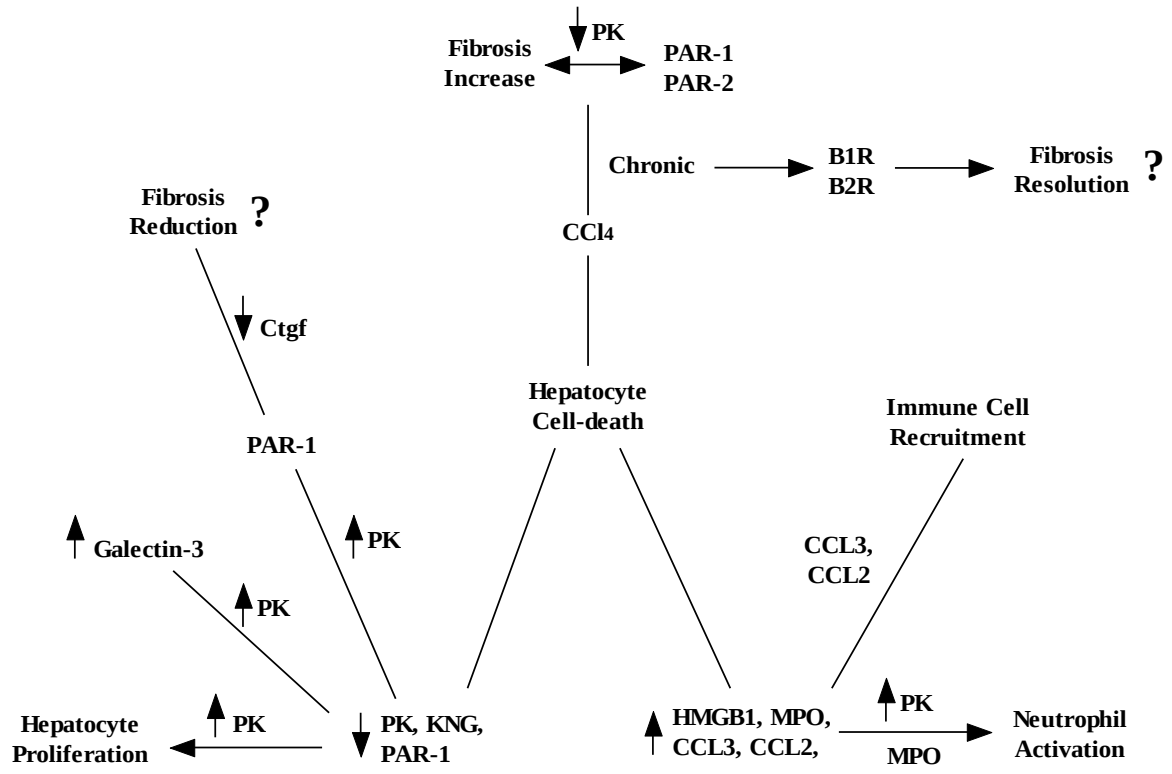

Figure 34: Schematic representation of the impact of the PKKS in fibrosis generation and resolution. ↑ (Increase), ↓ (decrease)

Overall, analysis by correlation identified strong associations between the *Kng1*, *F2r*, *F2rl1*, *Bdkrb1* and *Bdkrb2* gene to *Ccn2* and *Lgal3* genes and fibrotic area. Our gene expression analysis yielded a varying mode of hepatic PKSS gene mRNA levels and suggested an oscillating pattern of gene regulation. Could this be attributed to a rhythm of gene regulation subject to epigenetic mechanism? The plausible answers to this could be on transcriptional and epigenetic regulation of the PKSS genes.

The PKKS have a major impact on the cardiovasculature especially as they induce inflammation, coagulation and platelet aggregation. We studied the impact of a diabetic injured liver as a risk factor of cardiovascular disease. Based on this, we assessed gene expression of the hepatic PKSS. We observed no changes in their mRNA levels across all groups except for the *F2rl1* gene which was impacted by chronic injury in hepatic diabetic mice. Nevertheless, fibrosis was established in these mice as seen with fibrotic area, profibrotic markers, *Lgal3* and *Ccn2* genes, and MMPs. Inflammation seems to be unaffected as assessed via *Il1b* and *Tnfa* mRNA levels. Even the cell-death ligand gene, *Hmgb1* was unchanged. Yet, studies of hyperglycemia on the liver showed increased inflammation and immune cell infiltration (129). Why our study yielded no change in mRNA levels even in the diabetic group is surprising as studies showed the increase in PK activity in type 1 diabetic patients (163). One of the factors to be considered could be gene regulation over the time-point of diabetics and liver injury assessed because the fibrotic players were modified in this scenario. We may have to repeat this experiments with consideration on time-points of hyperglycemia stability and injury establishment and also, gene regulation over the PKKS.

Lastly, the PKKS is a mediator liver injury or fibrosis by being involved in inflammatory, immune cells' recruitment and activation, hepatocyte proliferation and fibrotic processes. PK, the primary activation of the system supposedly is involved in resolving injury and could be used as therapeutic target in alleviating or fibrotic resolution.

REFERENCES

1. Lee WM. Drug-Induced Hepatotoxicity N Engl J Med 2003;349.
2. Cullen JM. Mechanistic classification of liver injury. Toxicol Pathol. 2005;33(1):6-8.
3. Jaeschke H, Gregory J. Gores GJ, Cederbaum AI, Hinson jA, Pessayre D, Lemasters JJ. Mechanisms of Hepatotoxicity. Toxicological Sciences. 2002;65.
4. Henrion J, Schapira M, Luwaert R, Colin L, Delannoy A, Heller FR. Hypoxic hepatitis: clinical and hemodynamic study in 142 consecutive cases. Medicine (Baltimore). 2003;82(6):392-406.
5. Edward L. Krawitt MD. Medical Progress:Autoimmune Hepatitis. N Engl J Med 2006;354.
6. Ortega-Alonso A, Andrade RJ. Chronic liver injury induced by drugs and toxins. J Dig Dis. 2018;19(9):514-21.
7. Horvatits T, Drolz A, Trauner M, Fuhrmann V. Liver Injury and Failure in Critical Illness. Hepatology. 2019;70(6):2204-15.
8. Bissell DM, Gores GJ, Laskin DL, Hoofnagle JH. Drug-induced liver injury: mechanisms and test systems. Hepatology. 2001;33(4):1009-13.
9. Stadler M, Bollow E, Fritsch M, Kerner W, Schuetz-Fuhrmann I, Krakow D, et al. Prevalence of elevated liver enzymes in adults with type 1 diabetes: A multicentre analysis of the German/Austrian DPV database. Diabetes Obes Metab. 2017;19(8):1171-8.
10. Fisher K, Vuppalanchi R, Saxena R. Drug-Induced Liver Injury. Arch Pathol Lab Med. 2015;139(7):876-87.
11. Hussein R, El-Halabi M, Ghaith O, Jurdi N, Azar C, Mansour N, et al. Severe hepatotoxicity associated with the combination of spiramycin plus metronidazole. Arab J Gastroenterol. 2011;12(1):44-7.
12. Hussaini SH, O'Brien CS, Despott EJ, Dalton HR. Antibiotic therapy: a major cause of drug-induced jaundice in southwest England. European Journal of Gastroenterology & Hepatology. 2007;19(1).
13. De Valle MB, Av Klinteberg V, Alem N, Olsson R, Bjornsson E. Drug-induced liver injury in a Swedish University hospital out-patient hepatology clinic. Aliment Pharmacol Ther. 2006;24(8):1187-95.
14. Meier Y, Cavallaro M, Roos M, Pauli-Magnus C, Folkers G, Meier PJ, et al. Incidence of drug-induced liver injury in medical inpatients. Eur J Clin Pharmacol. 2005;61(2):135-43.

15. Vuppalanchi R, Liangpunsakul S, Chalasani N. Etiology of new-onset jaundice: how often is it caused by idiosyncratic drug-induced liver injury in the United States? Am J Gastroenterol. 2007;102(3):558-62; quiz 693.

16. Sgro C, Clinard F, Ouazir K, Chanay H, Allard C, Guilleminet C, et al. Incidence of drug-induced hepatic injuries: a French population-based study. Hepatology. 2002;36(2):451-5.

17. Bell LN, Chalasani N. Epidemiology of idiosyncratic drug-induced liver injury. Semin Liver Dis. 2009;29(4):337-47.

18. Fontana RJ. Acute liver failure due to drugs. Semin Liver Dis. 2008;28(2):175-87.

19. Licata A, Minissale MG, Calvaruso V, A. C. A focus on epidemiology of drug-induced liver injury: analysis of a prospective cohort. European Review for Medical and Pharmacological Sciences. 2017;21

20. Real M, Barnhill MS, Higley C, Rosenberg J, Lewis JH. Drug-Induced Liver Injury: Highlights of the Recent Literature. Drug Saf. 2019;42(3):365-87.

21. Guicciardi ME, Malhi H, Mott JL, Gores GJ. Apoptosis and necrosis in the liver. Compr Physiol. 2013;3(2):977-1010.

22. Tacke F, Luedde T, Trautwein C. Inflammatory pathways in liver homeostasis and liver injury. Clin Rev Allergy Immunol. 2009;36(1):4-12.

23. Cohen-Naftaly M, Friedman SL. Current status of novel antifibrotic therapies in patients with chronic liver disease. Therap Adv Gastroenterol. 2011;4(6):391-417.

24. Halilbasic E, Claudel T, Trauner M. Bile acid transporters and regulatory nuclear receptors in the liver and beyond. J Hepatol. 2013;58(1):155-68.

25. Stienstra R, Saudale F, Duval C, Keshtkar S, Groener JE, van Rooijen N, et al. Kupffer cells promote hepatic steatosis via interleukin-1beta-dependent suppression of peroxisome proliferator-activated receptor alpha activity. Hepatology. 2010;51(2):511-22.

26. Fernando DH, Forbes JM, Angus PW, Herath CB. Development and Progression of Non-Alcoholic Fatty Liver Disease: The Role of Advanced Glycation End Products. Int J Mol Sci. 2019;20(20).

27. Forbes SJ, Newsome PN. Liver regeneration - mechanisms and models to clinical application. Nat Rev Gastroenterol Hepatol. 2016;13(8):473-85.

28. Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. Best Pract Res Clin Gastroenterol. 2011;25(2):195-206.

29. Stravitz RT, Lee WM. Acute liver failure. The Lancet. 2019;394(10201):869-81.

30. Asada S, Kawaratani H, Mashitani T, Kaya D, Nishigori M, Kubo T, et al. Glycogenic Hepatopathy in Type 1 Diabetes Mellitus. Intern Med. 2018;57(8):1087-92.

31. Balakrishnan M, Garcia-Tsao G, Deng Y, Ciarleglio M, Jain D. Hepatic arteriolosclerosis: a small-vessel complication of diabetes and hypertension. Am J Surg Pathol. 2015;39(7):1000-9.

32. King RJ, Harrison L, Gilbey SG, Santhakumar A, Wyatt J, Jones R, et al. Diabetic hepatosclerosis: another diabetes microvascular complication? Diabet Med. 2016;33(2):e5-7.

33. Nazzari E, Grillo F, Celiento T, Picciotto A, Ferone D, Murialdo G, et al. Diabetic hepatosclerosis presenting with severe cholestasis. Diabetes Care. 2013;36(12):e206.

34. Wang K. Molecular mechanisms of hepatic apoptosis. Cell Death Dis. 2014;5:e996.

35. Malhi H, Guicciardi ME, Gores GJ. Hepatocyte death: a clear and present danger. Physiol Rev. 2010;90(3):1165-94.

36. Roychowdhury S, McMullen MR, Pisano SG, Liu X, Nagy LE. Absence of receptor interacting protein kinase 3 prevents ethanol-induced liver injury. Hepatology. 2013;57(5):1773-83.

37. Wang K. Molecular mechanisms of liver injury: apoptosis or necrosis. Exp Toxicol Pathol. 2014;66(8):351-6.

38. Malhi H, Gores GJ. Cellular and molecular mechanisms of liver injury. Gastroenterology. 2008;134(6):1641-54.

39. Jaeschke H, McGill MR, Ramachandran A. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metab Rev. 2012;44(1):88-106.

40. Moles A, Murphy L, Wilson CL, Chakraborty JB, Fox C, Park EJ, et al. A TLR2/S100A9/CXCL-2 signaling network is necessary for neutrophil recruitment in acute and chronic liver injury in the mouse. J Hepatol. 2014;60(4):782-91.

41. Hikita H, Takehara T, Kodama T, Shimizu S, Shigekawa M, Hosui A, et al. Delayed-onset caspase-dependent massive hepatocyte apoptosis upon Fas activation in Bak/Bax-deficient mice. Hepatology. 2011;54(1):240-51.

42. Khambu B, Yan S, Huda N, Yin X-M. Role of High-Mobility Group Box-1 in Liver Pathogenesis. International Journal of Molecular Sciences. 2019;20(21).

43. Malhi H, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology. 2006;43(2 Suppl 1):S31-44.

44. Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, et al. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell. 2008;135(7):1311-23.

45. Wu W, Liu P, Li J. Necroptosis: an emerging form of programmed cell death. Crit Rev Oncol Hematol. 2012;82(3):249-58.

46. Saeed WK, Jun DW. Necroptosis: an emerging type of cell death in liver diseases. World J Gastroenterol. 2014;20(35):12526-32.

47. Wang K, Lin B. Pathophysiological Significance of Hepatic Apoptosis. ISRN Hepatol. 2013;2013:740149.

48. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol. 2008;9(1):47-59.

49. Hoque R, Farooq A, Mehal WZ. Sterile inflammation in the liver and pancreas. J Gastroenterol Hepatol. 2013;28 Suppl 1:61-7.

50. Kubes P, Mehal WZ. Sterile inflammation in the liver. Gastroenterology. 2012;143(5):1158-72.

51. Wree A, McGeough MD, Inzaugarat ME, Eguchi A, Schuster S, Johnson CD, et al. NLRP3 inflammasome driven liver injury and fibrosis: Roles of IL-17 and TNF in mice. Hepatology. 2018;67(2):736-49.

52. Broderick L, De Nardo D, Franklin BS, Hoffman HM, Latz E. The inflammasomes and autoinflammatory syndromes. Annu Rev Pathol. 2015;10:395-424.

53. Rock KL, Latz E, Ontiveros F, Kono H. The sterile inflammatory response. Annu Rev Immunol. 2010;28:321-42.

54. Maher JJ. DAMPs ramp up drug toxicity. J Clin Invest. 2009;119(2):246-9.

55. Gao B, Seki E, Brenner DA, Friedman S, Cohen JI, Nagy L, et al. Innate immunity in alcoholic liver disease. Am J Physiol Gastrointest Liver Physiol. 2011;300(4):G516-25.

56. Brenner DA, Seki E, Taura K, Kisseleva T, Deminicis S, Iwaisako K, et al. Non-alcoholic steatohepatitis-induced fibrosis: Toll-like receptors, reactive oxygen species and Jun N-terminal kinase. Hepatol Res. 2011;41(7):683-6.

57. Smirne C, Rigamonti C, De Benedittis C, Sainaghi PP, Bellan M, Burlone ME, et al. Gas6/TAM Signaling Components as Novel Biomarkers of Liver Fibrosis. Dis Markers. 2019;2019:2304931.

58. Hara M, Inoue I, Yamazaki Y, Kirita A, Matsuura T, Friedman SL, et al. L59 TGF-β LAP degradation products serve as a promising blood biomarker for liver fibrogenesis in mice. Fibrogenesis & Tissue Repair. 2015; 8(17):1-10.

59. Bourbonnais E, Raymond VA, Ethier C, Nguyen BN, El-Leil MS, Meloche S, et al. Liver fibrosis protects mice from acute hepatocellular injury. Gastroenterology. 2012;142(1):130-9 e4.

60. Liu Y, Meyer C, Xu C, Weng H, Hellerbrand C, ten Dijke P, et al. Animal models of chronic liver diseases. Am J Physiol Gastrointest Liver Physiol. 2013;304(5):G449-68.

61. Weiskirchen R, Weiskirchen S, Tacke F. Recent advances in understanding liver fibrosis: bridging basic science and individualized treatment concepts. F1000Res. 2018;7.

62. Nakano Y, Kamiya A, Sumiyoshi H, Tsuruya K, Kagawa T, Inagaki Y. A novel deactivation factor of fibrogenic hepatic stellate cells induces regression of liver fibrosis in mice. Hepatology. 2019.

63. Park MR, Wong MS, Arauzo-Bravo MJ, Lee H, Nam D, Park SY, et al. Oct4 and Hnf4alpha-induced hepatic stem cells ameliorate chronic liver injury in liver fibrosis model. PLoS One. 2019;14(8):e0221085.

64. Ren S, Chen J, Wang Q, Li X, Xu Y, Zhang X, et al. MicroRNA-744/transforming growth factor beta1 relationship regulates liver cirrhosis. Hepatol Int. 2019.

65. Gressner OA, Lahme B, Demirci I, Gressner AM, Weiskirchen R. Differential effects of TGF-beta on connective tissue growth factor (CTGF/CCN2) expression in hepatic stellate cells and hepatocytes. J Hepatol. 2007;47(5):699-710.

66. Poisson J, Lemoinne S, Boulanger C, Durand F, Moreau R, Valla D, et al. Liver sinusoidal endothelial cells: Physiology and role in liver diseases. J Hepatol. 2017;66(1):212-27.

67. Bataller R, Brenner DA. Liver fibrosis. Journal of Clinical Investigation. 2005;115(2):209-18.

68. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev. 2008;88(1):125-72.

69. Wu Y, Li Z, Xiu AY, Meng DX, Wang SN, Zhang CQ. Carvedilol attenuates carbon tetrachloride-induced liver fibrosis and hepatic sinusoidal capillarization in mice. Drug Des Devel Ther. 2019;13:2667-76.

70. Chen X, Li XF, Chen Y, Zhu S, Li HD, Chen SY, et al. Hesperetin derivative attenuates CCl4-induced hepatic fibrosis and inflammation by Gli-1-dependent mechanisms. Int Immunopharmacol. 2019;76:105838.

71. Marina Barcena-Varela, Leticia Colyn, Fernandez-Barrena. MG. Epigenetic Mechanisms in Hepatic Stellate Cell Activation During Liver Fibrosis and Carcinogenesis. Int J Mol Sci 2019;20.

72. Kisseleva T, Cong M, Paik Y, Scholten D, Jiang C, Benner C, et al. Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. Proc Natl Acad Sci U S A. 2012;109(24):9448-53.

73. Troeger JS, Mederacke I, Gwak GY, Dapito DH, Mu X, Hsu CC, et al. Deactivation of hepatic stellate cells during liver fibrosis resolution in mice. Gastroenterology. 2012;143(4):1073-83 e22.

74. Boers W, Aarrass S, Linthorst C, Pinzani M, Elferink RO, Bosma P. Transcriptional profiling reveals novel markers of liver fibrogenesis: gremlin and insulin-like growth factor-binding proteins. J Biol Chem. 2006;281(24):16289-95.

75. Ignat SR, Dinescu S, Hermenean A, Costache M. Cellular Interplay as a Consequence of Inflammatory Signals Leading to Liver Fibrosis Development. Cells. 2020;9(2).

76. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. Annu Rev Pathol. 2011;6:425-56.

77. Hannivoort RA, Hernandez-Gea V, Friedman SL. Genomics and proteomics in liver fibrosis and cirrhosis. Fibrogenesis & Tissue Repair. 2012;5.

78. Pant A, Kopec AK, Luyendyk JP. Role of the blood coagulation cascade in hepatic fibrosis. Am J Physiol Gastrointest Liver Physiol. 2018;315(2):G171-G6.

79. Furukawa S, Yamaguchi M, Ooka A, Kikuchi H, Ishikawa T, Saito SY. Differentiation-inducing factor-1 prevents hepatic stellate cell activation through inhibiting GSK3beta inactivation. Biochem Biophys Res Commun. 2019;520(1):140-4.

80. Bruschi FV, Claudel T, Tardelli M, Starlinger P, Marra F, Trauner M. PNPLA3 I148M Variant Impairs Liver X Receptor Signaling and Cholesterol Homeostasis in Human Hepatic Stellate Cells. Hepatol Commun. 2019;3(9):1191-204.

81. PAU S, RAMÓN B, GUILLERMO F, MONTSERRAT M, LEANDRA N. R, JORDI C, et al. Bradykinin Attenuates Hepatocellular Damage and Fibrosis in Rats With Chronic Liver Injury. Gastroenterology. 2007;133(6):2019 28.

82. Wang Z, Li Q, Xiang M, Zhang F, Wei D, Wen Z, et al. Astragaloside Alleviates Hepatic Fibrosis Function via PAR2 Signaling Pathway in Diabetic Rats. Cell Physiol Biochem. 2017;41(3):1156-66.

83. Nault R, Fader KA, Kopec AK, Harkema JR, Zacharewski TR, Luyendyk JP. From the Cover: Coagulation-Driven Hepatic Fibrosis Requires Protease Activated Receptor-1 (PAR-1) in a Mouse Model of TCDD-Elicited Steatohepatitis. Toxicol Sci. 2016;154(2):381-91.

84. Rullier A, Gillibert-Duplantier J, Costet P, Cubel G, Haurie V, Petibois C, et al. Protease-activated receptor 1 knockout reduces experimentally induced liver fibrosis. Am J Physiol Gastrointest Liver Physiol. 2008;294(1):G226-35.

85. Knight V, Tchongue J, Lourensz D, Tipping P, Sievert W. Protease-activated receptor 2 promotes experimental liver fibrosis in mice and activates human hepatic stellate cells. Hepatology. 2012;55(3):879-87.

86. Kallis YN, Scotton CJ, Mackinnon AC, Goldin RD, Wright NA, Iredale JP, et al. Proteinase activated receptor 1 mediated fibrosis in a mouse model of liver injury: a role for bone marrow derived macrophages. PLoS One. 2014;9(1):e86241.

87. Shearer AM, Rana R, Austin K, Baleja JD, Nguyen N, Bohm A, et al. Targeting Liver Fibrosis with a Cell-penetrating Protease-activated Receptor-2 (PAR2) Pepducin. J Biol Chem. 2016;291(44):23188-98.

88. Zhang J, Li N, Yang L, Zang D, Yang P, Wang H, et al. Role of selective blocking of bradykinin B1 receptor in attenuating immune liver injury in trichloroethylene-sensitized mice. Cytokine. 2018;108:71-81.

89. Zhang J, Li N, Yang L, Xie H, Yang Y, Wang H, et al. Bradykinin contributes to immune liver injury via B2R receptor-mediated pathways in trichloroethylene sensitized mice: A role in Kupffer cell activation. Toxicology. 2019;415:37-48.

90. Frank Gieseler HU, Utz Settmacher, Morley D Hollenberg and Roland Kaufmann. Proteinase-activated receptors (PARs) – focus on receptor-receptor-interactions and their physiological and pathophysiological impact. Cell Communicatio & Signaling. 2013;11(86):1-26.

91. Heuberger DM, Schuepbach RA. Protease-activated receptors (PARs): mechanisms of action and potential therapeutic modulators in PAR-driven inflammatory diseases. Thromb J. 2019;17:4.

92. Li M, Qin XY, Furutani Y, Inoue I, Sekihara S, Kagechika H, et al. Prevention of acute liver injury by suppressing plasma kallikrein-dependent activation of latent TGF-beta. Biochem Biophys Res Commun. 2018;504(4):857-64.

93. Maria Kouyoumdjian, M´arcia Regina Nagaoka, Borges DR. Kallikrein–kinin system in hepatic experimental models. Peptides. 2005;26.

94. Marie Eve Moreau, Nancy Garbacki, Giuseppe Molinaro, Nancy J. Brown, François Marceau, Adam A. The KallikreinKinin System. Current and Future Pharmacological Targets. J Pharmacol Sci. 2005;99.

95. Kaplan AP, Ghebrehiwet B. The plasma bradykinin-forming pathways and its interrelationships with complement. Mol Immunol. 2010;47(13):2161-9.

96. Hillmeister P, Persson PB. The Kallikrein-Kinin system. Acta Physiol (Oxf). 2012;206(4):215-9.

97. Bader M. Kallikrein-kinin system in neovascularization. Arterioscler Thromb Vasc Biol. 2009;29(5):617-9.

98. Abdallah RT, Keum JS, El-Shewy HM, Lee MH, Wang B, Gooz M, et al. Plasma kallikrein promotes epidermal growth factor receptor transactivation and signaling in vascular smooth muscle through direct activation of protease-activated receptors. J Biol Chem. 2010;285(45):35206-15.

99. Dauren Biyashev, Fulong Tan, Zhenlong Chen, Kai Zhang, Peter A. Deddish, Ervin G. Erdös, et al. Kallikrein Activates B2R in the Absence of Kininogen. Am J Physiol Heart Circ Physiol. 2006;290(3).

100. Marceau F, Regoli D. Bradykinin receptor ligands: therapeutic perspectives. Nat Rev Drug Discov. 2004;3(10):845-52.

101. Bagher P, Segal SS. Regulation of blood flow in the microcirculation: role of conducted vasodilation. Acta Physiol (Oxf). 2011;202(3):271-84.

102. Gob E, Reymann S, Langhauser F, Schuhmann MK, Kraft P, Thielmann I, et al. Blocking of plasma kallikrein ameliorates stroke by reducing thromboinflammation. Ann Neurol. 2015;77(5):784-803.

103. Golias Ch, Charalabopoulos A, Stagikas D, Charalabopoulos K, A. B. The kinin system - bradykinin: biological effects and clinical implications. Multiple role of the kinin system - bradykinin. 124 HIPPOKRATIA. 2007;11(3).

104. Kaplan AP. Bradykinin-mediated diseases. Chem Immunol Allergy. 2014;100:140-7.

105. Murphey LJ, Malave HA, Petro J, Biaggioni I, Byrne DW, Vaughan DE, et al. Bradykinin and its metabolite bradykinin 1-5 inhibit thrombin-induced platelet aggregation in humans. J Pharmacol Exp Ther. 2006;318(3):1287-92.

106. Kolte D, Shariat-Madar Z. Plasma Kallikrein Inhibitors in Cardiovascular Disease: An Innovative Therapeutic Approach. Cardiol Rev. 2016;24(3):99-109.

107. <bachvarov1996.pdf>.

108. <ma1994.pdf>.

109. E. J. Mackie, C. N. Pagel, R. Smith, M. R. de Niese, S.-J. Song, Pike RN. Protease-Activated Receptors: A Means of Converting Extracellular Proteolysis into Intracellular Signals

IUBMB Life. 2002;53:277–81.

110. Zhao P, Metcalf M, Bunnett NW. Biased signaling of protease-activated receptors. Front Endocrinol (Lausanne). 2014;5:67.

111. Angela Russoa, Unice J. K. Sohb, May M. Painga, Puneeta Aroraa, JoAnn Trejoa. Caveolae are required for protease-selective signaling by protease-activated receptor–1

PNAS. 2009;106(15):6393–7.

112. Ludeman MJ, Kataoka H, Srinivasan Y, Esmon NL, Esmon CT, Coughlin SR. PAR1 cleavage and signaling in response to activated protein C and thrombin. J Biol Chem. 2005;280(13):13122-8.

113. Oe Y, Hayashi S, Fushima T, Sato E, Kisu K, Sato H, et al. Coagulation Factor Xa and Protease-Activated Receptor 2 as Novel Therapeutic Targets for Diabetic Nephropathy. Arterioscler Thromb Vasc Biol. 2016;36(8):1525-33.

114. Badeanlou L, Furlan-Freguia C, Yang G, Ruf W, Samad F. Tissue factor-protease-activated receptor 2 signaling promotes diet-induced obesity and adipose inflammation. Nat Med. 2011;17(11):1490-7.

115. McLean PG, Aston D, Sarkar D, Ahluwalia A. Protease-activated receptor-2 activation causes EDHF-like coronary vasodilation: selective preservation in ischemia/reperfusion injury: involvement of lipoxygenase products, VR1 receptors, and C-fibers. Circ Res. 2002;90(4):465-72.

116. Zhong B, Wang DH. Protease-activated receptor 2-mediated protection of myocardial ischemia-reperfusion injury: role of transient receptor potential vanilloid receptors. Am J Physiol Regul Integr Comp Physiol. 2009;297(6):R1681-90.

117. Lee SE, Jeong SK, Lee SH. Protease and protease-activated receptor-2 signaling in the pathogenesis of atopic dermatitis. Yonsei Med J. 2010;51(6):808-22.

118. Ando S, Otani H, Yagi Y, Kawai K, Araki H, Fukuhara S, et al. Proteinase-activated receptor 4 stimulation-induced epithelial-mesenchymal transition in alveolar epithelial cells. Respir Res. 2007;8:31.

119. Kopec AK, Joshi N, Towery KL, Kassel KM, Sullivan BP, Flick MJ, et al. Thrombin inhibition with dabigatran protects against high-fat diet-induced fatty liver disease in mice. J Pharmacol Exp Ther. 2014;351(2):288-97.

120. Kassel KM, Sullivan BP, Cui W, Copple BL, Luyendyk JP. Therapeutic administration of the direct thrombin inhibitor argatroban reduces hepatic inflammation in mice with established fatty liver disease. Am J Pathol. 2012;181(4):1287-95.

121. Kassel KM, Owens AP, 3rd, Rockwell CE, Sullivan BP, Wang R, Tawfik O, et al. Protease-activated receptor 1 and hematopoietic cell tissue factor are required for hepatic steatosis in mice fed a Western diet. Am J Pathol. 2011;179(5):2278-89.

122. Borensztajn K, von der Thusen JH, Peppelenbosch MP, Spek CA. The coagulation factor Xa/protease activated receptor-2 axis in the progression of liver fibrosis: a multifaceted paradigm. J Cell Mol Med. 2010;14(1-2):143-53.

123. Borensztajn K, Stiekema J, Nijmeijer S, Reitsma PH, Peppelenbosch MP, Spek CA. Factor Xa stimulates proinflammatory and profibrotic responses in fibroblasts via protease-activated receptor-2 activation. Am J Pathol. 2008;172(2):309-20.

124. Anstee QM, Wright M, Goldin R, Thursz MR. Parenchymal extinction: coagulation and hepatic fibrogenesis. Clin Liver Dis. 2009;13(1):117-26.

125. Calvaruso V, Maimone S, Gatt A, Tuddenham E, Thursz M, Pinzani M, et al. Coagulation and fibrosis in chronic liver disease. Gut. 2008;57(12):1722-7.

126. FABIO MARRA, GIUSEPPE GRANDALIANO, ANTHONY J. VALENTE, ABBouI HAE. Thrombin Stimulates Proliferation of Liver Fat-Storing Cells and Expression of Monocyte Chemotactic Protein-1: Potential Role in Liver Injury. Hepatology. 1995;22:780-7.

127. G V Papatheodoridis, E Papakonstantinou, E Andrioti, E Cholongitas, K Petraki, I Kontopoulou, et al. Thrombotic risk factors and extent of liver fibrosis in chronic viral hepatitis

Gut. 2003;52:404–9.

128. Yokoyama H, Masaki T, Inoue I, Nakamura M, Mezaki Y, Saeki C, et al. Histological and biochemical evaluation of transforming growth factor-beta

activation and its clinical significance in patients with chronic liver disease. Heliyon. 2019;5(2):e01231.

129. Lee YS, Eun HS, Kim SY, Jeong JM, Seo W, Byun JS, et al. Hepatic immunophenotyping for streptozotocin-induced hyperglycemia in mice. Sci Rep. 2016;6:30656.

130. Parveen K, Khan MR, Mujeeb M, Siddiqui WA. Protective effects of Pycnogenol on hyperglycemia-induced oxidative damage in the liver of type 2 diabetic rats. Chem Biol Interact. 2010;186(2):219-27.

131. Manna P, Das J, Ghosh J, Sil PC. Contribution of type 1 diabetes to rat liver dysfunction and cellular damage via activation of NOS, PARP, IkappaBalpha/NF-kappaB, MAPKs, and mitochondria-dependent pathways: Prophylactic role of arjunolic acid. Free Radic Biol Med. 2010;48(11):1465-84.

132. Frances DE, Ronco MT, Monti JA, Ingaramo PI, Pisani GB, Parody JP, et al. Hyperglycemia induces apoptosis in rat liver through the increase of hydroxyl radical: new insights into the insulin effect. J Endocrinol. 2010;205(2):187-200.

133. Dey A, Swaminathan K. Hyperglycemia-induced mitochondrial alterations in liver. Life Sci. 2010;87(7-8):197-214.

134. Chen M, Zheng H, Xu M, Zhao L, Zhang Q, Song J, et al. Changes in hepatic metabolic profile during the evolution of STZ-induced diabetic rats via an (1)H NMR-based metabonomic investigation. Biosci Rep. 2019;39(4).

135. Ou H, Fu Y, Liao W, Zheng C, Wu X. Association between Smoking and Liver Fibrosis among Patients with Nonalcoholic Fatty Liver Disease. Can J Gastroenterol Hepatol. 2019;2019:6028952.

136. Xie X, Yan D, Li H, Zhu Q, Li J, Fang YP, et al. Enhancement of Adiponectin Ameliorates Nonalcoholic Fatty Liver Disease via Inhibition of FoxO1 in Type I Diabetic Rats. J Diabetes Res. 2018;2018:6254340.

137. Sofiya Gancheva, Alessandra Bierwagen, Kirti Kaul, Christian Herder, Peter Nowotny, Sabine Kahl, et al. Variants in Genes Controlling Oxidative Metabolism Contribute to Lower Hepatic ATP Independent of Liver Fat Content in Type 1 Diabetes. Diabetes. 2016:1-34.

138. Hudacko RM, Sciancalepore JP, Fyfe BS. Diabetic microangiopathy in the liver: an autopsy study of incidence and association with other diabetic complications. Am J Clin Pathol. 2009;132(4):494-9.

139. Lui DT, Woo YC, Chow WS, Lee CH, Lee AC, Leung EK, et al. Glycogenic hepatopathy as an unusual etiology of deranged liver function in a patient with type 1 diabetes: A case report. Medicine (Baltimore). 2019;98(17):e15296.

140. van den Brand M, Elving LD, Drenth JP, van Krieken JH. Glycogenic hepatopathy: a rare cause of elevated serum transaminases in diabetes mellitus. Neth J Med. 2009;67(11):394-6.

141. Patita M, Nunes G, Alves de Matos A, Coelho H, Fonseca C, Fonseca J. Mauriac Syndrome: A Rare Hepatic Glycogenosis in Poorly Controlled Type 1 Diabetes. GE Port J Gastroenterol. 2019;26(5):370-4.

142. Medhioub M, Ayedi H, Chelbi E, Khsiba A, Hamzaoui L, Azzouz MM. Mauriac syndrome: An unusual presentation with portal fibrosis. Presse Med. 2019;48(6):718-20.

143. Elkrief L, Rautou PE, Sarin S, Valla D, Paradis V, Moreau R. Diabetes mellitus in patients with cirrhosis: clinical implications and management. Liver Int. 2016;36(7):936-48.

144. Sanyal A, Cusi K, Hartman ML, Zhang S, Bastyr EJ, 3rd, Bue-Valleskey JM, et al. Cytokeratin-18 and enhanced liver fibrosis scores in type 1 and type 2 diabetes and effects of two different insulins. J Investig Med. 2018;66(3):661-8.

145. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology. 2012;55(6):2005-23.

146. Targher G, Bertolini L, Padovani R, Rodella S, Zoppini G, Pichiri I, et al. Prevalence of non-alcoholic fatty liver disease and its association with cardiovascular disease in patients with type 1 diabetes. J Hepatol. 2010;53(4):713-8.

147. Xie Y, Song T, Huo M, Zhang Y, Zhang YY, Ma ZH, et al. Fasudil alleviates hepatic fibrosis in type 1 diabetic rats: involvement of the inflammation and RhoA/ROCK pathway. Eur Rev Med Pharmacol Sci. 2018;22(17):5665-77.

148. Liang T, Zhang Q, Sun W, Xin Y, Zhang Z, Tan Y, et al. Zinc treatment prevents type 1 diabetes-induced hepatic oxidative damage, endoplasmic reticulum stress, and cell death, and even prevents possible steatohepatitis in the OVE26 mouse model: Important role of metallothionein. Toxicol Lett. 2015;233(2):114-24.

149. Kim JY, Lee SH, Song EH, Park YM, Lim JY, Kim DJ, et al. A critical role of STAT1 in streptozotocin-induced diabetic liver injury in mice: controlled by ATF3. Cell Signal. 2009;21(12):1758-67.

150. Devi SS, Mehendale HM. The role of NF-kappaB signaling in impaired liver tissue repair in thioacetamide-treated type 1 diabetic rats. Eur J Pharmacol. 2005;523(1-3):127-36.

151. Sun Y, Jia Z, Yang G, Kakizoe Y, Liu M, Yang KT, et al. mPGES-2 deletion remarkably enhances liver injury in streptozotocin-treated mice via induction of GLUT2. J Hepatol. 2014;61(6):1328-36.

152. Wang J, Chakrabarty S, Bui Q, Ruf W, Samad F. Hematopoietic tissue factor-protease-activated receptor 2 signaling promotes hepatic inflammation and contributes to pathways of gluconeogenesis and steatosis in obese mice. Am J Pathol. 2015;185(2):524-35.

153. Simoes HG, Asano RY, Sales MM, Browne RA, Arsa G, Motta-Santos D, et al. Type 2 diabetes elicits lower nitric oxide, bradykinin concentration and kallikrein activity together with higher DesArg(9)-BK and reduced post-exercise hypotension compared to non-diabetic condition. PLoS One. 2013;8(11):e80348.

154. Harma MA, Dahlstrom EH, Sandholm N, Forsblom C, Groop PH, Lehto M, et al. Decreased plasma kallikrein activity is associated with reduced kidney function in individuals with type 1 diabetes. Diabetologia. 2020.

155. Teufel DP, Bennett G, Harrison H, van Rietschoten K, Pavan S, Stace C, et al. Stable and Long-Lasting, Novel Bicyclic Peptide Plasma Kallikrein Inhibitors for the Treatment of Diabetic Macular Edema. J Med Chem. 2018;61(7):2823-36.

156. Abdulaal M, Haddad NM, Sun JK, Silva PS. The Role of Plasma Kallikrein-Kinin Pathway in the Development of Diabetic Retinopathy: Pathophysiology and Therapeutic Approaches. Semin Ophthalmol. 2016;31(1-2):19-24.

157. Habib A, Chokr D, Wan J, Hegde P, Mabire M, Siebert M, et al. Inhibition of monoacylglycerol lipase, an anti-inflammatory and antifibrogenic strategy in the liver. Gut. 2019;68:522-32.

158. Bertheloot D, Latz E. HMGB1, IL-1alpha, IL-33 and S100 proteins: dual-function alarmins. Cell Mol Immunol. 2017;14(1):43-64.

159. El Gazzar M. HMGB1 modulates inflammatory responses in LPS-activated macrophages. Inflamm Res. 2007;56(4):162-7.

160. Man LL, Liu F, Wang YJ, Song HH, Xu HB, Zhu ZW, et al. The HMGB1 signaling pathway activates the inflammatory response in Schwann cells. Neural Regen Res. 2015;10(10):1706-12.

161. Paudel YN, Shaikh MF, Chakraborti A, Kumari Y, Aledo-Serrano A, Aleksovska K, et al. HMGB1: A Common Biomarker and Potential Target for TBI, Neuroinflammation, Epilepsy, and Cognitive Dysfunction. Front Neurosci. 2018;12:628.

162. Zhang C, Dong H, Chen F, Wang Y, Ma J, Wang G. The HMGB1-RAGE/TLR-TNF-alpha signaling pathway may contribute to kidney injury induced by hypoxia. Exp Ther Med. 2019;17(1):17-26.

163. Jaffa MA, Luttrell D, Schmaier AH, Klein RL, Lopes-Virella M, Luttrell LM, et al. Plasma Prekallikrein Is Associated With Carotid Intima-Media Thickness in Type 1 Diabetes. Diabetes. 2016;65(2):498-502.

164. Clermont A, Chilcote TJ, Kita T, Liu J, Riva P, Sinha S, et al. Plasma kallikrein mediates retinal vascular dysfunction and induces retinal thickening in diabetic rats. Diabetes. 2011;60(5):1590-8.

165. Feener EP, Zhou Q, Fickweiler W. Role of plasma kallikrein in diabetes and metabolism. Thromb Haemost. 2013;110(3):434-41.

166. Kolseth IB, Reine TM, Parker K, Sudworth A, Witczak BJ, Jenssen TG, et al. Increased levels of inflammatory mediators and proinflammatory monocytes in patients with type I diabetes mellitus and nephropathy. J Diabetes Complications. 2017;31(1):245-52.

167. Dallak M, Al-Ani B, Abdel Kader DH, Eid RA, Haidara MA. Insulin Suppresses Type 1 Diabetes Mellitus-Induced Ventricular Cardiomyocyte Damage Associated with the Inhibition of Biomarkers of Inflammation and Oxidative Stress in Rats. Pharmacology. 2019;104(3-4):157-65.

168. Niu S, Bian Z, Tremblay A, Luo Y, Kidder K, Mansour A, et al. Broad Infiltration of Macrophages Leads to a Proinflammatory State in Streptozotocin-Induced Hyperglycemic Mice. J Immunol. 2016;197(8):3293-301.

169. B. Martins, M. Kouyoumdjian, E. A. Limaos, Borges DR. The clearance rate of plasma kallikrein by the liver increases during the acute-phase response to inflammation

Agents Actions. 1992;37.

170. Toledo CF, Kouyoumdjian M, Lanzonit VP, Borges DR. Plasma kallikrein clearance by the liver of chronic carbon tetrachloride-treated rats. Journal of Gastroenterology and Hepatology. 1995;10.

171. Ueno A, Oh-ishi S. Roles for the Kallikrein-Kinin System in Inflammatory Exudation and Pain: Lessons From Studies on Kininogen-Deficient Rats Journal of Pharmacological Sciences. 2003;93:1-20.

172. Lafoz E, Ruart M, Anton A, Oncins A, Hernandez-Gea V. The Endothelium as a Driver of Liver Fibrosis and Regeneration. Cells. 2020;9(4).

173. KUNIHARU AKITA, ‡ MASATAKA OKUNO,* MASAMICHI ENYA,* SHOKO IMAI,* HISATAKA MORIWAKI,* NORIFUMI KAWADA,§ YASUHIRO SUZUKI,‡ and SOICHI KOJIMA‡. Impaired Liver Regeneration in Mice by Lipopolysaccharide Via TNF-␣/Kallikrein–Mediated Activation of Latent TGF-␤. Gastroenterology. 2002;123:.

174. Mitsuko Hara, Akiko Kirita, Wakako Kondo, Tomokazu Matsuura, Keisuke Nagatsuma, Naoshi Dohmae, et al. LAP degradation product reflects plasma kallikrein-dependent TGF-β activation in patients with hepatic fibrosis. SpringerPlus. 2014;3(221):1-11.

175. Ungefroren H, Gieseler F, Kaufmann R, Settmacher U, Lehnert H, Rauch BH. Signaling Crosstalk of TGF-beta/ALK5 and PAR2/PAR1: A Complex Regulatory Network Controlling Fibrosis and Cancer. Int J Mol Sci. 2018;19(6).