Therapeutic Potential of Autotaxin-Lipophosphatidic Acid (ATX-LPA) Signaling Inhibitor in Liver Diseases and Associated Complications

THESIS

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by

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CERTIFICATE

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Abstract

Background: Acute and chronic liver diseases are one of foremost reasons of mortality globally often lead to hepatic encephalopathy, hepatorenal syndrome, cholemic nephropathy, coagulation disorder etc. Although, there has been significant progress in drug discovery research, unfortunately the new drugs have shown limited efficacy in the clinic. Moreover, the ammonia built up in blood due to liver failure results in increased brain ammonia level and imparts neuroinflammation, which leads to a neuropsychiatric syndrome called hepatic encephalopathy. Furthermore, severe hyperbilirubinemia and jaundice associated with liver failure leads to acute kidney injury, namely cholemic nephropathy. Lysophosphatidic acid, a potent bioactive lipid signals through signals through six G-protein-coupled receptors is involved is several developmental, physiological, and pathological processes such as wound healing, differentiation, proliferation, migration, and survival. Autotaxin (ATX) is an ecto-nucleotide pyrophosphatase that catalyses the conversion of extracellular lysophospholipids into the lipid mediator lysophosphatidic acid. ATX inhibitor has shown the potential in several proliferative and inflammatory conditions. Based on the literature we chose to investigate the role of ATX-LPA signalling in acute and chronic liver failure and associated complications such as hepatic encephalopathy and cholemic nephropathy.

Method: We initially investigated the involvement of ATX-LPA signalling in the pathogenesis of acute and chronic liver disease. We employed moue model of thioacetamide (TAA) induced acute liver failure and assessed liver function, blood and brain ammonia, behavior parameters such as grading score locomotor activity, cognitive function (novel object recognition test) and neuroinflammatory markers in brain samples. After completion, we evaluated lysophosphatidic acid levels in plasma and brain samples. We further tested the therapeutic potential of an ATX inhibitor in TAA

induced acute liver failure model and determined the above-mentioned parameters. We also evaluate the potential of ATX inhibitor in ammonium chloride induced release of inflammatory mediators in astrocytes and microglial cells.

In subsequent experiments, we explored the role of ATX-LPA signalling in rat model of bile duct ligation (BDL) indued chronic liver disease and associated hepatic encephalopathy. We evaluated, liver function, LPA levels in plasma and brain, blood and brain ammonia, behavior parameters locomotor activity, cognitive function (novel object recognition test and neuroinflammatory markers in brain samples. Later we tested a potent ATXi inhibitor for its potential in alleviating progression of liver fibrosis and hepatic encephalopathy.

Furthermore, we examined the prospective of ATX inhibitor in lessening cholemic nephropathy in bile duct induced chronic liver disease model in rats.

Results: In mouse model of acute liver failure, plasma levels of AST, ALT and TBIL were found to be elevated significantly following TAA administration. Significant increase in blood ammonia levels due to acute liver failure led to rise in brain ammonia level, which mounted neuroinflammation and resulted in hepatic encephalopathy characterized by a series of neuropsychiatric symptoms such as impaired grading score, locomotion, and cognition. Although ATX inhibitor failed to protection from TAA induced liver injury, it alleviated neuroinflammation as evident from decrease in inflammatory cytokine (TNF-α, IL-1β & IL-6) levels. It also improved the neuropsychiatric symptoms namely grading score, locomotor activity and cognitive function supported by significant elevation in BDNF level. To delineate the mechanism of action, we investigated the effect of ATX inhibitor in ammonium chloride induced neuroinflammation in astrocytes and glial cells. Interestingly, it exhibited significant decrease in inflammatory cytokines release and oxidative stress markers.

In BDL induced model of chronic liver disease, AST, Alt and TBIL were found to be elevated. Increased brain ammonia level because of increased blood ammonia levels resulted in development of hepatic encephalopathy as illustrated by abnormal locomotor activity, cognitive deficits. Ammonia induced neuroinflammation was quite apparent as inflammatory cytokines were found elevated in the brain samples of BDL animals. A potent ATX inhibitor CBT-295 showed favourable pharmacokinetic properties in rats. The therapeutic potential was evaluated in BDL induced chronic liver disease in rats. It demonstrated improvement in progression of fibrosis as liver collagen deposition was significant decreased following chronic treatment. Furthermore, it also improved the neuropsychiatric symptoms of hepatic encephalopathy in BDL rats.

As reported, BDL animals also developed cholemic nephropathy as evident from impaired renal function. Plasma creatinine and blood urea levels were significantly increased compared to sham animals. Kidney hydroxyproline content as marker of renal fibrosis was significantly higher in BDL rats. Interestingly, LPA levels were found increased in kidney samples of BDL rats. Unfortunately, ATX inhibitor failed to protect injury in BDL rats as no significant change was observed in renal function and renal hydroxyproline content were observed compared to vehicle treatment group.

Conclusion: ATX-LPA signalling was found to be involved in the pathogenesis of acute liver injury and development of hepatic encephalopathy. Although ATX inhibitor failed to protect from liber injury, it alleviates ammonia induced neuroinflammation in the brain samples and thereby alleviating neuropsychiatric symptoms of hepatic encephalopathy. Furthermore, reduction of neuroinflammation by ATX inhibitor was confirmed in ammonium chloride induced inflammatory cytokines release in astrocytes and microglial cells. Role ATX-LPA was also found evident in the progression of biliary fibrosis and ATX inhibitor hindered this progression. It also protected the development of neuroinflammation and alleviated development of neuropsychiatric symptoms of

hepatic encephalopathy. But it could not safeguard development of renal impairment associated with chronic liver disease. However, more potent ATX inhibitor can be evaluated for efficacy in cholemic nephropathy. Therefore, inhibition of ATX-LPA signalling can proved to be a multimodal therapeutic approach acute and chronic liver disease and encompassing associated hepatic encephalopathy and renal dysfunction.

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Abbreviations

ALF- Acute liver failure

ALT- Alanine transaminase

AST- Aspartate aminotransferase

ATX- Autotaxin

ATXi- ATX inhibitor

BDL- Bile duct ligation

BDNF- Brain derived neurotrophic factor.

CCI- Chronic constriction injury

CLD- Chronic liver disease

CNS- Central nervous system

CSF- Cerebrospinal fluid

CTGF: connective tissue growth factor

ECM- Extracellular matrix

FXR- Farnesoid X receptor

GABA- Gamma-amino butyric acid

GLP-1- Glucagon-like peptide-1

HE- Hepatic encephalopathy

HRS- Hepatorenal syndrome

HSC- Hepatic stellate cells

HSCs- Hepatic stellate cells

LPA- Lysophosphatidic acid

NAFLD- Non-alcoholic fatty liver disease

NASH- Non-alcoholic steatohepatitis

NO- Nitric oxide

NSAIDs- Nonsteroid anti-inflammatory drugs

PPAR-γ- Peroxisome proliferator activated receptor

PUFA- Polyunsaturated fatty acids

TAA- Thioacetamide

TBIL- Total bilirubin

UUO- Unilateral ureter obstruction

Chapter 1. Introduction and Literature Review

1.1. Liver disease

Hepatic disorers is one of the foremost reason of deaths globally which encompasses an extended array of ailments having diverse or indefinite etiology. Approximately 1.32 million or 2 to 4% of all annual mortality globally took place because of cirrhosis (Seto and Susan Mandell 2021). As per the report published by WHO in 2017, in India as well, dealths due to hepatic disease touched to 259,749 i.e. 2.95% of total deaths, accounting for 1/5th (18.3%) of all cirrhosis mortality globally (Mishra *et al.* 2020). Non-alcoholic fatty liver disease (NAFLD) is known to be the leading hepatic disorders globally, involving 25% of total global inhabitants. Viral hepatitis is also a key reason for causing cirrhosis and triggers increased disease load throughout the world. Based on 6754 studies published between 2013, and 2016, viral hepatitis because of hepatitis C virus infection has affected about 71 million individuals on the other hand hepatitis B virus (HBV) a the foremost reason for producing cirrhosis leading to growth of disease burden throughout the world with estimated occurrence of 257 million in 2016 (Rancoule *et al.* 2015) (Seto *et al.* 2018).

1.1.1. Acute liver failure (ALF)

Acute liver failure (ALF) being an extreme consequence of sudden liver injury can become fatal. With an occurance of about 10 cases per million persons per year, acute liver failure exhibits great challenges in therapeutic management. Several injuries to hepatocytes lead to abrupt increase of aspartate transaminase and alanine transaminase, hepatic encephalopathy, and coagulation defects (Williams *et al.* 1993). The lack of pre-existing hepatic ailment differentiates acute liver failure out of deteriorated chronic liver disorder or acute episode of chronic liver diseases (Alexander *et al.* 1989). Major etiology of ALF encompassess acetaminophen, herbal and dietary supplements induced liver injury, liver impaired perfusion, hepatitis A, B and C related and autoimmune hepatitis, and drug-induced liver injury. ALF is detected through assessment of transaminases, current

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medication, and previous viral exposure if any. Although recent developments in the therapies have shown decrease in mortality, liver transplantation in most of the patient still confirmed to be a better option to medicine (Stravitz and Lee 2019). Acute liver failure is a very uncommon but alarming ailment mostly observed in patients who is not already suffering from liver disorders. The clinical symptoms generally involve liver dysfunction, abnormal liver markers, impaired coagulation and hepatic encephalopathy leading to multiorgan failure and eventually death in majority of cases (Strauss *et al.* 2001). Public health precautions such as vaccination and personal hygiene are some of the measures which can reduce the occurance of liver infections. However, xenobiotic-induced hepatotoxicity has been reported to be the foremost reason of acute liver failure (O'Grady 2005)

1.1.2. Chronic liver disease

Chronic liver disease in the is defined as a disorder of the liver in which a process of gradual damage and regeneration of the liver parenchyma results in development of fibrosis and subsequently cirrhosis if not halted (Blachier *et al.* 2013). "Chronic liver disease" implies to a liver disease which continues over a period of six months. It includes a wide range of liver pathologies comprising chronic hepatitis, liver fibrosis, liver cirrhosis, and hepatocellular carcinoma (Tsochatzis *et al.* 2014).

1.1.2.1. Liver fibrosis:

Fibrosis occurs due to chronic liver injury from several insults which includes toxins (alcohol), drugs, viral infections like hepatitis B and C, and hyperlipidaemia (non-alcoholic fatty liver disease). These insults mount an inflammatory response leading to increased production and accumulation of extracellular matrices, and reduced regeneration and wound healing reactions (Nathwani *et al.* 2019). Being a complicated and aggressive, it involves engagement and platelets activation, kupffer cell, hepatic stellate and other extracellular matrix generating cells like portal fibroblasts, hepatocytes, cholangiocytes,

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and bone marrow derived cells. Hepatic fibrosis is an important predictive indicator in chronic liver disorder, regardless of etiologies. Normally, progresses over years and typically takes 20 to 40 years, however it can become very aggressive, as evident in children with from bile duct atresia, medicine-related hepatic toxicity, HCV infection in combination with HIV, and liver transplanted patients infected with HCV. If not intervened, fibrosis finally leads to cirrhosis, which is characterised by significant alteration of hepatic microarchitecture (Hernandez-Gea and Friedman 2011).
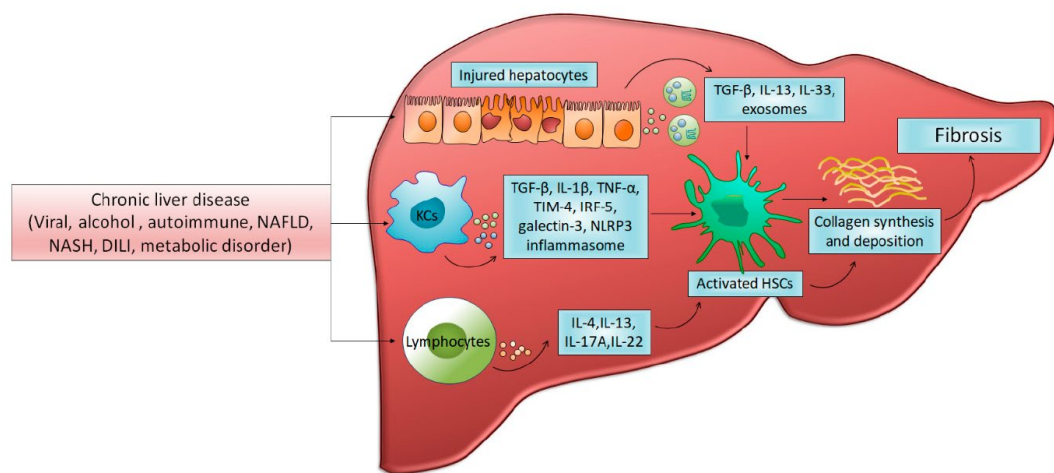
Figure 1. Mechanisms of liver fibrosis (Khanam, Saleeb, and Kottilil 2021)

1.1.2.2. Liver cirrhosis:

Fibrosis takes place as result of covering or substitution of damaged parenchyma cells by extracellular matrices. Fibrosis occurs out of contineous normal wound healing process progressing to an abnormal fibrogenesis due to connective tissue generation and deposition. The progression of fibrosis depends on on the cause of hepatic disorder, environment, and patient related factors (Schuppan and Afdhal 2008). Fibrosis eventually progresses to cirrhosis if not halted, which is charaterized by alteration of the liver arrangement of blood vessels in regenerative nodules of hepatic parenchyma enclosed in fibrotic septa. The clinical outcomes of cirrhosis are greater deaths rates, morbidity, portal hypertension linked ailments, and poor life quality (Pinzani 2015). In cirrhosis, it mostly results in thrusting of the hepatic and artery blood flow straight way into the liver outlay,

eluding interchange between liver sinusoids and liver cells. Histology of cirrhotic liver presents as blood vesels rich fibrotic septa which joins portal zones together and link with central veins, resulting to formation of liver cell islands that are encircled by fibrotic septa devoid of central vein. The serious outcomes of hepatic cirrhosis include hepatic dysfunction, an elevated hinderance within hepatic vasucalature leading to portal hypertension and ultimately progresses to liver cancer. The circulation related aberration associated cirrhosis such as dilation of vasulature around viscera, constriction of renal blood vessels leading to reduced blood & water to kidneys resulting in salt holding, elevated ventricular ejection is directly linked to changes in the liver blood vessels and portal hypertension. Cirrhosis plus realted circulatory defects until now were considered as permanent damages but recent advances in research advocates that lessening or to an extent reversal of cirrhosis is feasible (Schuppan and Afdhal 2008) (Desmet and Roskams 2004) (Wanless *et al.* 2000).
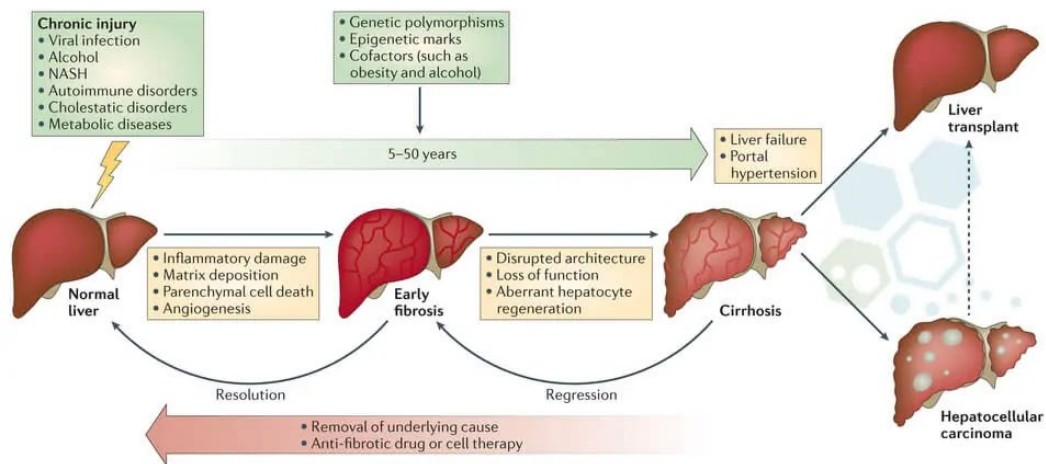
Figure 2: Types of hepatic diseases (https://stemcellthailand.org/therapies/liver-disease-cirrhosis-hepatocyte/)

1.1.2.3. Hepatocellular carcinoma (HCC):

Hepatocellular carcinoma, the primary haptic tumor has proved to be a most prominent reason of cancer associated mortality globally. In US, HCC stands the 9th leading reason of mortality related to cancer (Bruix and Sherman 2011). Although there have been

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advancements in prophylactic measures, examination, and discovery of techniques for both diagnosis and treatment, incidence and mortality are still rising. Cirrhosis is the most prominent cause leading to HCC. Hepatitis B and C infections are the other causative elements for the development of cirrhosis. Alcohol consumption as well is an additional threat as alcohol abuse is rising relatively more than hepatitis C (Singal and Anand 2007). Inspite of availability of multiple therapeutic options, liver transplantation or partial hepatectomy are the only curative procedures till date. HCC is an aggressive cancer that takes place greatly in cirrhotic patients. HCC can be circumvented if appropriate measures are taken, which comprices hepatitis B immunization, testing of blood products, sterile injection techniques, proper medicines and rehabilitation of alcoholics and drug addicts practicing parenteral administration, and timely intervention with antiviral drugs (Balogh *et al.* 2016).

1.2. Complications of liver failure:

Cirrhosis eventually leads to portal hypertension in addition to liver failure. Cirrhosis itself or together with portal hypertension may bring in various ailments, like peritonitis, ascites, varices, encephalopathy, live cancer, hepatopulmonary syndrome, and cogulation disorder (Nusrat *et al.* 2014). Hepatorenal syndrome (HRS) is also a critical consequence of hepatic cirrhosis often exhibits prediction (Ng *et al.* 2007). Kidney damage may also occur in patient with severe jaundice which is called as as cholemic nephropathy (Krones *et al.* 2018).

1.2.1. Hepatic encephalopathy:

Hepatic encephalopathy (HE), a disease of altered performation of brain mostly takes place late phase of hepatic disorders. The precise etiology of HE remains unclear. However, the current assumption on pathophysiology suggests the involvement of neurotoxins, neuroinflammation and modified neurotransmission because of metabolic alteration due of hepatic failure, change in CNS energy turnover, generalized

inflammation, and alteration in the BBB. HE is known to cause a sequence of broad neuropsychiatric indications (Blei *et al.* 2002). Approximately 20 to 80% of patient with cirrhosis shows symptoms of HE. Around 10 to 14% patients present evident HE during the diagnosis of cirrhosis. This incidence rises to 16 to 21% with deterioration of cirrhosis and about 10 to 50% in case of patients suffering from transjugular intrahepatic portosystemic shunt (TIPS). Mostly, about 30% to 40% of patients with cirrhosis gets affected from HE (Elsaid and Rustgi 2020). But acute hepatic failure accounts about 20% of mortality probably because of hepatic encephalopathy. Approximately 50% of mortality with liver cirrhosis occurs in a year due to severe HE. HE can exhibit a wide spectrum of neurologic, neuropsychiatric, and musculoskeletal manfestations. These symptions remain unnoticed in many incidences (Córdoba *et al.* 1998). At initiat phase, patients may only feel subtle indications, such as altered day and night cycles. As disease advances, patients may experience symptoms such as apathy, irritability, and disinhibition. If these disorders are not intervened immediately, it may eventually lead to cognitive impairments such as disorientation, amnesia, speech alteration, confusion, and finally coma. In addition to neurological abnormalities, HE also affects the musculoskeletal activities. However, minimal HE may demonstrate minor symptoms of muscle coordination, like handwriting changes etc (Lizardi-Cervera *et al.* 2003).

Several components are involved in the pathogenesis of HE, which comprise ammonia (NH3), Inflammation causing cytokines, manganese accumulation in basal ganglia, gamma-amino butyric acid (GABA) etc. Latest investigations too advocate the roles for microbial flora plus aromatic amino acids in the pathophysiology of HE. Although the aetiology of HE is complex, and several altered signalling pathways lead to neuronal cells abnormality, the axact mechanism is remains still unclear. After detailed investigation, NH3 is considered as the major causation component for pathogenesis of HE. NH3 is generated from gut by break down of dietary proteins consumed in diet by microbiota.

NH3 is mostly detoxified in liver followed by renal eleminated and to a smaller amount through the muscles (Tapper *et al.* 2015). As liver function is compromised cirrhotic patient, NH3 does not get detoxified, and it enters the systemic circulation. NH3 freely crosses the blood-brain barrier and astrocytes in brain convert glutamate to glutamine with the help of glutamine synthetase. In astrocytes, deposition of glutamine produces an osmotic imbalance resulting in swelling of astrocyte and generation of reactive oxygen species. This process leads to impairment of cerebral function. The alteration of gut flora and increased gut absorptivity as apperent in cirrhosis too lead to increased production of several cytokines, which enhances the BBB penetration leading to cerebral edema. Furthermore, NH_3 also acts on gamma-aminobutyric acid receptors of astrocytes and results in stimulation and production of neurosteroids, and it in turn contribute to the episodes of HE as well (Gerber and Schomerus 2000). Additionally, as severe HE is linked to elevated NH3 levels, recent investigations advocate that interference with gut microflora can considered to be an essential factor in HE. Since cirrhosis ultimately leads to a decompensated state, the microbiota gets converted into dysbiosis state, resulting in increased inflammation and cholestasis, thereby lowering bile acid production, and consequently causing an increased growth of disease-causing microbes because the gut bile acids levels are reduced (Elwir and Rahimi 2017).

1.2.1.1. Hepatic encephalopathy and neuroinflammation

HE is a most prominent syptoms of central nervous system of critical acute and chronic hepatic disorders. Though increased NH3 level is the major factor for pathogenesis of HE, as latest reports have revealed a substantial role of inflammation of CNS in the pathophysiology of acute as well as chronic, HE (Jayakumar *et al.* 2015), Wright *et al* in 2007 reported that increased levels of serum TNF-α, interleukin-1b (IL-1b) and interleukin-6 (IL-6) in parallel with brain cytokine penetration in acute HE patients indicated the involvement of a neuroinflammation in HE (Wright *et al.* 2007). Microglial

and astrocytes get activated promptly with the advancement of acute liver failure and consequently cause encephalopathy and brain edema. ALF because of either depletion hepatic vascularization or noxiuos hepatic injury leads to triggering of microglial cells and simultaneous increase in brain inflammatory cytokines levels including TNF-a, IL-1b, and IL-6 (Jiang *et al.* 2009).

1.2.1.2. Role of Microglia in HE:

Microglia activation has been proved to be an important reason for the pathological process of HE irrespective of causes. There are clinical reports showing involvement of stimulation of microglial cells as ionized calcium binding adaptor molecule 1 (Iba-1) was observed realatively elevated more in the cortex of individual who had HE than devoid of HE (Zemtsova *et al.* 2011). Furthermore, complete mRNA examination revealed an overexpression of indicators of inflammatoion causing M1 as well as anti-inflammatory M2 traits of microglial cells, which indicates that M1 along with M2 microglial cells might be responsible for causing HE in cirrhosis patients (Görg *et al.* 2013). In conclusion, these clinical data advocates involvement of microglia activation in HE (McMillin and DeMorrow 2017).

1.2.1.3. Role of Astrocytes in HE:

the utmost abundant glial cell, astrocytes in the brain are important as it provides growth and metabolic aid to the neurons, facilitates neuronal-glial communication. It is also important for blood brain barrier function. Increased ammonia concentrations during HE changes the morphology of astrocytes and leads to development of big light nuclei recognised as Alzheimer T II astrocytosis. Alzheimer Type II astrocytic nuclei are ample as apparent by auto-radiography in animal model methionine sulfoxamine induced hyperammonemia rats (Brumback and Lapham 1989). Hyperammonemia results in upregulation of glutamine synthesis in the astrocytes, causing elevated osmotic pressure there by produces astrocyte swelling. Astrocytes swelling can lead to brain edema

deteriorating the hepatic encephalopathy symptoms. Recent investigation suggests astrocytes are actively implicated in the etiology of hepatic encephalopathy as it causes oxidative stress, increased production of glutamine and glutamine synthase, imbalance in H_2O content in brain, and lactate turnover (Jaeger *et al.* 2019).

1.2.1.4. Cytokines involved in pathogenesis of HE:

Cirrhotic individual as well as disease models of HE shows generalized inflammation which further worsens neurological pathology. It is and evident that inflammation causing impetus work simultaneous with NH_3 to cause the central nervous system ailments of acute liver failure and chronic liver disorders (Butterworth 2013). TNF-α, being a strong inflammatory cytokine has been proved to promote microglial cell activation in various models of neuroinflammation. Serum levels of TNF-α are found to be elevated as a measure of the severity of HE in both patients and preclinical models of liver disorders (Odeh 2007) (Jiang *et al.* 2009). Furthermore, the presence of TNF-α gene polymorphisms has been accepted to impact the consequences of individual with ALF. In patients suffering from HE, serum TNF-α concentrations are elevated in animal model of azoxymethane induced ALF. Amlioration of TNF-α pathway by intervention with TNF-α antibody, etanercept attenuated generalized inflammation, lowered neuropsychiatric impairment, because of hindered microglial activation in the cerebral cortex (Chastre *et al.* 2012). Therefore, the results advocate the theory that TNF-α, may also trigger microglia cells and related CNS dysfunction in liver failure cases. Furthermore, in agreement of this hypothesis, neurological ailments occuring in bile duct ligated mice might be the outcome of monocyte enrolment because of TNF-α signalling which is mediated through microglial activation. Particularly, TNF-α signalling in periphery provokes microglia to generate monocyte chemoattractant protein-1 (MCP-1), which therefore facilitates monocyte recruitment in the brain. Other pro-inflammatory mediators in addition to TNF-α can also be involved in causing HE. Interleukin-1β and stimulation of microglial cells are enhanced

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in rats following the portacaval shunt. Sildenafil, a phosphodiesterase type 5 inhibitor has shown reduction in neuroinflammation and microglia activation (Agusti *et al.* 2017). In azoxymethane induced hyperammonemia model, cortical Interleukin-1β, Interleukin--6, TNF-α along with MCP-1 were found upregulated. Treatment with TGF β1 antibodies or knocking down TGF-β receptor 2 in neurons of azoxymethane model resulted in attenuation of microglia activation and normalized the levels of IL-1β, IL-6, TNF-α and MCP-1, indicating that TGFβR2-mediated signalling is involved in causing neuroinflammation in HE (McMillin *et al.* 2019). Surprisingly, although treatment of the gamma aminobutyric acid antagonist, bicuculine lowered the level of Interleukin-1β nevertheless did not affect microglial stimulation in NH4Cl induced hyperammonia (Malaguarnera *et al.* 2019). Therefore, more studies are required in this area for better understand this pathogenesis.

1.2.2. Cognitive impairment in HE:

Minimal hepatic encephalopathy (MHE) leads to mild memory deficits such as attention deficit and alteration in short term memory, problem solving, reason, task flexibility, decision-making etc. HE is a complicated neurobehavioral condition caused because of distorted CNS function associated with hepatic cirrhosis. Approximately 33 to 55% of individuals with cirrhosis devoid of overt HE symptoms exhibits MHE alongside mild cognitive impairment (García-García *et al.* 2017). The combined effect of hyperammonemia and inflammation in patients with cirrhosis, exhibit not only cognitive impairment, but also attention and co-ordination deficits. The cognitive dysfunction results in memory and learning deficits because of impairment of structural and functional hippocampus (García-García *et al.* 2018). Early diagnosis of MHE shows a surprisingly high incidence of mild cognitive and psychomotor impairment (Felipo 2013). In agreement, memory loss is also evident in mouse model of thioacetamide induced ALF (Yuan *et al.* 2020). CLD itself causes neuroinflammation that found to be involved in

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cognitive deficits in hepatic encephalopathy. Mature brain derived neurotrophic factor (BDNF) and its precursor expression and its uphill transcription factors are significantly reduced in brain of BDL rats. Altogather, these results support the fact that neuroinflammation and reduced BDNF and its transcription factor related to its signalling pathway might be substially involved in the pathogenesis of memory deficit related to HE (Dhanda *et al.* 2018) (Kawai *et al.* 2012)Click or tap here to enter text..

1.2.3. Motor dysfunction in HE:

Motor dysfunction is an important clinical attribute in cirrhotic patients and minimal hepatic encephalopathy (Mechtcheriakov *et al.* 2006). HE occurs either from ALF or CLD which results in various complications, along with locomotor deficit. Bile duct ligated rats causes CLD and demonstrates diminished locomotiom, more inactive time, reduced rearings, prolonged stay in fixed place and reduced stay in the centre of the cage (Leke *et al.* 2012). Similarly, individuals with minimal hepatic encephalopathy demonstrate poor locomotor activity out of individuals devoid of MHE. MHE illustrates reduced motor control also known as hypokinesia (Martín-Valenzuela *et al.* 2020).

1.2.4. Cholemic nephropathy:

Cholemic nephropathy (CN) is caused by severe renal damage due to excessive increased bilirubin level (Tinti *et al.* 2021). The pathophysiology of CN is still not very clear. It is trigerred by injury created by cholephiles and bile casts accumulation in kidney which results in epithelium injury and occlusion of kidney tubules. Classically, of bile accumulation in CN, leads to interstitial nephritis (Fickert and Rosenkranz 2020). CN evidently corresponds to a most underrated though essential reason of kidney impairment in impaired bile flow or late stage of hepatic disorders with hyperbilirubimia. CN illustrates kidney dysfunction attributed to typical histological changes which includes intratubular bile cast accumulation and damage to the epithelium of remal tubules extended towards distant nephron parts (Krones *et al.* 2018). Increased remal elimination

of bile acids in cholestasis occurs due to redundant extent of bile salts surpassing the utmost limit for reabsorption by renal tubules leading to increased secretion which may result in kidney damage (Tinti *et al.* 2021). Bile acids is also known directly to induce oxidative stress in cell membranes of renal tubules, which eventually causes secreation of vasocontrictive factors that subsequently impact kidney dysfunction via constriction of renal blood vessels and thereby lowering GFR (Fickert *et al.* 2013). Furthermore, reduced albumin levels may lower the blood volume. The decline in the kidney perfusion increases bilirubin in kiney tubules and results biliary casts formation (Elsom 1937). The probable renal toxic effect of bilirubin leads to bilirubin deposition in mitochondria and eventually leading to attenuation of oxidative phosphorylation which results in reduced ATP activity. This leads to dysfunstion of mitochondria and enhanced penetratability of cell membranes, causing imbalance in electrolyte levels and cellular mass (Elias *et al.* 1985).
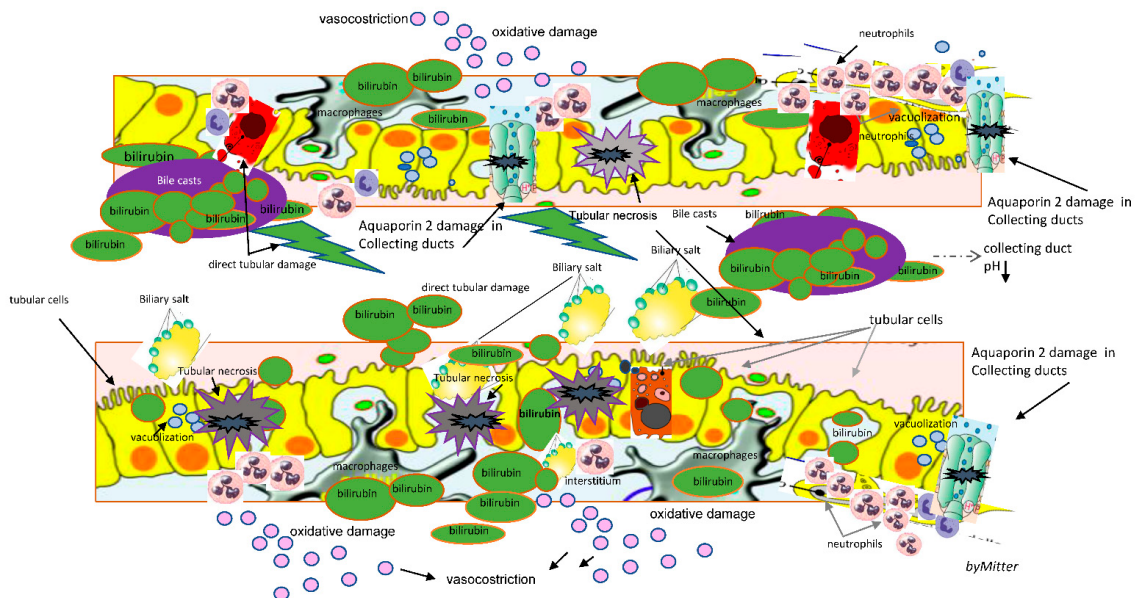
Figure 3: Illustration of pathological progression of cholemic nephropathy (Tinti *et al.* 2021).

1.2.5. Hepatorenal Syndrome:

Hepatorenal syndrome (HRS) is a life threating symptom associated with liver cirrhosis. It takes place due to excessive renal vasoconstriction because of alteration in viscreral and

systemic circulations along with generalized and kindney vasoconstriction (Ng *et al.* 2007). This complication is possibly the ultimate outcome of greately reduced perfusion of arteries as a concequence to vasodilation arteries in the visceral blood vessels along with the kidney flow as most blood vessels other than splanchnic vascular beds are constricted. The prediction appers to be very poor, particularly when when the renal failure occurs very aggressively (Lautrette *et al.* 2009).
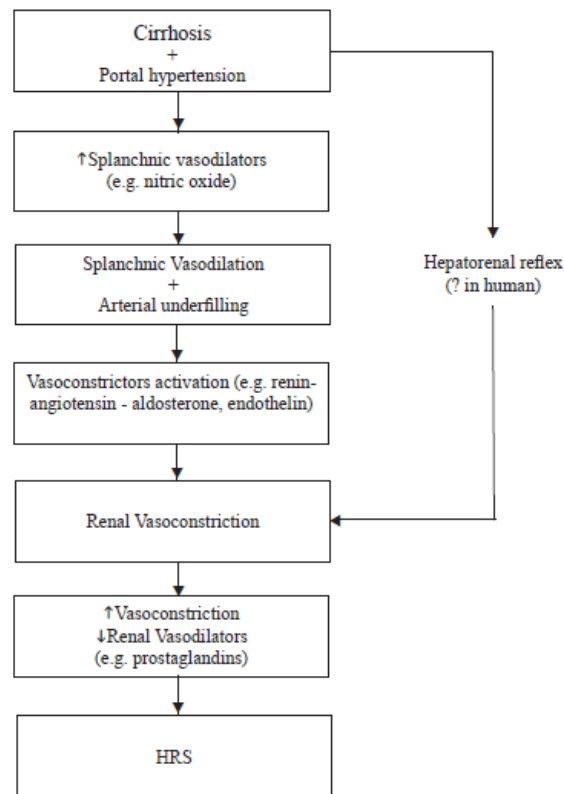
Figure 4: Pathogenesis of Hepatorenal syndrome (Ng *et al.* 2007) (Arroyo *et al.* 2002).

1.2.6. Coagulation disorder:

The liver contributes significantly to the clotting process, and acute and chronic liver diseases are aften leads to coagulation disorders due to several reasons. It causes reduced synthesis of clotting and inhibitor factors, reduced removal of activated factors, platelet depletion, hyperfibrinolysis etc (Soultati and Dourakis 2006). The possibility of bleeding leads to increase the chances of illness along with deaths associated with liver disorders undergoing surgeries or invasive procedures. Unpredicted coagulation defects are

prevalent in person with acute liver desease related to pregnancy or undergoing liver transplantation(Kujovich 2015).

1.2.7. Ascites:

Ascites is defined as accumulation of fluid in peritoneal cavity. This is very common in patient with liver diseases. The two important reasons responsible for development of ascites are hypertension portal vein and sodium along with water holding. Structural changes due to progressive fibrosis are most importantant mechanism that causes intrahepatic resistance in portal flow in liver cirrhosis (Fleming *et al.* 2010). Furthermore, as hepatic stellate cells undergo phenotypic changes, liver sinusoidal endothelium also show important involvement in causing ascitis. Once stellate cells get trigerred, these become contractive, its enrolment surrounding the newly made sinusoidal vascular bed increases the resistance in blood vessels (Nusrat *et al.* 2014). Reduced generation and availability of NO in liver of individual with cirrhosis also increases constriction of blood vessels. Constriction of blood vessels is expected to contrubute around 25% of the enhanced resistance in the liver. Elevated pressure build in portal vein is sensed by gut microcirculation which produces angiogenic factors (VEGF etc.), that in turn activates the progression of portosystemic side by side brances by unblocking previous vasculatures or forming new vessels. Once the pressure in the portal vein increases more, generation of NO synthase in endothelium increases leading to excessive generation of nitric oxide resulting in vasodilatation of arteries in visceral circulation. Eventually, portal blood flow gets elevated, thereby worsening blood pressure the portal vein. Portal as well as systemic parallel brances further allow dilators of blood vessells such as NO, PGI2 and endocannabinoids to reach the blood circulation resulting in a state of effective reduced blood volume. This stimulates adrenergic system to increase the reintake of Na^+ from proximal, distal tubules, loop of Henle and collecting duct through activation of RAAS system, leading to increased Na^+ reintake from distal tubule and collecting duct. Sodium

reabsorption by kidney and consequent water removal because of relaese of arginine along with vasopressin activity through V2 receptor present in collecting duct leads to water accumulation resulting in edema and ascites associated with cirrhosis (Cárdenas and Arroyo 2003) (Aithal *et al.* 2021).

1.3. Management of liver fibrosis

Liver fibrosis is one of the foremost reasons of illness and deaths globally. Even though there are reports advocating, reversal of early fibrosis, the exact mechanism of attenuation of fibrosis is still unclear (Zoubek *et al.* 2017). Therefore, the significance of research and development for ant-fibrotic drug has increased in recent years. Many approaches have been developed so far as to hinder the incidence and occurance of hepatic fibrosis, which includes anti-inflammatory therapy and protecting liver, inhibiting liver stellate cells stimulation and production, lowering of extracellular matrix overexpression, and increasing ECM brakedown. Furthermore, gene treatment may be an encouraging anti-fibrotic approach as evident in recent years (Tan *et al.* 2021).
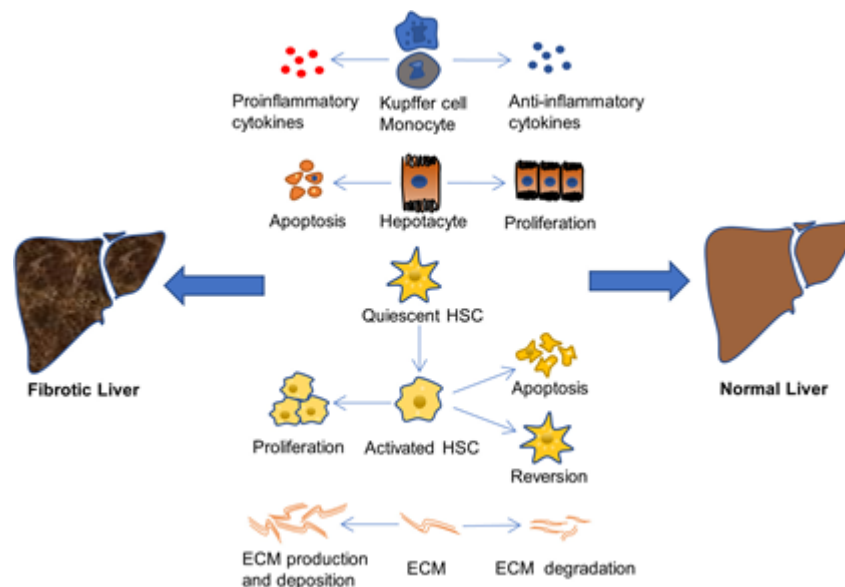
Figure 5: Therapeutic approaches for liver fibrosis (Roehlen *et al.* 2020).

The treatment choices mostly aim causative components of hepatic fibrosis to speed up the restoration of hepatic damage, or estblish equilibrium of liver metabolic function, such as anti-viral, anti-apoptotic, and regulators of lipid turnover etc (Zhang *et al.* 2023a).

Biological interference:

LOXL2 suppression of in liver cancer following cirrhosis has been shown to augment the effect of sorafenib and 5-fluorouracil in liver cancer cells. However, this approach did not produce promising results in some clinical trials (Gong *et al.* 2022).

1.3.1. Drug therapy:

A liver stearoyl-CoA desaturase blocker, Aramchol, has been found effective in reversing non alcoholic steatohepatis and hepatic fibrosis in preclinical model, in addition to reduction in liver lipids and fibrosis in clinical trials (Ratziu *et al.* 2022).

1.3.2. Anti viral drugs:

These drugs in patient with hepatic viral contagion have attenuated hepatic inflammation and liver cell mortality to reduce the hepatic damage, leading to ratard the development of Hepatic fibrosis. Medicines like faldaprevir, ribavirin (Wu *et al.* 2016), and peginterferon alfa-2a were found effective in clinics for therapy of hepatic fibrosis (Zhang *et al.* 2023a).

1.3.3. Cenicriviroc:

CCR 2 plus 5 dual blockers, was found promising in reducing liver fibrosis thereby avoiding deterioration of NASH in clinics (Clinicaltrials.gov, NCT02217475) (Ratziu *et al.* 2020).

1.3.4. Cholangitis therapy:

Obeticholic acid and ursodeoxycholic acid proved to be the lone medications which got approval for the treatment of primary biliary cholangitis, which subsequently leads to development liver fibrosis if to halted (Bernal *et al.* 2023).

1.3.5. Cyclophilin blockers:

A pan blocker of cyclophilin, CRV431, proved to be effective in amliorating liver fibrosis in CCl4 and high fat diet induced non alcoholic steatohepatitis. NV556 as well efficacy inreducing fibrosis non alcoholic steatohepatitis models such as STAM model and MCD models. Furthermore, it also attenauted transforming growth factor-β1 stimulated hepatic stellate cells triggering in *in vitro* studies (Serrano *et al.* 2019).

1.3.6. FGF regulators or analogues:

Pegbelfermin, a PEGylated human FGF21 analogue, markedly reduced accumulation of hepatic lipid content in individual suffering from non alcoholic steatohepatitis and did not show any major side effect. It lowered hepatic fibrosis in individual with obesity and type 2 diabetes (Sanyal *et al.* 2018).

1.3.7. FXR agonists:

Obeticholic acid, an farnesoid X receptor ligand, considerably inhibited hepatic fibrosis thereby improved biomarkers of non-alcoholic steatohepatitis as well as in biopsy sample (Younossi *et al.* 2019). Tropifexor, another farnesoid X receptor ligand, also markedly reduced cholestasis induced hepatic injury and fibrosis and the content of small heterodimer partner in livers of piglets, however it also inhibits cholesterol 7α-hydroxylase. Furthermore, tropifexor also enhances the colonies of bile acid-forming microbes and later the amino acid ratio in the gut and lowers gut barrier damage in bile duct ligated piglets (Xiao *et al.* 2021).

1.3.8. Gal-3 inhibitors:

GB1211, a Gal-3inhibitor, inhibits the epithelium differentiation into myofibroblasts and macrophage thereby reduces liver fibrosis (Zetterberg *et al.* 2022).

1.3.9. Glucagon-like peptide-1 (GLP-1) receptor agonist:

GLP-1 analogues showed attenuation of Hepatic lipd deposition, hepatic damage, and insulin resistance in mouse model of NASH. Clinical trial demonstrated that liraglutide

treatment reduced hepatic fibrosis development in non-alcoholic steatohepatitis patients (Cegla 2016).

1.3.10. Pan-caspase inhibitor:

Emricasan, a pan-caspase inhibitor, amliorates hepatocyte apoptosis and inflammatory reaction and lowers pressure in portal vein in rat model of CCl4-induced liver injury (Frenette *et al.* 2021).

1.3.11. PPAR agonists:

PPAR-γ agonist thiazolidinedione reduced liver fibrosis in BDL rats by inhibiting hepatic stellate cell stimulation and fibrosis by reducing causative factors, such as transforming growth factor-β, PGDF, and CTGF etc (Alatas *et al.* 2020).

1.3.12. Natural products or herbal medicines:

Herbal medicines may have potential for the treatment of liver fibrosis. Yinchenhao decoction, a traditional chinese medicine has shown reduction in DMN-induced liver fibrosis in rats by inhibiting hepatocyte apoptosis (Cai *et al.* 2019).

1.3.13. Dietary regulation or supplementation consumption of PUFA:

19,20-epoxy docosapentaenoic acid, an endogenous metabolite of PUFA has inhibited hepatic fibrosis in mouse non-alcoholic steatohepatitis models. It acts by increasing G-protein-coupled receptors to amliorate hepatic inflammatory reaction and fibrosis. Addition of docosahexaenoic acid (omega-3 fatty acid), also lowers hepatic inflammatory response and retards hepatic fibrosis in HFD-induced liver fibrosis model through G-protein coupled receptor-120, through a free fatty acid receptor signalling (Zhang *et al.* 2023b).

1.3.14. Probiotics:

Lactobacillus rhamnosus inhibits hepatic inflammatory reaction and fibrosis markedly by lowering the generation of liver bile salts in mice with bile duct ligation (Liu *et al.* 2020).

1.3.15. Antioxidant and anti-inflammatory agents:

Natural products supplementation containing antioxidant as well as anti-inflammatory components reduces CLD. β-sitosterol plus silymarin have been proved to cause improvement in liver fibrosis and incidence of cancer development (Zhang *et al.* 2023a).

Thus, the goal of current treatment approaches for hepatic fibrosis remains directed to inhibit the early triggering factors for hepatic inflammation, liver cell apoptosis and reactive oxygen species generation. But reversal of liver fibrosis is slow and most often difficult to halt the progression to fibrosis or cirrhosis. For the advance phase of hepatic cirrhosis and hepatocellular cancer, the only cure is liver transplantation. Intervention with anti-fibrotic treatments as well as biological, drugs, alteration in food habbit etc. are required to hinder the advancement of hepatic fibrosis and cirrhosis to life-threatening stage.

1.4. Management of hepatic encephalopathy

The choice of treatment for HE depends on seriousness of the disease. NH_3 is major culprit, and the target of therapy is to reduce the generation of ammonia and increasing the elimination of NH_3 from the circulation. But NH_3 turnover is complicated, and it is controlled by various tissues namely liver, muscles, kidneys, and brain. Furthermore, absence of therapy for other trigerring mediators associated in the pathogenesis of HE, namely oxidative stress, inflammation, or other cerebral alterations, is a foremost hindrence in the treatment of HE. Some of the most recent and upcoming therapies for HE is discussed below (Hadjihambi *et al.* 2018).
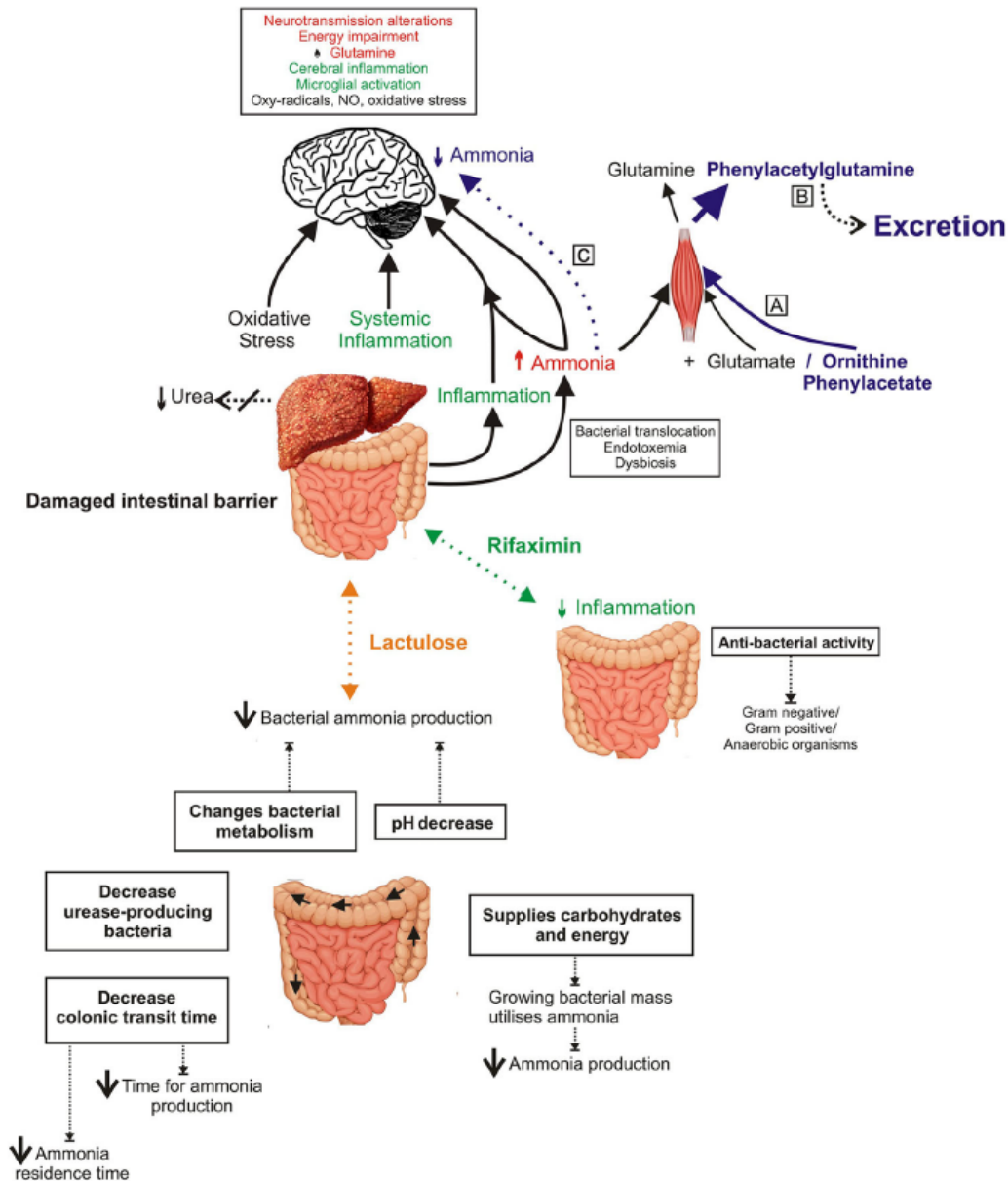
Figure 6: Mediators involved in causing hepatic encephalopathy and therapeutic modalities (Hadjihambi *et al.* 2018).

1.4.1. Disaccharides and polyethylene glycol

The well-known treatment, Lactulose and lactitol acts by reducing the absorption of NH_3 from gut to the circulation. Lactulose produces a high osmolar condition and acts as a purgative and eventually hinders absorption of NH_3 in gut (Gluud *et al.* 2013). However, extreme use of lactulose can lead to complications, like dehydration, reduction in Na^+ content and itching around anus. Furthermore, efficacy of lactulose is not very promising in clinics, which restricts the use of lactulose for prophylaxis (Bajaj *et al.* 2010).

1.4.2. Antibiotics

Antibiotics are also used for treatment of HE. Neomycin, which is an absorbable antibiotis has been earlier tried widely in the treatment of overt hepatic encephalopathy (Conn *et al.* 1977). Rifaximin, showing very less absorption is the extensively used for the treatment of hepatic encephalopathy. As patients tolerate rifaximin better, it is often used in combination with lactulose for better outcome (Falavigna *et al.* 2017). However, it increases the likelihood of adverse effect of Clostridium difficile infection (Rifaximin Therapy and Clostridium difficile Infection Diagnostic Testing for Clostridium difficile Infection in Patients With Inflammatory Bowel Disease 2013).

1.4.3. L-ornithine and L-aspartate

LOLA acting as substrate for the ornithine cycle enhances urea production in liver cells around portal tract. It also triggers glutamine synthesis by stimulating glutamine synthetase in liver cells around veins and muscular tissues. However, there are reports in which use of LOLA was found unsuccessful in lowering the NH_3 levels and the seriousness of hepatic encephalopathy in acute liver failure (Acharya *et al.* 2009) (Bai *et al.* 2013). Moreover, while LOLA primarily reduces blood ammonia levels, even in end-stage liver disease, its effects is short lived as a reflex hyperammonemia is often observed after stopping the drug (Hadjihambi *et al.* 2014).

1.4.4. Ornithine phenylacetate

Ornithine phenylacetate (OP) triggers the formation of glutamate and the elimination of glutamine. OP enhances glutamine synthetase action in external organs. Consequently, the enhanced glutamine synthesis causes in a net reduction of plasma NH_3 (Ventura-Cots et al. 2013). However, a latest report suggests that in which 38 cirrhotic patients were enrolled with GI bleeding showed that OP was finely tolerated but it could not decrease plasma ammonia levels significantly (Ventura-Cots *et al.* 2016).

1.4.5. Glycerol phenylbutyrate (HPN-100)

Glycerol phenylbutyrate (GPB) offers unconventional mechanism for NH_3 elimination and waste N_2 elimination in the form of phenylacetyl glutamine which allows to lower total circulatory blood glutamine and, subsequently, NH_3 production by glutaminase (Rockey *et al.* 2014). It has exhibited some efficacy in clinical trials, which suggests its therapeutic capability in prevention of hepatic encephalopeathy.

1.4.6. Albumin administration and dialysis

Albumin shows efficacy in HE patients as it has antioxidant properties and can reduce ROS. However, albumin treatment in individual with phase II or more hepatic encephalopeathy was not efficacious in lowering acuteness, NH_3 levels, ROS or cytokines, however it extended the life expectancy (Hassanein *et al.* 2007).

1.4.7. Portosystemic shunt occlusion

Large portosystemic shunts avoid the liver, and result in increased levels of NH_3 and hepatic encephalopeathy. Although embolization of these shunts corrects, hepatic encephalopeathy instantly, it is merely successful in the presence of partial liver function (Stoddard and Chun 2015) (An *et al.* 2014).

1.4.8. AST-120

AST-120 is an orally administered microspherical carbon, which acts by adsorbing ammonia. A study on AST-120 treatment in rodents with CLF treated showed reduced ammonia levels, corrected cerebral edema and motor function, but failed to show an effect on generalized ROS production (Bosoi *et al.* 2011).

1.4.9. Probiotics

Probiotic treatments target to adjust the intestinal conditions thereby lowering the ammonia production in the colon. Probiotics therapy always reduce circulatory NH_3 and result in alleviating hepatic encephalopeathy but fail to show significant efficacy (Dalal *et al.* 2017).

1.4.10. Nutrition and branched-chain amino acids.

Maintaining muscle mass is thought to be very important in patient with HE, as it removes ammonia from circulation. On the otherhand, when enough protein is administered to the patient, protective effect is observed in managing hyperammonemia and HE (Córdoba *et al.* 2004). Branched-chain amino acids are thought to improve nutritional status and seem to be effective in hepatic encephalopeathy. But, BCAA have unexpectedly increased blood ammonia levels in some cases (Dam *et al.* 2011). Oral BCAA was found to be useful in the treatment of HE but failed to show improvement in overall mortality or nutrition condition (Gluud *et al.* 2013).

1.4.11. Future approaches

Although liver transplantation is the only cure, it is not everytime feasible. Some treatments are at the discovery stage and seems promising for the therapy of hepatic encephalopeathy and these includes Minocycline acts by lowering microglial cell stimulation following brain injury and leads to reduction in water content in brain along with plasma and CSF NH_3 levels. NSAIDs such as Ibuprofen, indomethacin and Phosphodiesterase-5 inhibitors like sildenafil have been demonstrated some protective effect in mouse and rats with liver failure and mild hepatic encephalopeathy. Although several mechanisms have been proposed for the pathogenesis of HE, most of the therpies targets to reduce the elevated NH_3 as well as inflammation. Still, therapies for minimal HE is a need of the hour, and a great intensive effort is required to investigate this condition presisely to dicover new therapies (Hadjihambi *et al.* 2018).

1.5. Management of cholemic nephropathy

Ursodeoxycholic acid reduces kidney injury progression by lowering hyperbilirubinemia. Ursodiol also decreases the absorption of bile acids from gut and may attenuate hyperbilirubinemia. Plasma exchange is also a proved to be a treatment option in eliminating bile acids and toxins (Ocon *et al.* 2019). In addition, efficacy of terlipressin

has been proved for cholemic nephropathy. Althogh, several therapies have been discovered, efficacy of these therapies is limited (Krones *et al.* 2015) and there is unmet need for new therapeutic modalities for management of cholemic nephropathy.

1.6. Management of hepatorenal syndrome

The foremost goal in the therapy of HRS is to stabilize the kidney impairmant until the patients undergo liver transplantation. Total blood volume needs to be improved. However, 0.9% NaCl and artificial plasma expanders have found have not shown efficacy. It has been evident that albumin is the utmost effective amongst all volume expanders. Following albumin treatment, incidence of Type 1 HRS has found to be decreased (Arroyo *et al.* 2002). Ornipressin and terlipressin, vasoconstrictive agents have also shown effect on intestinal circulation relatively more out of renal and other vascular systems. Combination of terlipressin and albumin is desired in individuals, who are not put in intensive care unit. Nonetheless, non-existence of effective therapy often leads to renal transplantation, which stays the only treatment choice for extreme cases of HRS (Shah 2001).

1.7. ATX–LPA receptor signalling

Lysophosphatidic acid (LPA) is a potent biological active lipid that acts through six G-protein coupled receptor (GPCR) and exerts developmental, physiological, and pathological processes (Lin, Herr, and Chun 2010). Lysophosphatidic acid (LPA) is a widespread lysophospholipid and foremost lipid signalling agents derived from cell membrane. LPA, an autocrine as well as paracrine mediator works through six GPCRs LPA1 to 6, and exhibits various cellular activities encompassing wound healing, differentiation, proliferation, migration, and survival (Valdés-Rives and González-Arenas 2017). The most plentiful plasma LPA species are in order 18:2, 18:1, 18:0, 16:0, 20:4. Despite acyl-LPA 18:2 is the abundant type, LPA 18:1 is the commonly used in current research (Sano *et al.* 2002) (Choi *et al.* 2010). Abonormal LPA signalling may lead to a

series of ailments namely neurodevelopmental and neuropsychiatric disorders, pain, heart disease, bone abnormalities, fibrosis, cancer, sterility, and obesity. The investigations highlight the prospective of LPA receptor subtypes and associated signalling pathway to offer new therapeutic approaches (Yung *et al.* 2014).

Autotaxin (ATX), is a lysophospholipase D and breks down extracellular lysophospholipids into the lipid signalling molecule, lysophosphatidic acid (LPA) (Perrakis and Moolenaar 2014) (Im 2015). ATX has extensive tissue distribution, with reasonably high levels in liver, blood, brain (primarily in the choroid plexus and embryonic floor plate), kidney, and lymphatics(Perrakis and Moolenaar 2014).
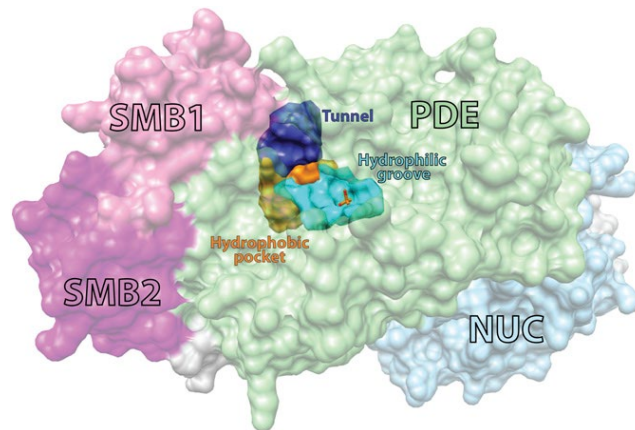
Figure 7: The structure of autotaxin (Perrakis and Moolenaar 2014).
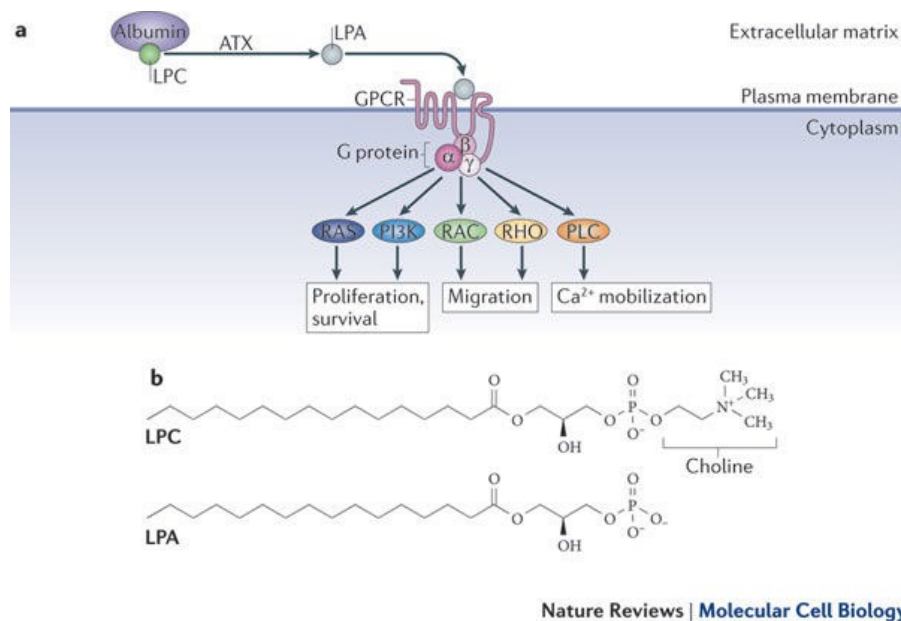
Figure 8: ATX–LPA receptor signalling (Moolenaar and Perrakis 2011).

ATX, an extracellular phosphodiesterase has been proved to be an apealing and greatly druggable target. As the turnover of LPA is very fast, plasma LPA levels can be lowered by more than 95% following with a strong ATX inhibitor. Furthermore, since LPA1 to 3 are involved in majority of pathogenesis, it is easier to inhibit the LPA signalling by hindering LPA synthesis than focussing on specific receptor antagonist. Whereas, ATX inhibition leads to effective reduction of unsaturated and polyunsaturated LPA species, the saturated LPA are lowered to lesser extent as their production is mostly mediated by PLA2 activity. Hence, LPA production by minimal sources like PLA2 might be good enough to maintain the physiological activities, while greater levels of unsaturated LPA species from upregulated ATX produces the pathological cascades (Benesch *et al.* 2016). Inspite of the fact that embryo of ATX knockout (KO) dies in utero (Koike *et al.* 2010) and the expression of ATX and LPA receptors are widespread (Benesch *et al.* 2016), genetic abolition of autotaxin in mature mouse, and prolonged ATX inhibiton with very active inhibitor did not show any adverse effect, ruling out potential safety issues of autotaxin as therapeutic targeting (Katsifa *et al.* 2015).

1.7.1. ATX-LPA Signalling and Chronic Liver Disease:

Serum ATX is proved to be a marker for the seriousness of hepatic disorders and a predictic marker in cirrhotic dual. Patients suffering from cirrhosis exhibited higher levels of ATX levels than normal individual. Low ATX levels is associated with longer survival of patients with liver diseases (Wunsch *et al.* 2016). It is still not clear whether elevation in ATX levels with progression of cirrhosis is a cause or effect. Although LPA is known to trigger stellate cells and liver cell proliferation, as major cause of extracellular matrix (ECM) generation in liver (Nakagawa *et al.* 2016), the role of ATX in etiology of liver cirrhosis is yet to be investigated. We hypothesize early treatment with ATX inhibitor might retard the progression from fibrosis to cirrhosis by lowing circulating levels of LPA and subsequently liver collagen deposition.

1.7.2. ATX-LPA Signalling in Brain:

Cirrhosis often is a lethargic disease, and most patients do not experience symptoms untill the episode of decompensation takes place. One of the serious consequences of cirrhosis is encephalopathy which is considered the major cause of deaths (Heidelbaugh and Bruderly 2006). Interestingly, serum ATX levels in cirrhotic individual with HE was found to be higher than patients without encephalopathy (Pleli *et al.* 2014). Though, level of ATX and LPA in brain could never be measured and corelated with severity of HE associated with liver cirrhosis.

Role of ATX-LPA signalling has been found to be prominent in neuroinflammation. Increase in LPA in the CSF and brain has been reported in human patients and mice undergone to traumatic brain injury (Yung *et al.* 2015). Furthermore, antagonising LPA with the specific mouse anti-LPA mAb amliorated neurological injury by lowering IL-6 levels and degree of injury (Crack *et al.* 2014). It also reduced microglia induced inflammatory reaction and killing of neurons, resulting in enhanced survivance of neurons as crucial for the injury and subsequently recovery of neurological activities (Goldshmit *et al.* 2012). Individual with multiple sclerosis exhibits higher levels of serum LPA (Balood *et al.* 2014) and pharmacological inhibition of ATX resulted in amelioration of inflammation associated with MS. Furthermore, ibuprofen, a NSAID, lowered the stimulation of microglia cells and reinstated memory and locomotor activirties in rats with hyperammonemia following bile duct ligation (Brück *et al.* 2011).

As neuroinflammation is evident in pathogenesis of hepatic encephalopathy (Jayakumar *et al.* 2015), we hypothesize ATX inhibitor might alleviate HE by lowering neuroinflammation.

1.7.3. ATX-LPA signalling and kidney disease:

Unilateral ureteral obstruction (UUO) leads to in upregulation of LPA1 receptor in kideny. The LPA levels following incubation of kidney explants in conditioned media from UUO

animals were also found significantly increased. Lpar KO mice exhibited diminished renal fibrosis in this model. Similarly, wild-type mice treated with LPA1 antagonist Ki16425 decreased fibrosis and markedly lowered kidney levels of the fibrosis causing connective tissue growth factor (CTGF) and transforming growth factor β (TGFβ) (Pradere *et al.* 2007) . LPA also triggers connective tissue growth factor (CTGF) expression in kidney fibroblasts (Chen *et al.* 2008). Furthermore, the fibroblasts accumulation remains a crucial step in the pathogenesis of kidney fibrosis, and LPA accelerates fibrosis by controlling various fibroblast functions. Autotaxin (ATX) being a most important enzyme, catalyses production of LPA which is associated in the promotion of kidney fibrosis vis LPA-induced accumulation of fibroblast in UUO model (Sakai *et al.* 2019). However, the mechanism of pathogenesis of cholemic nephropathy is different than in UUO model. The involvement of ATX-LPA pathway is yet to be explored in renal injury coexisting with liver cirrhosis. We assume that activity of ATX and level of LPA would be increased in renal tissue in mouse model of cirrhosis and inhibitor of ATX-LPA pathway might protect cholemic nephropathy. Both renal ATX protein levels and its activity was found to be higher with the advancement of fibrosis in tied kidney in renal interstitial fibrosis induced by unilateral ureteral obstruction (UUO) in mouse.

1.7.4. Involvement of ATX-LPA signalling in acute liver failure induced hepatic encephalopathy

HE is the most frequent problem of hepatic disorders such as ALF and liver cirrhosis and affecting up to 40% of liver cirrhosis patients. HE presents as a range of neurobehavioral signs, including memory impairment, locomotor deficit and change in character and perception. Interruption of the ornithine cycle after hepatic failure results in to increase in blood NH_3 level, and it enters the brain and causes deleterious effects (Jaeger *et al.* 2019). Treatment of HE remains an unmet need, and efforts should be directed towards development of new therapies.

1.7.5. Involvement of ATX-LPA signalling of in the pathogenesis of chronic liver disease and associated hepatic encephalopathy and hepatorenal syndrome.

Liver disease accounts about 2 million mortalities annually in the world, where cirrhosis is responsible for one million deaths and rest due to viral hepatitis and hepatocellular carcinoma (HCC). Cirrhosis at present has become the 11th most common cause of death worldwide (Asrani *et al.* 2019). Hepatic fibrosis is the main reason of cirrhosis. Liver fibrosis occurs due to excessive accumulation of collagen and other ECM that occur in most types of chronic liver diseases. The main causes of liver fibrosis are chronic hepatitis C infection, excessive alcohol consumption, and nonalcoholic fatty liver disease (NAFLD) (Seto and Susan Mandell 2021) (Hernandez-Gea and Friedman 2011). If not stopped, liver fibrosis often progresses to cirrhosis and eventually complications such as ascites, renal failure, hepatic encephalopathy, and variceal bleeding. Decompensated cirrhosis often results in short survival and liver transplantation becomes the only choice of effective therapy (Bataller and Brenner 2005).

Among liver cirrhosis associated complication, hepatic encephalopathy (HE) is a serious neurobehahioral syndrome with variable clinical symptoms ranging from subtle impairment in mental state, coma to death (Hadjihambi *et al.* 2018). There are several factors involved in patholophysiology of HE namely hyperammonia, inflammatory cytokines, oxidative stress and microbiota and aromatic amino acids (Elwir and Rahimi 2017).

Although medical and surgical advancement towards curing chronic hepatic disorders and extending the life expectancy of individuals has advanced in recent years, the existing therapeutic approaches for the treatment of cirrhosis are targetted on the prophylaxis or treatment of definite clinical ailments like fibrosis, ascites, GI bleeding, and HE. Therefore, need of novel drugs affecting specific key points in the complicated

pathogenesis processess of deteriorated chronic liver disease is immediately required to prevent the clinical decompensation (Toniutto 2022).

1.8. Autotaxin

Autotaxin (ATX), an extracellular enzyme which is liable for the ctalysis of lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) and choline. LPA triggers several cell signalling pathways once it binds to receptor and stimulation of its GPCRs. LPA signalling drives several cellular activities, which involves wound healing, differentiation, proliferation, migration, and survival (Valdés-Rives and González-Arenas 2017). Recently inhibition of ATX activity is recognized as a potential therapeutic intervention for several diseases including fibrotic diseases, cancer, pain, and inflammation (Perrakis and Moolenaar 2014).

Table-1: Therapeutic potential of ATX inhibitors

1.	A type IV Autotaxin inhibitor ameliorates acute liver injury and non-alcoholic steatohepatitis in mice. Model: Mouse model of CCl4 induced acute liver injury, cirrhosis, MCD diet-induced NASH model Treatment regime: Intraperitoneal administration ATX inhibitor, Cpd17 (5mg/kg) Signalling pathways: ATX-LPA signaling	(Booijink *et al.* 2022)
2.	Autotaxin inhibition to the rescue in stroke Model: Mouse model of MCAO induced stroke Treatment regime: Astrocyte specific genetic deletion Signalling pathways: ATX-LPA signaling	(Crunkhorn 2022)
3.	BBT-877, a Potent Autotaxin Inhibitor in Clinical Development to Treat Idiopathic Pulmonary Fibrosis Model: Mouse model of bleomycin in pulmonary fibrosis Treatment regime: Twice oral administration ATX inhibitor BBT-877 (10 & 30mg/kg) for 14 days Signalling pathways: ATX-LPA signaling	(Lee *et al.* 2019)
4.	Characterization of the properties of a selective, orally bioavailable autotaxin inhibitor in preclinical models of advanced stages of liver fibrosis Model: Rat model of CCl4 induce liver fibrosis & Choline-deficient L-amino acid-defined diet-induced NASH Treatment regime: Twice daily administration of ATX inhibitor Ex_31 at 15 mg/kg for 4 weeks Signalling pathways: ATX-LPA signaling	(Baader *et al.* 2018)
5.	Differentiating Characteristics of Cudetaxestat (BLD-0409), a Non- Competitive Autotaxin Inhibitor Under Development to Treat Idiopathic Pulmonary Fibrosis Model: Mouse model of bleomycin in pulmonary fibrosis Treatment regime: Once daily oral administrationof ATX inhibitor BLD-0409 at 3, 10 and 30 mg/kg for 14 days Signalling pathways: ATX-LPA signaling	(Wong *et al.* 2022)

6.	Pharmacological profile and efficacy of GLPG1690, a novel ATX inhibitor for COPD treatment	(Blanqué *et al.* 2015)
	Model: Mouse model of tobacco smoke (TS) induced COPD	
	Treatment regime: Once daily dose of ATXi GLPG1690 (3, 10 and 30 mg/kg b.i.d., p.o.) for 11 days	
	Signalling pathways: ATX-LPA signaling	
7.	Strong reversal of the lung fibrosis disease signature by autotaxin inhibitor GLPG1690 in a mouse model for IPF	(Ongenaert M, Dupont S, Blanqué R, Brys R, van der Aar E 2016)
	Model: Mouse bleomycin (BLM) lung fibrosis model	
	Treatment regime: Once daily dose of ATXi GLPG1690 (3, 10 and 30 mg/kg b.i.d., p.o.) for 14 days	
	Signalling pathways: ATX-LPA signaling	
8.	Effect of autotaxin inhibition in a surgically induced mouse model of osteoarthritis	(Datta *et al.* 2020)
	Model: Mouse model of osteoarthritis induced by destabilization of medial meniscus	
	Treatment regime: Intra-articular injection of 2.5 ng of ATXi PF-8380	
	Signalling pathways: ATX-LPA signaling	
9.	Inhibition of autotaxin activity ameliorates neuropathic pain derived from lumbar spinal canal stenosis.	(Uranbileg *et al.* 2021)
	Model: Mouse model of neuropathic pain induced by L5 DRG compression.	
	Treatment regime: Once oral dose of ATXi ONO-8430506 at 30mg/kg for 28 days.	
	Signalling pathways: ATX-LPA signaling	
10.	Inhibitors of the Autotaxin-Lysophosphatidic Acid Axis and Their Potential in the Treatment of Interstitial Lung Disease: Current Perspectives	(Mulholland *et al.* 2020)
	Review on potential of inhibitors of the ATX-LPA sigalling to alleviated Idiopathic pulmonary fibrosis.	
	Signalling pathways: ATX-LPA signaling	

11.	Inhibition of Autotaxin and Lysophosphatidic Acid Receptor 5 Attenuates Neuroinflammation in LPS-Activated BV-2 Microglia and a Mouse Endotoxemia Model	(Joshi *et al.* 2021)
	Model: Mouse model of LPS induced endotoxemia and neuroinflammation	
	Treatment regime: Oral administration of PF8380 (30 mg/kg body weight)	
	Signalling pathways: ATX-LPA signaling	
12.	Inhibition of Autotaxin with GLPG1690 Increases the Efficacy of Radiotherapy and Chemotherapy in a Mouse Model of Breast Cancer	(Tang *et al.* 2020)
	Model: Mouse model of 4T1 induced breast cancer	
	Treatment regime: Once daily administration of GLPG1690 at 50 & 100 mg/kg	
	Signalling pathways: ATX-LPA signaling	
13.	ONO-8430506: A Novel Autotaxin Inhibitor That Enhances the Antitumor Effect of Paclitaxel in a Breast Cancer Model.	(Iwaki *et al.* 2020)
	Model: Mouse model of MDA-MB-231 induced breast cancer	
	Treatment regime: Once daily administration of ONO-8430506 at 30 or 100 mg/kg twice daily for 32 days.	
	Signalling pathways: ATX-LPA signaling	
14.	Pharmacological characterization of a potent inhibitor of autotaxin in animal models of inflammatory bowel disease and multiple sclerosis	(Thirunavukkarasu *et al.* 2016)
	Model: Carrageenan induced paw inflammation in rats, f LPS-induced endotoxemia in mouse & DSS-induced inflammatory bowel disease model in mouse, MOG-induced EAE mouse model	
	Treatment regime: Twice daily oral administration of ATXi, Compound-1 at 3, 10 or 30 mg/kg.	
	Signalling pathways: ATX-LPA signaling	
15.	Selective Inhibition of Autotaxin is Efficacious in Mouse Models of Liver Fibrosis	(Bain *et al.* 2016)

	Model: CCl4 induced liver injury & STAM™ mouse model of non-alcoholic steatohepatitis Treatment regime: Twice daily oral administration of PAT-50 at 10 or 30mg/kg. Signalling pathways: ATX-LPA signaling	
16.	Strong reversal of the lung fibrosis disease signature by autotaxin inhibitor GLPG1690 in a mouse model for IPF. Model: Mouse bleomycin (BLM) lung fibrosis model Treatment regime: Twice daily oral dose of ATXi GLPG1690 at 30 mg/kg for 14 days. Signalling pathways: ATX-LPA signaling	(Ongenaert M, Dupont S, Blanqué R, Brys R, van der Aar E 2016)
17.	The Autotaxin Inhibitor GLPG1690 Attenuates Bleomycin-Induced Pulmonary Fibrosis in Mice Model: Mouse bleomycin (BLM) lung fibrosis model Treatment regime: Twice daily oral dose of ATXi GLPG1690 at 30 & 60 mg/kg for 14 days Signalling pathways: ATX-LPA signaling	(Murgo *et al.* 2019)
18.	Autotaxin/lysophosphatidic acid signaling mediates obesity-related cardiomyopathy in mice and human subjects Model: HFD induced obesity related cardiomyopathy Treatment regime: PF-8380 (30 mg/kg/day) for 8 weeks Signalling pathways: ATX-LPA signaling	(Weng *et al.* 2019)
19.	Effect of BBT-877, a novel inhibitor of ATX, on a mouse model of type 1 diabetic nephropathy Model: STZ-induced diabetic mouse model Treatment regime: Twice daily dose 10 mg/kg, 30 mg/kg, and 90 mg/kg for 8 weeks Signalling pathways: ATX-LPA signaling	(Lee *et al.* 2022)

Autotaxin (ATX) is a secreted glycoprotein ubiquitous in biological matrices is mainly responsible for LPA production (Katsifa *et al.* 2015). Role of ATX-LPA signalling has been found evident in neuroinflammation (Crack *et al.* 2014) (Schmitz *et al.* 2017). Elevation of LPA in the CSF fluid and brain has been reported in human and mice undergone

traumatic brain injury (Yung *et al.* 2015). (Zahednasab *et al.* 2014) reported that ATX activity was significantly higher in MS and other patients diagnosed with neuroinflammatory disorders. Both Autotaxin and Lysophosphatidic acid are greatly increased in the CNS. Excessive levels and activity of ATX with related alteration in LPA signalling is known to be involved in the pathogenesis of Alzheimer's disease (Ramesh *et al.* 2018). Furthermore, autotaxin is strongly up-regulated in reactive astrocytes adjacent to the lesion following neurotrauma (Savaskan *et al.* 2007). Therefore, reports indicate involvement of ATX-LPA signalling in etiology of neuroinflammatory disorders.

Recent report advocates that inflammatory reaction shows significant involvement in advancement of hepatic encephalopathy. Deranged nitrogen metabolism because of increased ammonia level in brain mounts neuroinflammation and found to be responsible for neurobehavioral symptoms (Jayakumar *et al.* 2015). Elevated NH_3 level leads to generation of ROS in cerebral cortex, thus also participate in causing to the severeity of hepatic encephalopathy, as evident in ALF (Bosoi and Rose 2013). A collaborative relation between NH_3, ROS and reactive nitrogen species and inflammation causing cytokines leads to astrocyte swelling resulting in cerebral edema edema associated acute hepatic encephalopathy as reported by (Rama Rao *et al.* 2010). The ammonia triggers both astrocytes and microglia and mounts inflammatory responses due to synthesis of proinflammatory cytokines and oxidative stress, which leads to neurotoxicity (Guerra *et al.* 2012) (Claeys *et al.* 2021).

Interestingly, serum autotaxin concentration in cirrhotic patients with hepatic encephalopathy were found higher than patients without encephalopathy (Pleli *et al.* 2014), but the role of ATX-LPA signalling within the brain in the pathophysiology of HE is still not known so far precisely.

In the present study, we have demonstrated the role of ATX-LPA signalling in the pathogenesis of HE and its beneficial effect of pharmacological inhibition of ATX in the animal model of HE.

In recent years, lysophosphatidic acid (LPA) was found to be a rich biologically active phospholipid, with several activities both in growth and in pathological conditions. LPA is the minutest biologically active lipid which shows strong extracellular signaling via its action with its six perticular GPCRs (LPAR1-6), facilitating main processes, such as cell proliferation, migration, and cytoskeletal reorganization (Henrique *et al.* 2021). Recently inhibition of ATX activity is recognized as a potential therapeutic intervention for several ailments such as fibrotic diseases, cancer, pain, and inflammation (Perrakis and Moolenaar 2014). The involvement of the ATX in liver disease has been confirmed by increased levels of ATX mRNA and protein levels in human and murine livers with induced injury (Katsifa *et al.* 2015). Furthermore, level of autotaxin was found to be higher in cirrhotic individual with hepatic encephalopathy (Pleli *et al.* 2014).

Targeting ATX/LPA signalling may prove to be a multimodal approach not only to halt the progression of chronic liver diseases but also prevent clinical decompensation after cirrhosis (Manuscript and Morphogenesis 2014).

Therefore, the goal of this study was to assess the effect of novel autotaxin inhibitor in bile duct ligation induced liver cirrhosis and associated hepatic encephalopathy.

Chapter 2. Gaps in existing research

Serum ATX indicates the severity of liver disease, helps in prognosis of cirrhotic patients, and found to be an early warning indicator in patients with cirrhosis. Furthermore, elevation in serum levels of ATX and LPA in overt CLF reveals the involvement of ATX-LPA in clinical decompensation of liver disease. It is still not clear whether elevation in ATX levels with progression of cirrhosis is a cause or effect. Although LPA stimulated stellate cell and liver cell proliferation, the major factors for ECM accumulation in liver (Nakagawa et al., 2016), the involvement of ATX in pathogenesis of ALF and CLF has not been investigated. Furthermore, role of ATX-LPA signalling in the etiology in biliary cirrhosis remains unexplored.

Cirrhosis often is an indolent disease, and most patients remain symptomless until the occurrence of decompensation. One of the serious consequences of cirrhosis is encephalopathy and is the major cause of deaths. Interestingly, serum autotaxin concentration in cirrhotic individual with hepatic encephalopathy was observed elevated in HE than patients without encephalopathy. However, level of ATX and LPA in brain were never measured and corelated with severity of HE associated with liver cirrhosis.

Hepatic encephalopathy (HE) is a major neuropsychiatric disorder of abrupt liver disease that appears in both ALF and CLD. Although incresaed brain ammonia level is known as the foremost causative agent in this disorder, latest investigations have revealed a substantial involvement of neuroinflammation in the pathology of acute as well as chronic HE. In recent report, elevated levels of serum TNF-α, IL-1β and IL-6 in association with a brain cytokine efflux in patients with acute HE advocates the presence of neuroinflammation in HE. Furthermore, stimualtion of microglia cells takes place early in the phase of ALF and was found to be elevated further as encephalopathy and brain edema becomes evident. ALF due to either depletion of liver vasculatures in rat or toxic hepatic injury in the mouse result in microglial stimulation and concomitantly elevation brain concentrations of proinflammatory cytokines, which includes TNF-α, IL-1β, and IL-6

(Jiang et al., 2009). Alevated NH_3 concentrations induce brain oxidative stress also participate to cause severe HE, as seen in ALF. A collaborative interaction between NH_3, ROS & RNS and inflammation causing cytokines in the astrocyte swelling leads to brain edema associated AHE has been reported. The ammonia triggers both astrocytes and microglia and triggers inflammatory responses due to synthesis of proinflammatory cytokines and oxidative stress, which leads to neurotoxicity. Nevertheless, involvement of ATX-signalling in pathogenesis of neuroinflammation associated with liver cirrhosis yet to be explored.

HE is a complicated neurobehavioral dysfunction as result of altered neurological function associated with liver cirrhosis. Approximately 33 to 55% of cirrhotic individual devoid of noticeable clinical signs of HE presents MHE with mild memory impairment. In individual with with cirrhosis, the certain levels of hyperammonemia in addition to inflammation is related not only to cognitive impairment, but also in attention deficienct and impaired coordination memory loss and loss of learning ability related to structural and functional hippocampal dysfunctions. However, role of ATX-LPA signalling on disrupted cognitive and motor activities remains to be investigated.

Cholemic nephropathy, also called as bile cast nephropathy, apparently shows an extensively undervalued but essential trigger of kidney deficiency in cholestasis or progressive liver disorders with hyperbilirubinemia. Unilateral ureteral obstruction UUO caused overexpression of LPA1 receptor. Furthermore, mice treated with the LPA1 blocker Ki16425 likewise attenuated fibrosis and considerably lessened kidney levels of the CTGF TGFβ. LPA has exhibited to stimulate CTGF expression in kidney fibroblast cells. However, the mechanism of pathogenesis of cholemic nephropathy is different than in UUO model. The role of ATX-LPA pathway has not been explored so far in renal injury coexisting with liver cirrhosis.

Chapter 3. Objectives

Based on the literature review and gaps in existing research we hypothesized that

1. To measure of LPA levels in plasma & brain with functional assessment in BDL induced chronic liver disease and thioacetamide induced acute liver failure models.

2. To study the therapeutic benefit of ATX inhibitors primary biliary cirrhosis and associated HE in BDL induced liver cirrhosis and thioacetamide induced acute liver failure & HE in mice.

3. To dissect out the role of ATX inhibitor on.

   o Ammonia induced neuroinflammation in astrocytes.

   o Ammonia induced neuroinflammation in microglial cells.

4. To study the involvement of ATX-LPA signaling in chronic liver disease induced kidney injury & effect of ATX inhibitor alleviating kidney injury.

Working objectives:

1. To assess the engagement of ATX-LPA pathway in the pathogenesis of acute and chronic liver diseases (CLD) and CLD induced hepatic encephalopathy and renal injury.

   A. To evaluate time course change of biomarker, LPA levels in plasma, brain and kidney with progression of chronic liver disease.

   B. To evaluate the change in biomarker, LPA levels in plasma, brain, and kidney with functional assessment in acute liver failure.

2. To dissect out the mode of action of ATX in pathogenesis of hepatic encephalopathy.

   A. Role of ATX-LPA signalling in neuroinflammation in glial cells.

   B. Role of ATX-LPA signalling in oxidative stress in glial cells.

3. To study the therapeutic potential of ATX inhibitor (ATXi) on liver diseases and associated complications.

To accomplish the proposed objectives, we performed following experiments.

1. Induction of acute liver injury with thioacetamide in mice and evaluation of LPA levels in plasma, brain, and kidney.

2. Evaluation of therapeutic potential of ATXi in TAA induced acute liver injury.

3. Evaluation of therapeutic potential of ATXi in acute liver injury induced hepatic encephalopathy.

4. The effect of ATXi in ammonium chloride induced neuroinflammation and oxidative stress in astrocyte and microglial cells.

5. Induction of bile duct ligation induced liver fibrosis/cirrhosis in rats.

6. Time course study of LPA levels in plasma and brain with progression of disease.

7. Assessment of ATXi for efficacy in bile duct ligation induced biliary fibrosis and associated hepatic encephalopathy in rats.

8. The therapeutic potential of ATXi in progression of liver fibrosis and subsequent hepatic encephalopathy in rats.

9. Induction of bile duct ligation induced renal injury and levels of LPA with progression of disease.

10. Evaluation of ATXi in BDL induced cholemic nephropathy in rats.

Chapter 4. Materials and Methods

Table-2: List of the materials used for conducting this study.

Material	Catalogue	Company
Thioacetamide	163678	Sigma
PF-8380	SML0715	Sigma
Mouse TNF-alpha Quantikine ELISA Kit	MTA00B	R&D Systems, Inc.
Mouse IL-1 beta Quantikine ELISA Kit	MLB00C	R&D Systems, Inc.
Mouse IL-6 Quantikine ELISA Kit	M6000B	R&D Systems, Inc.
C18:0 LPA	857128	Avanti Polar Lipids
C16:0 LPA	857223	Avanti Polar Lipids
C17:0 LPA	857324	Avanti Polar Lipids
Sodium chloride	S9888	Sigma
Dextrose	G7021-1KG	Sigma
Alanine transaminase	120178	Erba Mannheim
Aspartate aminotransferase	120179	Erba Mannheim
Total bilirubin	120224	Erba Mannheim
Creatinine	120228	Erba Mannheim
Blood urea nitrogen	120214	Erba Mannheim
Heparin sodium	1000IU	Biological E Limited
Trichloroacetic acid	T6399	Sigma
Potassium carbonate	209619	Sigma
Acetonitrile LC-MS GRADE	3040	DUKSAN PURE CHEMICALS
Methanol, OptimaA® LC/MS grade	AAB-A456-4	Thermo Fisher
Ammonium acetate	73594-100G-F	Sigma
Polar-RP column	00N-4336-B0-CE	Phenomenex

Luna RP C18	00N-4252-B0-CE	Phenomenex
Magnesium chloride	M8266	Sigma
Potassium chloride	P9541	Sigma
4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride	A8456	Sigma
Leupeptin	L2884	Sigma
Pepstatin	1190	Sigma
Igepal	542334	Sigma
DTT	10708984001	Sigma
RIPA buffer	89900	Thermo Scientific
Ethylenediaminetetraacetic acid	ED2P	Sigma
Sodium deoxycholate	D6750	Sigma
Triton X-100	T8787	Merck
Sodium orthovanadate	450243	Sigma
Protease Inhibitor Cocktail	78437	Thermo
BCA Protein Assay Kit	23227	Thermo
PVDF Membrane	1620177	BioRad
Nonfat-Dried Milk	M7409	Sigma
Rabbit anti-BDNF	S.C.-546	Santa Cruz Biotechnology
HRP-conjugated anti-rabbit IgG	31460	GE Healthcare Bio- Sciences
Mouse anti-actin (Clone AC-15)	A5441	Sigma
PrimeScript™ 1st strand cDNA Synthesis Kit	6110A	Takara Bio
Ammonium chloride	254134	Sigma
2′,7′−dichlorofluorescin diacetate	D6883	Sigma
Butylated hydroxytoluene	W218405	Sigma

Thiobarbituric acid	T5500	Sigma
Trichloroacetic acid	T8657	Sigma
CBT-295		TCG Lifesciences Pvt. Ltd.
Isoflurane	Aerrane	Baxter
Hematoxylin	H3136	Sigma
Tris Buffered Saline, with Tween 20, pH 8.0	T9039	Sigma

4.1. Section 1:

To assess the engagement of ATX-LPA pathway in the pathogenesis of acute liver diseases (ALD) and ALD induced hepatic encephalopathy (HE). To study the therapeutic potential of ATX inhibitor on acute liver diseases and associated HE.

❖ **Materials**

Thioacetamide and PF-8380 were purchased from Sigma-Aldrich. LPA 18:0 was procured from Avanti Polar Lipids, USA. The Quantikine mouse TNF-α, IL-1β, and IL-6 levels immunoassay kits were purchased from R&D Systems.

❖ **Experimental procedure**

Laboratory Animals: The experiment was performed on male CD1 mice weighing 35 ± 4 g (TCG Lifesciences Pvt., Ltd., India). During the study, the animals were housed in a group of 3−4 per cage under a controlled environment (22 ± 3 °C, relative humidity of $50 \pm 20\%$, and the light/dark cycle of 12 h light/12 h dark cycles). The animals were supplied water and standard rodent diet ad libitum. The study was conducted with a protocol approved by the Institutional Animal Care Committee (Protocol mumber: TCGLS/IAEC/2020/58/M-HEP/001/011).

Biomarker Study: Liver injury was induced by intraperitoneal injection of thioacetamide (TAA) at a dose of 100 mg/kg in physiological saline every morning for three consecutive

days. The control group received only saline intraperitoneally. The animals also received ringer lactate solutions along with 250 μL of 5% dextrose water to prevent hypoglycemia, renal complications, and electrolyte inequality till the end of the experiment (Sarhan *et al.* 1993). Clinical signs and behavioral parameters were assessed 24 h after the last dose of TAA. Blood samples were collected, and ammonia levels were measured. Plasma samples were analysed for biochemical parameters such as ALT, AST, and total bilirubin and lysophosphatidic acid (LPA) levels. Brain samples were collected for cytokine analysis.

Efficacy Study: In the first cohort of animals, thioacetamide (TAA) was dissolved in physiological saline and administered intraperitoneally (i.p.) once every morning for three successive days, at a dose of 100 mg/kg. The vehicle 0.5% methyl cellulose was used for ATX inhibitor PF-8380 and administered twice daily to the control and disease groups, while the treatment group was administered with the ATX inhibitor PF-8380 (30 mg/kg; p.o.) (Joshi *et al.* 2021) (Weng *et al.* 2019) twice daily for 3 days starting from the day of the first TAA injection. The animals also received ringer lactate solutions along with 250 μL of 5% dextrose water to prevent hypoglycemia, renal failure, and electrolyte imbalance till the end of the experiment. Clinical signs and behavioral parameters were assessed 24 h after the last dose of TAA. Blood samples were collected, and ammonia levels were measured. Plasma samples will be analysed for biochemical parameters such as ALT, AST, and total bilirubin and LPA levels. The cerebral cortex and hippocampus samples were collected for determination of cytokine, LPA, and BDNF levels.
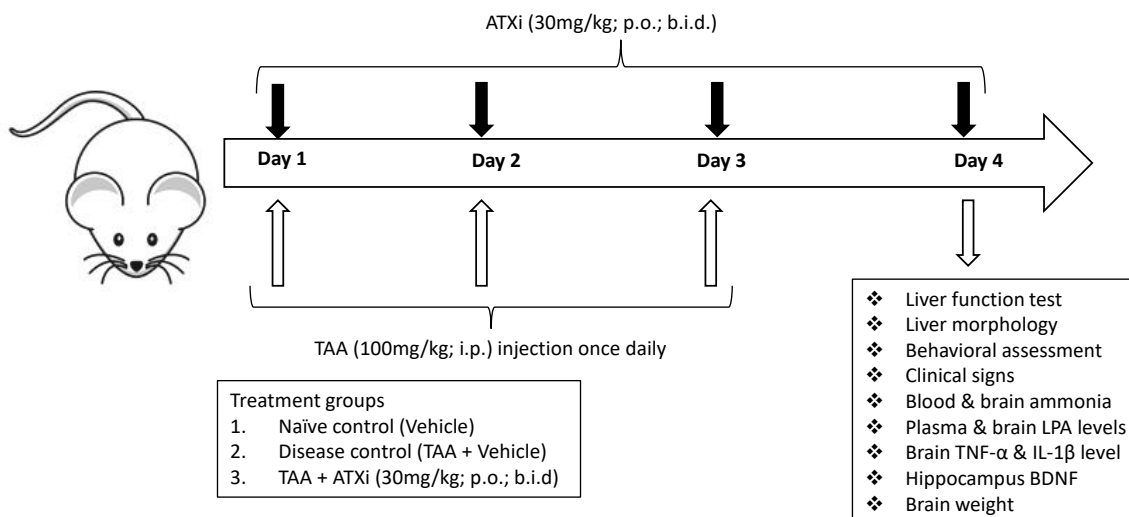
Behavioral parameters

Clinical Grading Score: The body weight of individual animals was monitored, and clinical signs were recorded every day. The beginning of clinical signs of encephalopathy in TAA-treated mice was assessed by the scoring method represented in Table (Farjam *et al.* 2012).

Table 1: Clinical grading scores of the animals' behavior.

Clinical grade	Definition
0	Normal behavior
1	Mild lethargy
2	Decreased motor activity, poor gesture control, diminished pain perception
3	Sever ataxia, no spontaneous righting reflex
4	No righting reflex, no reaction to pain stimuli

Spontaneous Locomotor Activity: Open-field locomotor and behavioral activities were recorded using Linton AM1053 X, Y, Z IR activity monitors. The lomotor activities were recorded over 15 min. Different activity parameters such as rearing counts, active time, mobile time, and distance travelled were measured. Behavioral and locomotor measurements were recorded 24 h after the last dose of TAA. The mortality rate of animals, the daily animal weight loss, and the clinical grade were evaluated.

Novel Object Recognition Test (NOR Test): For accessing learning and memory functions, the novel objection recognition test was used following 3 days of treatment. Mice were acclimatized in a 38 cm × 38 cm × 38 cm chamber for 2 days. Each animal was left to

explore the empty chamber for 5 min once a day. The NOR test was performed on day 3, wherein the mice were familiarized with two similar objects kept at the opposite corners of the chamber for 5 min. One hour after the familiarization, the mice were subjected to a test in the chamber with one familiar object replaced with one novel object. The animals were allowed to explore the objects for 5 min. The exploration time of each object was recorded. After every test, the chamber was wiped with ethanol (75% v/ v). Sniffing and touching the objects were considered as exploratory behaviours. The discrimination index was calculated using the formula difference between the time taken to explore new and familiar objects/total time (Yuan *et al.* 2020).
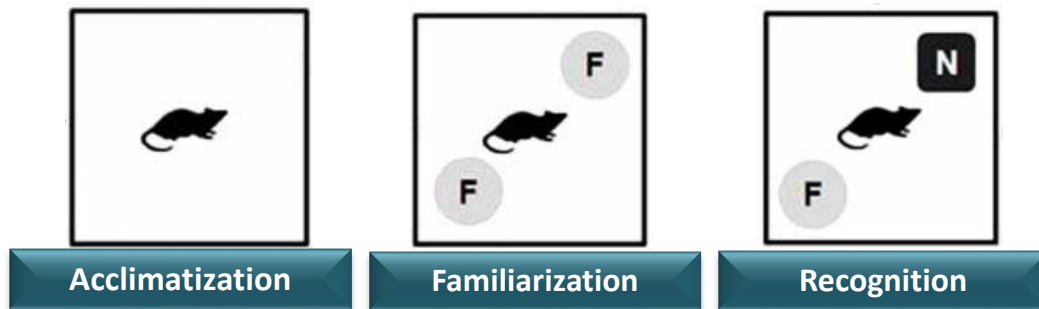
Rotarod Test. The rotarod test (IITC apparatus) was performed after 3 days of treatment with ATXi. The test was carried out with a speed of 10 rpm and cutoff of 300 s. Each animal was tested three times with an intermittent gap of 10 min between two successive tests. The time of fall was captured automatically for further analysis (Farahmandnejad *et al.* 2020).

Plasma preparation: Blood was collected in heparinized polypropylene tubes and centrifuged for 15 min at 1650g at 4 °C. The plasma samples were stored at −80 °C for further analysis.

Brain collection: After recording the behavioral parameters, the mice were euthanized and cerebral cortex samples were rapidly isolated, snap-frozen in liquid nitrogen, and stored at −80 °C.

Liver function test: Determination of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was carried out by the kinetic method. The total bilirubin (TBIL) was estimated by the colorimetric method. All the tests were performed using commercially available diagnostic kits (Erba Mannheim, Germany on Erba Mannheim biochemistry semi-autoanalyzer).

Determination of plasma and brain ammonia: Blood ammonia concentrations were analysed using a Pocketchem BA (Manufacturer name: ARKARY, Instrument code: 20503, model no. PA-4140, SN-41012016). Around 100 mg of cerebral cortex tissue was homogenized in 3 mL of trichloroacetic acid (6%, w/v) on ice. The lysed tissue was centrifuged at 17,000g for 10 min at 4 °C to collect the supernatant, which was further neutralized with potassium carbonate (2 mol/L, pH = 7). The harvested brain tissue supernatant was then used to analyse the ammonia content using a Pocketchem BA.

Measurement of plasma lysophosphatidic acid: LPA C18:0 was analysed by the LC-MS/MS method as reported by Murph et al. (2007). In brief, 20 µL of the sample was extracted with 80 µL of acetonitrile/methanol containing C17:0 LPA at 400 ng/mL as an internal standard. Then, 70 µL of the supernatant was removed, and 70 µL of 10 mM ammonium acetate was added before analysis. Quantitation experiments were performed by two-dimensional LC-MS/MS using an API4000 mass spectrometry system of AB-Sciex integrated with a CTC-PAL autosampler and a Shimadzu 20AD LC system. Typically, 20 µL of the sample was injected onto a Synergy 4 µ Polar-RP column (30 mm × 3 mm) with 10 mM ammonium acetate (pH ~ 9.5) as an aqueous phase (A) and 90:10 acetonitrile: A with 10 mM ammonium acetate as an organic phase (B). After 1.2 min, the valve was switched, and the captured analytes were eluted for 1.6 min.

Estimation of the ATXi (PF-8380) concentration in plasma samples: Plasma levels of the proprietary standard and ATXi were measured using LC-MS/MS methods. An API4000 mass spectrometry system of AB-Sciex integrated with a CTC-PAL autosampler and a

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Shimadzu 20AD LC system was used. A total of 20 μL of the sample was injected onto a Luna RP C18 (2.0 mm × 30 mm, 5 μ; Phenomenex) with H2O with 5mM ammonium acetate as an aqueous phase (A) and MeCN:MeOH: H2O 40:40:20 (v/v/v) with 5 mM ammonium acetate as an organic phase (B).

Determination of Brain TNF-α and IL-1β Levels: All brain samples were immediately frozen in liquid nitrogen and stored at −80 °C until assay was performed. Brain tissue was homogenized for 1 min in a buffer containing 10 mM HEPES-KOH, pH 7.9, buffer containing 1.5 mM MgCl2, 10mM KCl, 0.5 mM DTT, 1.0 mM AEBSF, 1 g/mL leupeptin, 1.25 g/mL pepstatin, and 0.1% Igepal. Each homogenate was then sonicated for 15 s on ice followed by centrifugation at 12,000g for 10 min at 4 °C. The supernatant was then analysed for TNF-α and IL-1β levels using ELISA (R&D Systems) kits as per the manufacturer's protocol.

Western Blotting: Hippocampi of both sides were collected for each animal (n = 6-8 for each treatment group). Hippocampus samples were homogenized using cold RIPA buffer 10 mM Tris, pH 7.2, 158 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% SDS, 1% sodium deoxycholate, 1% Triton-X, 1 mM Na3VO4, and 1% protease inhibitor cocktail. The total protein concentration of the supernatant from the homogenate was determined using BCA (Bradford) assay. A total of 25 μg of the sample was loaded in each lane. Once protein was separated, it was transferred to a PVDF membrane, and western blot analysis was performed for BDNF and β-actin. In brief, 5% non-fat dry milk, in Tris-buffered saline Tween 20 (TBST), was used to block the membranes for 1 h. The membrane was then incubated for 2 h using rabbit anti-BDNF (N-20, catalog #s.c.-546; Santa Cruz Biotechnology) (1:5000) in 5% milk/TBST at room temperature. Three washes with TBST were performed. Following this step, HRP-conjugated anti-rabbit IgG (1:10,000; GE Healthcare Bio- Sciences) in 5% milk/TBST was added and incubated for 1 h. Then, an ECL-Plus kit and reagents (GE Healthcare Bio-Sciences) were used to

visualize the protein bands. β-actin was considered a loading control and probed using mouse anti-actin (1:200,000; clone AC-15; Sigma). Band intensity was determined using ImageJ software. The levels of BDNF were normalized to actin levels. Statistical significance was determined by Student's t-test using GraphPad Prism version 5.0. (Lauterborn *et al.* 2007).

<u>Determination of TNF-α, IL-1β, and IL-6 mRNA Expression and ROS and TBARS Levels in C8-D1A (Mouse Astrocytes) and N9 Cell (Mouse Microglial Cells) Assays.</u>

<u>Cell Culture and treatment</u>: C8-D1A 441 and N9 cells were cultured in Dulbecco's modified Eagle medium (DMEM) and RPMI (pH 7.2, 10% FBS, penicillin/ streptomycin) at 37 °C and 5% CO2. Cells were seeded at densities of 2×10^6 cells/cm2 in 6-well plates. After 16−18h, the cells were starved in serum-free media for 2 h followed by the pre-treatment with different concentrations of ATXi (500 nM and 1 μM). After the pre-treatment, the cells were stimulated with 5 mM ammonia (NH4Cl). C8-D1A and N9 cells were stimulated for 5 and 10 h, respectively, at 37 °C.

Briefly following the treatment, cellular total RNA was extracted from cells using the MN NucleoSpin RNA plus isolation kit (Cat. No. 740984.5). One microgram of RNA was converted into cDNA using the PrimeScript first-strand cDNA kit from Takara (#6110A). qRT-PCR was performed with the SYBR Green PCR Kit (#RR820A, Takara) in the LightCycler 480 Real-Time PCR system with cycling conditions of 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 60°C for 40 s, and a melt curve analysis. All reactions were performed in triplicates and normalized with GAPDH as the reference gene. The relative gene expression of each sample was calculated using the $2^{-\Delta\Delta Ct}$ method.

<u>Measurement of Reactive Oxygen Species (ROS)</u>: Intracellular ROS generation in cells treated with NH4Cl, ATXi PF-8380, and a combination of both was assessed using 2′,7′−dichlorofluorescin diacetate (DCFDA; Molecular Probes) as previously described with some modifications. Briefly, the cells were incubated with DCFDA (5 nM) for 30

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min at 37 °C and washed twice with 1× PBS, and then, fluorescence intensity was quantified using a SpectraMax iD3 microplate reader (Molecular Devices Corporation) (excitation, 485 nm; emission, 530 nm).

Determination of Cytokine Levels: The C8-D1A cell line and N9 cells were seeded at densities of 2 × 106 cells per well in 6-well plates. The following day culture medium was removed, and the cells were pretreated for 1 h with indicated doses of ATXi at 37 °C and 5% CO2 in DMEM without serum. Subsequently, 5 mM ammonia (NH4Cl) was added in the presence or absence of ATXi for 24 h at 37 °C in DMEM without serum. Supernatants from all samples were immediately frozen at −80 °C until ELISA was carried out.

TBARS Levels: For performing the TBARS assay, 1×10^5 cells/well were seeded in a 12-well plate and allowed to grow for 24 h. The cells were incubated with NH4CL, NH4CL + ATXI (500 nM and 1 μM), and only ATXI (500 nM and 1 μM). After the completion of the incubation period, the cell supernatant was mixed with TBARS solution containing 0.67% 483 of TBA, 20% of TCA, and 0.04% of BHT. The MDA standard was prepared in the same manner. The samples and standard were heated at 95 °C in a hot-water bath for 20 min. The mixture was transferred to 96-well plates, and absorbance was measured on a SpectraMax iD3 microplate reader (Molecular Devices Corporation) at 532 nM wavelength.

Statistical Analysis: The data were expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 software. Either unpaired t-test or ANOVA followed by the Tukey test was used to determine the statistical significance. It was considered a significant difference when $p < 0.05$.

4.2. Section 2:

To evaluate the engagement of ATX-LPA pathway in the pathogenesis of chronic liver diseases (CLD) and CLD induced hepatic encephalopathy. To evaluate the therapeutic potential of an ATX inhibitor on chronic liver diseases and HE.

Materials

CBT-295 was synthesized in the department of medicinal chemistry, TCG Lifesciences Pvt. Ltd. LPA 18:1 and LPA 16:0 were procured from Avanti Polar Lipids, USA. The Quantikine Mouse TNF-α and IL-6 immunoassay kits were purchased for R&D system, USA.

Laboratory animals:

The experiment was performed on male Sprague Dawley rats weighing 275±25 g (TCG Lifesciences Pvt. Ltd., India). During the course of study, animals were housed in a group of 3-4 per cages under a controlled environment (22 ± 2° C, relative humidity 50 ± 10% and 12-hr light/12-hr dark cycles). Animals were supplied water and standard rodent diet *ad libitum*. The study was performed under a protocol approved by the Institutional Animal Care Committee.

Experimental procedure

Pilot study for biomarker assessment: Liver cirrhosis was induced by in male Sprague-Dawley rats by bile duct ligation (BDL) (Bosoi *et al.* 2011). Animal were anesthetized by isoflurane (3%) inhalation anesthesia. Common bile dust was isolated 100µl of formalin was injected intracholedochally to avoid dilation of the ligated bile ducts. Following formalin injection, bile duct was ligated at places. Sham surgery was performed for control rats in which the bile duct was isolated and formalin injection, ligation, or resection was not carried out. Liver function test, clinical signs, and behavioral parameter such as spontaneous motor activity, contextual fear conditioning and novel object recognition test

were assessed on day 14, 28 and 42 post BDL surgery. Blood samples were collected & ammonia levels will be measured. Plasma samples were analysed for biochemical parameters such as ALT, AST, total bilirubin, albumin, and lysophosphatidic acid (LPA) levels.
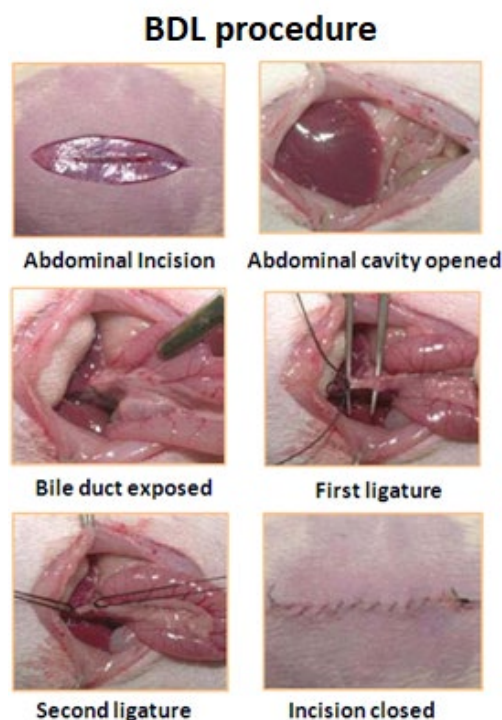
Bile duct ligation procedure

Pharmacokinetic study: CBT-295 was administered to male Sprague Dawley rats (200-250g) intravenously (i.v) at 1 mg/kg body weight or orally at 10 mg/kg body weight (n=3/route of administration). 100µl of blood samples were collected through saphenous vein puncture at 0.83, 0.25, 0.5, 1, 2, 4, 8 and 24 h post-dose following intravenous administration and 0.25, 0.5, 1, 2, 4, 8 and 24 h following oral administration. Blood samples were collected in heparin coated capillaries. Plasma was separated by centrifugation and stored at −20 °C until bioanalysis was performed. The compound concentrations in plasma were determined by LC-MS/MS analysis (API 4000, Applied Biosystems, USA). A calibration curve was prepared ranging concentration from 1.22–1250 ng/ml with lower level of quantitation 1.22ng/ml. The pharmacokinetic parameters

were calculated by WinNonlin software using non-compartmental analysis method. The study was performed under a protocol approved by the Institutional Animal Care Committee (Protocol mumber: TCGLS/IAEC/2021/60/R-BDL/001/027).

<u>Tolerability study</u>: Male Sprague Dawley rats of body weight 250±25g (n=5/gr) were administered either vehicle or CBT-295; b.i.d; p.o. for 5 days. Body weight was monitored every day. On day 5, blood samples were collected, and RBC, WBC, AST, ALT, and creatinine levels were measure.

<u>Pharmacodynamic study</u>: Following bile duct ligation or sham surgery animals were assigned to control (sham), vehicle and CBT-295 treatment groups. Control and vehicle groups received vehicle (0.5% Methylcellulose with 0.5% tween 80 while CBT-295 was administered orally twice daily at 20mg/kg for 28 days. At the end of treatment, behavioral parameters were assessed. Blood samples were analysed for ammonia levels. AST, ALT, and total bilirubin levels were measured in plasma sample. Brain samples were analysed for TNF-α levels. Liver samples were preserved in formalin for histological analysis.

Behavioral parameters

<u>Spontaneous locomotor activity:</u> Open field behavioural and locomotor activities were recorded using the Linton AM1053 X, Y, Z IR Activity Monitors. The activities were recorded over a 15-minute. Different activity parameters such as rearing counts, active time, mobile time and distance travelled were measured. Behavioral and locomotor measurements were recorded following 28 days of treatment.

<u>Novel object recognition test:</u> The learning and memory function was measured using a novel objection recognition test. Rats were acclimatized in a 38x38x38 cm chamber for 2 days. Each animal was made to explore the empty chamber for 5 min once in a day. NOR test was performed on day 3 where rats were allowed to explore two similar objects that were kept at opposite corners of the chamber for 5 min. This phase is called as the familiarization phase. Following a 1h retention interval, the rats were subjected to a test

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in the chamber with a familiar and a novel object. Animals were allowed to explore the objects for 5 min. The exploration time at each object was recorded. After every test the arena was wiped with Ethanol (75% v/v). Sniffing and touching of the objects were considered as exploratory behaviours. Discrimination index was calculated using the formula; difference between new & familiar object exploration/total time (Yuan *et al.* 2020).

Contextual fear conditioning (CFC): CFC was performed in a fear conditioning chamber (Coulbourn Instruments, USA). CFC comprised of two phases, training, and testing. A day before training, the animals were acclimatized in the chamber for 5min. During training each animal was kept in the conditioning chamber for 5 seconds for context conditioning. After training, a foot-shock (unconditioned stimulus) of 1.5mA for 2 seconds was given for conditioned stimulus-unconditioned stimulus pairing. Thirty seconds thereafter, the animal was placed back into its home cage and the conditioned stimulus chamber was cleaned with 70% alcohol. Twenty-four hours after the conditioning session, each animal was again exposed to the conditioning chamber for 5min, during which no shock was delivered (only conditioned stimulus). During this period, the freezing time was recorded. Freezing behaviour is defined as the absence of visible movement of the body except respiration associated movements (Das *et al.* 2020).
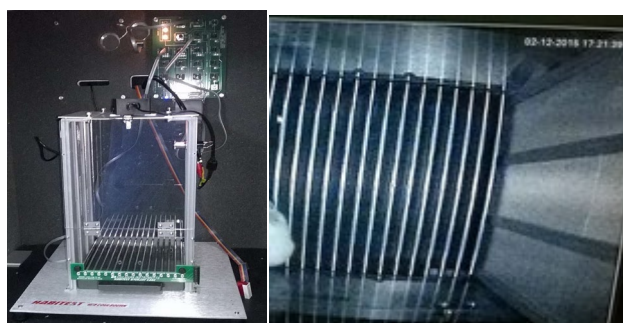
Contextual fear condition apparatus

Plasma preparation: Blood was collected in heparinized polypropylene tubes and centrifuged for 15 min at 1650 g at 4°C. The plasma samples were stored at -80°C.

Brain collection: Rats were euthanized at end of the study. Cerebral cortex and hippocampus samples were rapidly isolated snap frozen in liquid nitrogen and stored at -80°C.

Liver function test: Determination of plasma Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were carried out by kinetic method. The total bilirubin (TBIL) was estimated by colorimetric method. All the tests were performed using commercially available diagnostic kits (Erba Mannheim, Germany on Erba Mannheim biochemistry semi auto analyser).

Determination of Plasma and Brain Ammonia: Blood ammonia concentrations were analysed by Pocketchem BA (Manufacturer name: ARKARY, Instrument code: 20503, Model No.PA-4140, SN-41012016). For the determination of brain ammonia content, the sample (100 mg) of the forebrain (cerebral cortex) was collected, homogenized, and deproteinized in 3mL of ice-cooled lysis solution (Trichloroacetic acid, 6%, w/v, 4°C). After centrifugation (17,000g, 10 minutes, 4°C), the supernatant was collected and neutralized with potassium carbonate (100 µL of $KHCO_3$; 2 mol/L, pH = 7). Afterward, brain ammonia content was assessed using Pocketchem BA.

Measurement of plasma lysophosphatidic acid: LPA C16:0 and C18:1 was analysed by using an LC-MS/MS method as reported by Murph et al. (2007). In brief, 20 µl of sample was extracted with 80µl of acetonitrile/methanol containing C17:0 LPA at 400ng/ml as internal standard. Then, 70µl supernatant was removed and 70µl of 10mM ammonium acetate was added before analysis. Quantitation experiments were performed by two-dimensional LC-MS/MS by using an API4000 mass spectrometry system of AB-Sciex integrated with CTC-PAL auto sampler and Shimadzu 20AD LC system. Typically, 20µl of sample was injected onto a Synergy 4µ Polar-RP column (30x3 mm) with 10mM ammonium acetate (pH ~9.5) as aqueous phase (A) and 90:10 Acetonitrile: A with 10mM

ammonium acetate as organic phase (B). After 1.2 min, the valve was switched, and the captured analytes were eluted onto a 1.6 minute.

Determination of brain TNF-α level: All samples were immediately frozen in liquid nitrogen and stored at -80°C until assay was performed. Brain tissue was homogenized (7 v/w ratio) for 1 minute in a homogenization buffer containing 10 mM HEPES-KOH, pH 7.9 buffer containing 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 1.0 mM AEBSF, 1g/mL leupeptin, 1.25g/mL pepstatin, and 0.1% Igepal. Each sample was subjected for sonication for 15 seconds. Homogenates were centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant solution was collected and TNF-) was measured in duplicate. 50 µL of supernatant was added to the well and cytokine levels were measured using a specific ELISA (R&D Systems) kit as per manufacturer's protocol. The protein level was measured by the Bradford (1976) method. The total protein concentrations were determined using spectrophotometric assay at 540 nm in mg/ml by interpolating OD values from standard curve prepared with bovine serum albumin.

Liver histology: Formalin-fixed liver tissue was embedded in paraffin, and sections were stained with Sirius red. The staining solutions consisted of picro-sirius red solution (0.5 g sirius red in 500 ml of Saturated aqueous solution of picric acid) and acidified water (5 ml glacieal acetic acid to 1 litre of distilled water). The tissue sections are first deparaffinized with three washes with xylene. Subsequently the sections are hydrated with descending grades of alcohols (absolute alcohol: two changes, 95 % alcohol: two changes, 80 % alcohol: one change then 70 % alcohol: one change. Then the sections were stained with picro-sirius red for one hour. The sections were then washed with two changes of acidified water. After this step, the sections were dehydrated with increasing concentration of alcohol (70%, 80%, 95% followed by absolute alcohol). The sections were subsequently cleared with 3 washes of Xylene. The stained slides were allowed to drain excess of xylene. Then the slides were mounted by placing two drops of DPX mountant on cover

slips and by quickly inverted the slides over the cover slips so that tissue section was sandwiched between glass slide and coverslip. Sirius red-stained sections were photographed using a Leica DM1000 microscope. Histological evaluation of the liver sections was performed by the same pathologist in a blinded manner. Six areas in each section were randomly chosen to quantify the Sirius red staining using an Image J software and the average value of the 6 areas was used to evaluate fibrosis level.

Statistical analysis: The data were expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 software. Either unpaired t test or ANOVA followed by Tukey Test was used to determine statistical significance. It was considered a significant difference when $P<0.05$.

4.3. Section 3:

To evaluate the engagement of ATX-LPA pathway in the pathogenesis of chronic liver diseases (CLD) induced and renal injury. To evaluate the therapeutic potential of an ATX inhibitor on CLD induced renal disorder.

Materials

CBT-295 was synthesized in the department of medicinal chemistry, TCG Lifesciences Pvt. Ltd. LPA 18:1 and LPA 16:0 was procured from Avanti Polar Lipids, USA. ALT, AST, TBIL, Creatinine and blood urea nitrogen kits were procured from Erba Mannheim, Germany.

Laboratory animals:

The experiment was performed on male Sprague Dawley rats weighing 275±25 g (TCG Lifesciences Pvt. Ltd., India). During study, animals were housed in a group of 3-4 per cages under a controlled environment (22 ± 2° C, relative humidity 50 ± 10% and 12-hr light/12-hr dark cycles). Animals were supplied water and standard rodent diet *ad libitum*.

The study was performed under a protocol approved by the Institutional Animal Care Committee (Protocol mumber: TCGLS/IAEC/2021/60/R-BDL/001/027).

Experimental procedure

Pilot study for biomarker assessment: Liver cirrhosis was induced by in male Sprague-Dawley rats by bile duct ligation (BDL) (Bosoi *et al.* 2011). Animal were anesthetized by isoflurane (3%) inhalation anesthesia. Common bile dust was isolated 100µl of formalin was injected intracholedochally to avoid dilation of the ligated bile ducts. Following formalin injection, bile duct was ligated at places. Sham surgery was performed for control rats in which the bile duct was isolated and formalin injection, ligation, or resection was not carried out. Liver function and kidney function tests were assessed on day 14, 28 and 42 post BDL surgery.

Pharmacodynamic study: Following bile duct ligation or sham surgery animals were assigned to control (sham), vehicle and CBT-295 treatment groups. Control and vehicle groups received vehicle (0.5% Methylcellulose with 0.5% tween 80 while CBT-295 was administered orally twice daily at 20mg/kg for 28 days. At the end of treatment, plasma samples were analysed for ammonia levels. AST, ALT, total bilirubin, creatinine and blood urea nitrogen levels.

Liver function test: Determination of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was carried out by the kinetic method. The total bilirubin (TBIL) was estimated by the colorimetric method. All the tests were performed using commercially available diagnostic kits (Erba Mannheim, Germany on Erba Mannheim biochemistry semi-autoanalyzer).

Kidney function test: Determination of plasma creatinine Blood urea nitrogen was carried out by the colorimetric method. All the tests were performed using commercially available diagnostic kits (Erba Mannheim, Germany on Erba Mannheim biochemistry semi-autoanalyzer).

Measurement of plasma lysophosphatidic acid: LPA C16:0 and C18:1 was analysed by using an LC-MS/MS method. In brief, 20 µl of sample was extracted with 80µl of acetonitrile/methanol containing C17:0 LPA at 400ng/ml as internal standard. Then, 70µl supernatant was removed and 70µl of 10mM ammonium acetate was added before analysis. Quantitation experiments were performed by two-dimensional LC-MS/MS by using an API4000 mass spectrometry system of AB-Sciex integrated with CTC-PAL auto sampler and Shimadzu 20AD LC system. Typically, 20µl of sample was injected onto a Synergy 4µ Polar-RP column (30x3 mm) with 10mM ammonium acetate (pH ~9.5) as aqueous phase (A) and 90:10 Acetonitrile: A with 10mM ammonium acetate as organic phase (B). After 1.2 min, the valve was switched, and the captured analytes were eluted onto a 1.6 minute.

Kidney hydroxyproline content: Renal hydroxyproline content was measured using Ehrlich's reagent (p-dimethyl amino benzaldehyde, 15 g in n-propanol/perchloric acid; 2:1 v:v). Briefly, kidney homogenate (1 ml of 10% w:v in KCl) was digested in 1 ml of 6 N HCl (at 120°C for 24 h). Then, an aliquot (25µl) of digested tissue was added to a Petri dish and treated with citrate-acetate buffer (25µl, pH = 6) and dried at room temperature (25°C). Then, 0.5 ml of chloramines-t-solution (56 mM) was added and incubated at 25°C for 20 minutes. Afterward, 0.5 ml of Ehrlich's reagent was added, and the mixture was incubated in a 65°C water bath for 15 minutes. The absorbance was assessed at $\lambda = 550$ nm (Spectramax plate reader, Molecular Device) (Ommati *et al.* 2021).

Chapter 5. Results and Discussion

5.1. Section 1:

Evaluation of the engagement of ATX-LPA pathway in the pathogenesis of acute liver diseases (ALD) and ALD induced hepatic encephalopathy. To study the therapeutic potential of an ATX inhibitor on acute liver diseases and HE.

Hepatic encephalopathy is a range of neurobehavioral impairment in individuals with liver disease (Farmer and Mulakkan 1990). Although the significant progress in understanding the several pathogenic signalling patheays involved in HE, the therapeutic choices are still limited (Hadjihambi *et al.* 2018). There there is urgent need of new therapies.

In our first study, we evaluated the role of ATX inhibitor in the animal model of HE. We initiated the investigation with objective to assess the involvement of ATX-LPA in the pathogenesis of HE. The animal model of HE was developed by intraperitoneal TAA injection for 3 consecutive days (Sarhan *et al.* 1993). We chose mice as they are more sensitive to TAA as the extent of liver injury is more than rats which is evident from comparatively higher liver injury in mice than rat which makes mouse a preferred species for TAA induces liver injury (Papadakis GZ, Millo C 2008). The mice exhibited significant elevation of liver enzymes. The ALT and AST levels were found to be significantly elevated ($p < 0.001$) compared to control animals (Figure 9A and 9B respectively). Lesions in the liver of TAA injected animals were quite evident (Figure 9C).
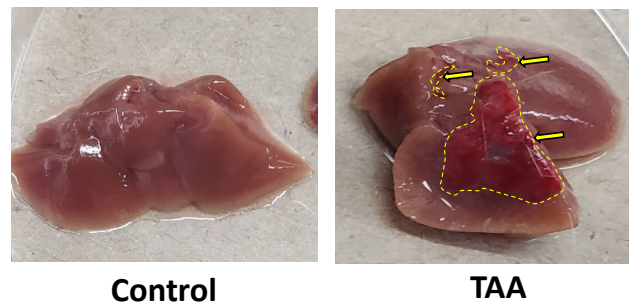
(A)   (B)

(C)
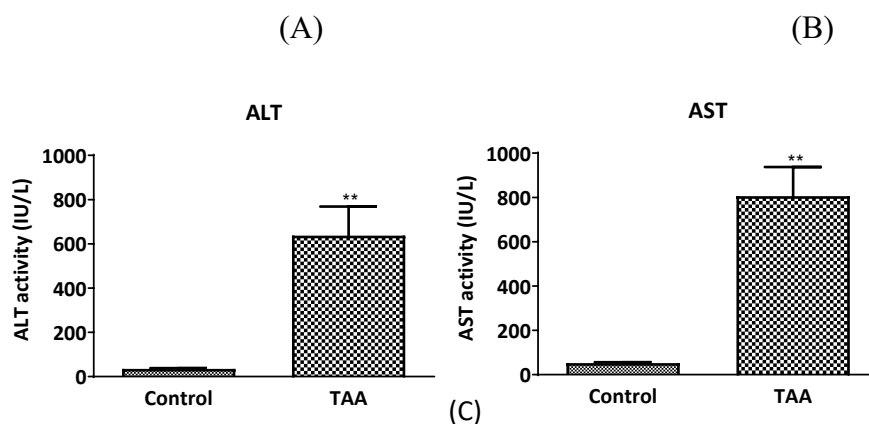
Control   **TAA**

Figure 9: Biochemical changes in TAA induced acute liver failure in mice. Changes in plasma (A) ALT and (B) AST levels. (C) Liver morphology of control animal & TAA treated mice. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as ** ($p<0.01$) vs control mice.

In individuals with cirrhosis, hepatic dysfunction impairs liver metabolism of ammonia, and portal hypertension results in pushing of ammonia rich portal blood to the systemic circulation (Elwir and Rahimi 2017). Ammonia penetrates into the brain very quickly and considered to be major culprit for the pathogenesis of HE causes neuropsychiatric complications (Prakash and Mullen 2010). In agreement, we observed that TAA treated animals showed more than two-fold increase in blood ammonia ($p<0.001$) with respect to control animals (Figure 10A). We determined the cortical brain ammonia levels and found significantly higher in TAA treated mice (Figure 10B).

Animals with HE demonstrates disturbed spontaneous locomotor and exploratory activities in open field test because of altered behavior (Leke *et al.* 2012) (Farjam *et al.* 2012). Furthermore, TAA mice exhibit a significant change in HE related behavioral parameters related to HE as reported by (Farjam *et al.* 2012). In our experiment as well, we observed similar results. TAA treatment showed significant increase in clinical grading score ($p<0.01$) (10D) in TAA animals in comparison with control animals. Spontaneous locomotor activity parameter, distance travelled was also found markedly lower ($p<0.01$) in TAA animals (Figure 10D) which is in agrremment with published reports (El-Marasy

et al. 2019) (DeMorrow *et al.* 2021) (Bai *et al.* 2023). Three out of ten animals died in TAA treated group while no mortality was observed in control group.
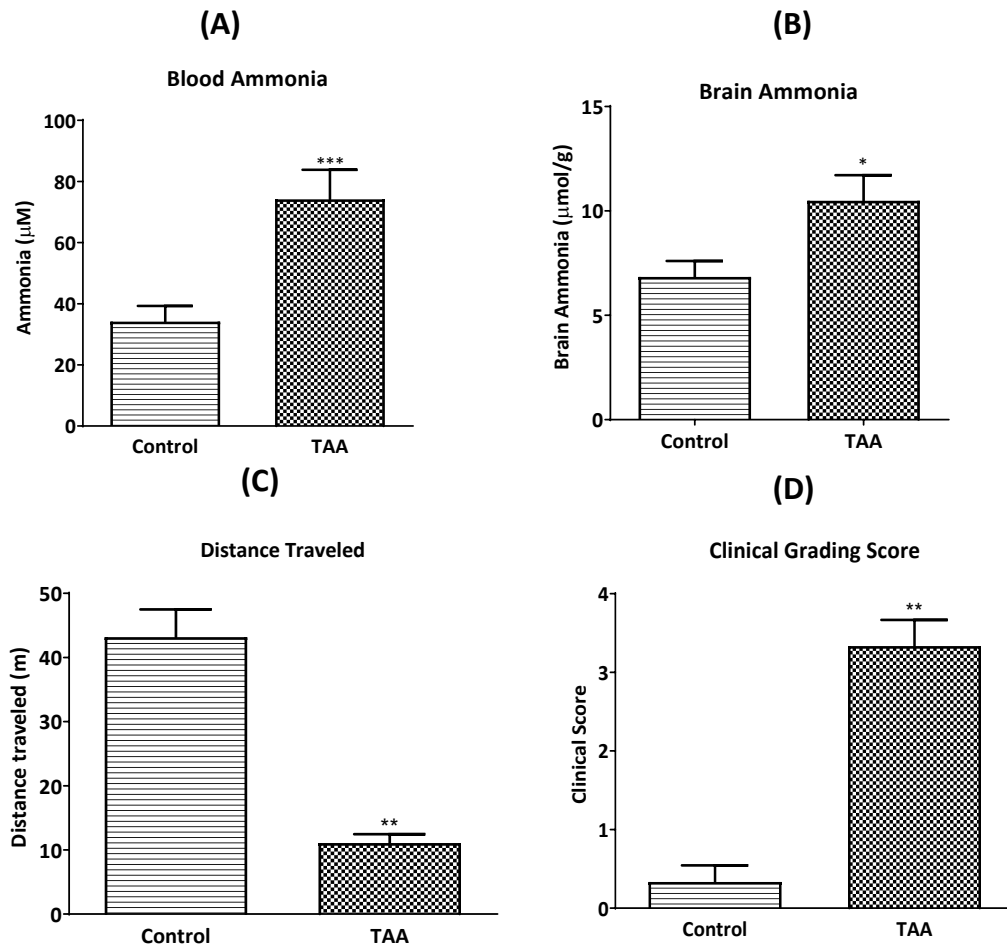

Figure 10: Acute liver failure induced hyperammonemia and behavioral changes in TAA treated mice. Changes in (A) Blood ammonia, (B) Brain ammonia, Change in HE related clinical score; (C) Spontaneous locomotor activity, (D) HE related clinical grading score. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as *($p < 0.05$) and ** ($p < 0.01$) vs control mice.

There has been persistent finding of neuroinflammatory processes in individuals with acute and chronic liver disorders (Jayakumar *et al.* 2015). Furthermore, reports suggests that TNF-α is increased in animal model of neuroinflammation caused by traumatic brain injury and treatment with TNF-α antibody improves neurobehavioral abnormalities (Rowe *et al.* 2018) (Dadsetan *et al.* 2016). Brain edema has been observed in individuals with acute liver failure due to neuroinflammation (Tong and Norenberg 2010). In this line of

thought, we measured inflammatory marker TNF-α in the cerebral cortex and found three-fold elevation (p<0.01) in TAA animals (Figure 11 A). We chose cerebral cortex as oxidative stress in cerebral cortex in two types of HE models of thioacetamide (TAA) and bile duct ligation (BDL) attribute to bradykinesia or decreased locomotor deficits (Bai *et al.* 2023). Therefore, cortical of brain was considered. Brain weight as an indicator of cerebral edema was also significantly increased (p<0.05) in TAA mice with respect to control group (Figure 11B).

(A)   **(B)**
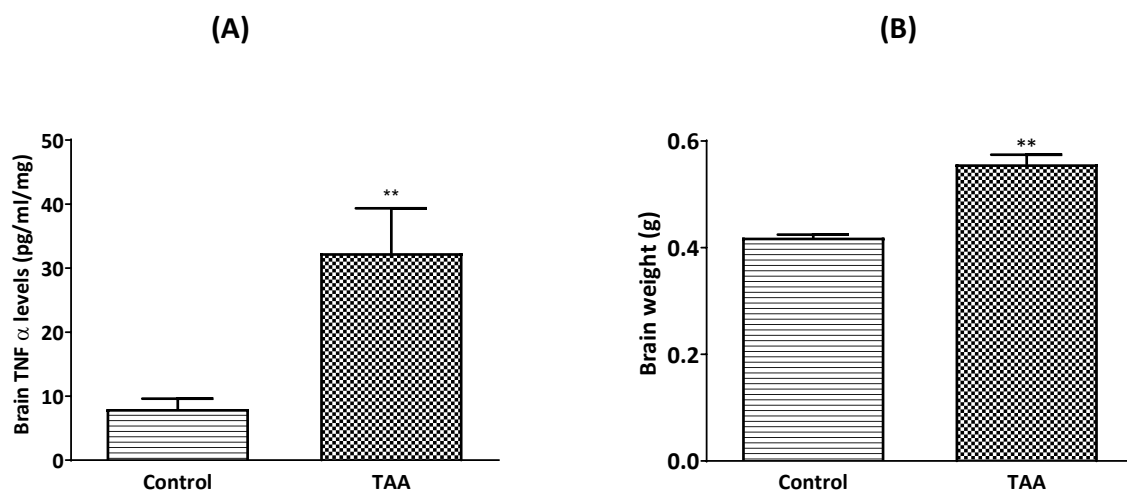

Figure 11: Ammonia induced neuroinflammatory changes in TAA treated mice. Changes in (A) Brain TNF-α and (B) Brain weight. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as ** (p<0.01) vs control mice.

Role of LPA has been well established in several neurological disorders namely, MS, traumatic brain injury, hydrocephalous, AD etc (Henrique *et al.* 2021). Interestingly LPA 18:0 is overwhelmingly the common species in the brain of mouse following neuroinflammation induced by traumatic brain injury (Crack *et al.* 2014). We asked if LPA has any role in the pathogenesis of HE as well. We chose LPA 18:0 as it has been found to be elevated in neuroinflammatory conditions. We measured the levels in the plasma of TAA animals and found marked increase (p<0.001) compared to control animals (Figure 12A). LPA level was also found to be elevated by two-fold (p<0.01) in cerebral cortex

homogenate of TAA mice (Figure 12B). Therefore, from the outcome of this study we could delineate that neuroinflammation was evident in the cortical region of brain in HE mice and the elevated cortical level of LPA indicates involvement in the pathogenesis HE.
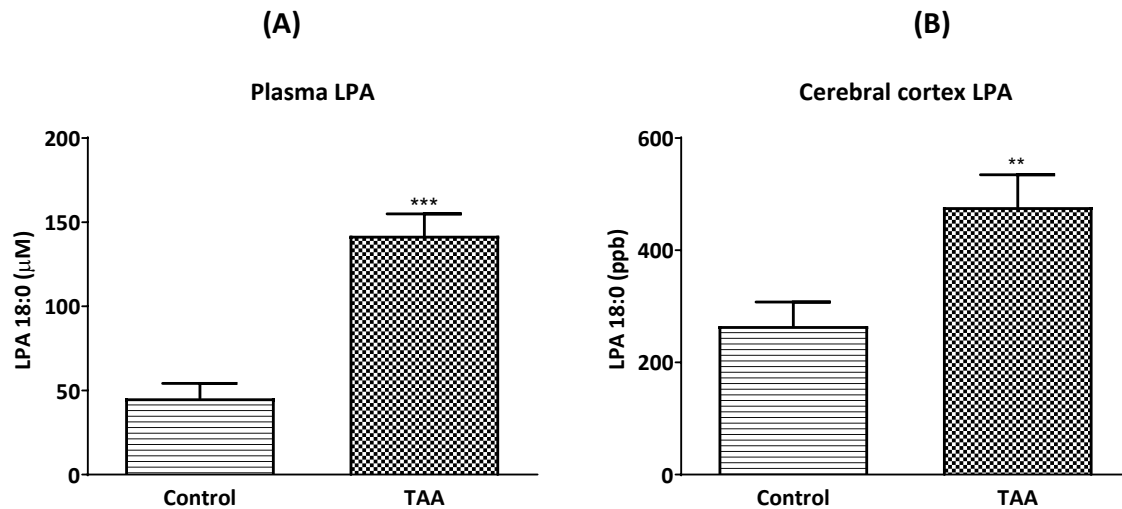

Figure 12: Changes in LPA levels following treatment with TAA. Change in (A) Plasma LPA and (B) Cerebral cortex LPA level. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as ** (p<0.01), *** (p<0.001) vs control mice.

In subsequent experiments, we evaluated the efficacy of ATX inhibitor in TAA induced acute liver failure in mice. Although, ATX KO mice show embryonic lethality and expression of ATX in adult life the ubiquitous questions the suitability of ATX as a drug target (Koike *et al.* 2010) (Benesch *et al.* 2016) (van Meeteren *et al.* 2006) (Fotopoulou *et al.* 2010). Surprisingly, genetic deletion of ATX mature animals, as well as inhibition of ATX for longer period with potent inhibitor, are well tolerated, chances of target related toxicity are minimal (Katsifa *et al.* 2015). Therefore, we selected ATX inhibitor, PF-8380 as it shows favourable pharmacokinetic profile such adequate oral bioavailability and exposures required for in vivo testing of autotaxin inhibition along with good brain penetration (Gierse *et al.* 2010). Several reports advocated the role of LPA in liver

pathology (Booijink *et al.* 2022) (Fujimori *et al.* 2018). However, most pharmacological interventions failed so far in alleviating liver injury. Therefore, the potential of small molecule ATX inhibitors for the treatment of patients with NASH and late stages of liver fibrosis remains questionable (Baader *et al.* 2018). We did not observe improvement in liver lesions with ATXi, PF-8380 treatment in morphometric analysis in agreement with reports (Baader *et al.* 2018). PF-8380 treatment had no marked effect on liver markers ALT, AST & TBIL with respect to vehicle treated group (Figure 13A, 13B and 13C respectively)

Study the therapeutic potential of an ATX inhibitor on acute liver diseases and associated complications.
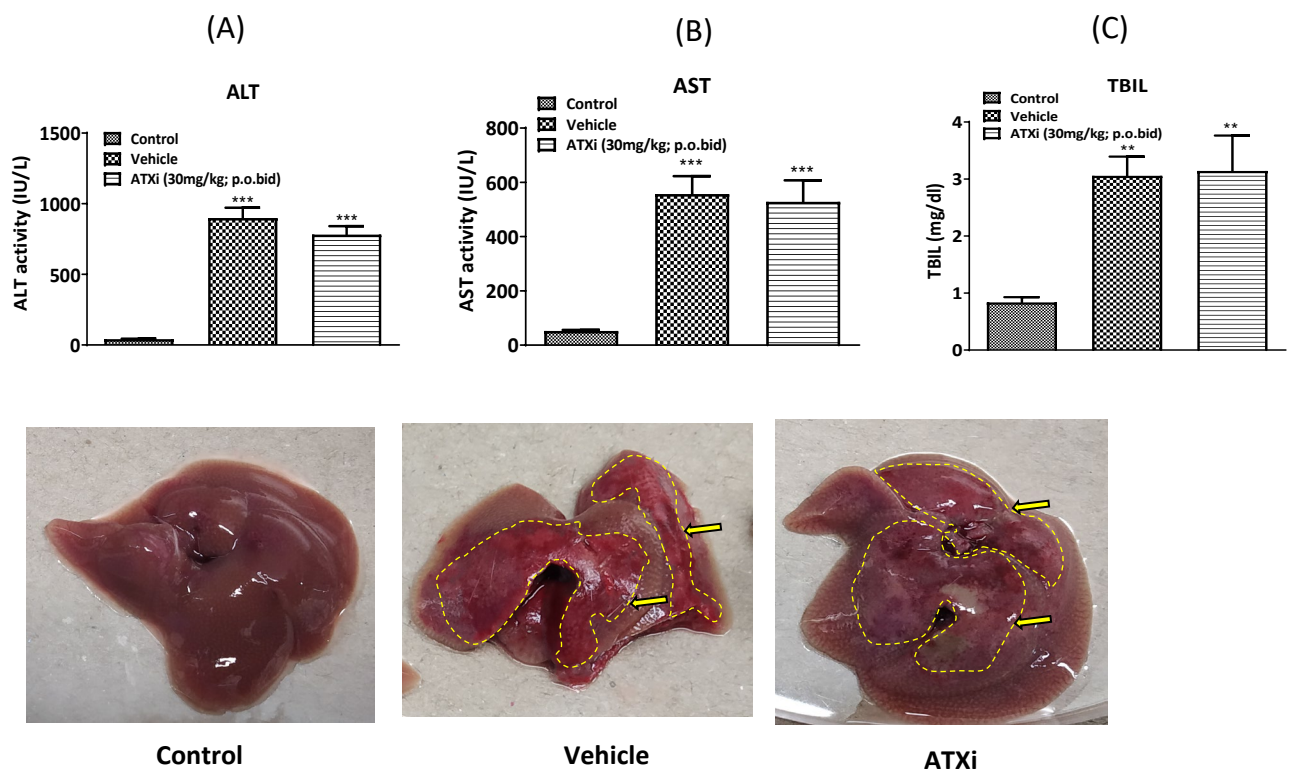

Figure 13: Effect of ATXi on liver function test in TAA treated mice. Change in plasma (A) ALT, (B) AST and (C) total bilirubin level with ATXi treatment. ATXi was administered b.i.d for 3 days. (D) Morphometric analysis of liver. Data represented as mean ± SEM, n=6-10 per group. Statistical significance is shown as ** (p<0.01), *** (p<0.001) vs control mice.

However protective role of ATXi has been reported in several inflammatory conditions. Compound-1, an autotaxin inhibitor significantly inhibited disease activity score in the DSS model of IBD (Thirunavukkarasu *et al.* 2016). Ziritaxestat is an oral, autotaxin inhibitor had reached Phase 3 clinical trial for lung fibrosis (Blanqué *et al.* 2015). More interestingly, ATX-LPA signalling has been ascertained in pathogenesis of neuroinflammatory conditions (Choi *et al.* 2010). Efficacy of ATX inhibitor has been proved in experimental model of multiple sclerosis (Thirunavukkarasu *et al.* 2016). In addition, Lpathomab is a first in this class, humanized monoclonal antibody targeting lysophosphatidic acid (LPA), and has shown good efficacy in animal of traumatic brain injury and undergone clinical trial. Lpathomab^{TM} lowered IL-6 levels, an inflammatory cytokine which was related well with consequence follwing TBI (Crack *et al.* 2014). This led to conclude that pharmacological intervention of ATX-LPA signalling may have neuroprotective effects. In this study, PF-8380 produced significant reduction in LPA levels in plasma, cerebral cortex, and hippocampus homogenates (Figure 14A, 14B and 14C). Cortical region of brain was considered as oxidative stress in cerebral cortex in two types of HE models of thioacetamide (TAA) and bile duct ligation (BDL) attribute to bradykinesia or decreased locomotor deficits (Bai *et al.* 2023). Furthermore, in patients with cirrhosis, the combination of hyperammonemia and neuroinflammation are related not only to cognitive impairment and impaired coordination memory loss and learning inability related to structural and functional hippocampal dysfunctions. Neuroinflammation often leads to disrupted neurogenesis in the dentate gyrus region of the hippocampus and for a decrease in the hippocampal level of brain derived neurotrophic factor (BDNF) (Magen et al. 2009). This region is known to play a crucial role in learning and memory (Stawicka *et al.* 2021) (Magen *et al.* 2009). Hence, we considered

hippocampus as well for estimation of BDNF as a marker for learning and memory in TAA model.
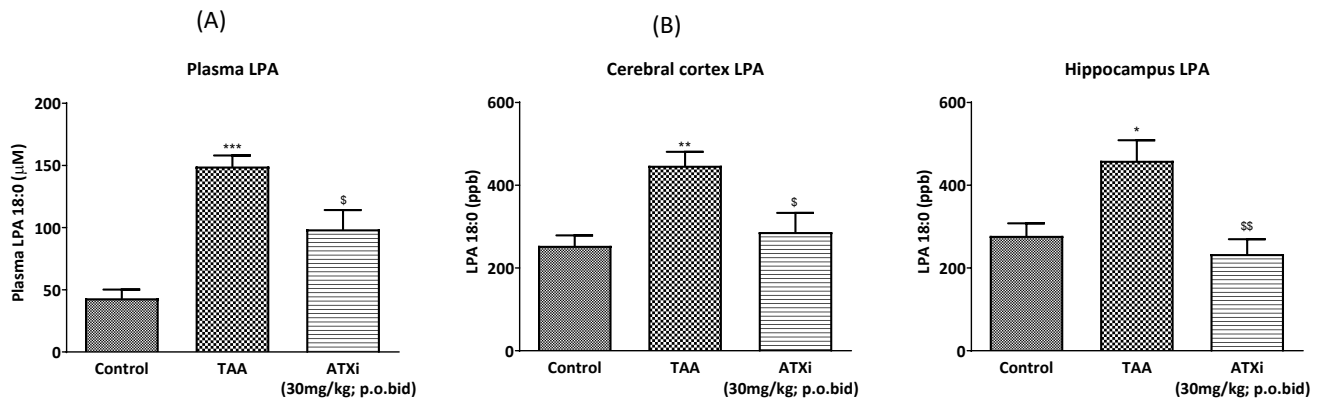

Figure 14: Effect of ATXi on (A) Plasma LPA, (B) Cerebral cortex LPA levels and (C) Hippocampus LPA levels in TAA treated mice. ATXi was administered b.i.d for 3 days. Data represented as mean ± SEM, n=6-10 per group. Statistical significance is shown as *** (p<0.001), ** (p<0.01), * (p<0.05) vs control mice or $ (p<0.05) and $$ (p<0.01) vs vehicle treated group.

The blood and brain ammonia levels remained unaltered with ATXi treatment (15A and 15B), which was obvious as PF-8380 treatment did not ameliorate liver injury in TAA treated mice.
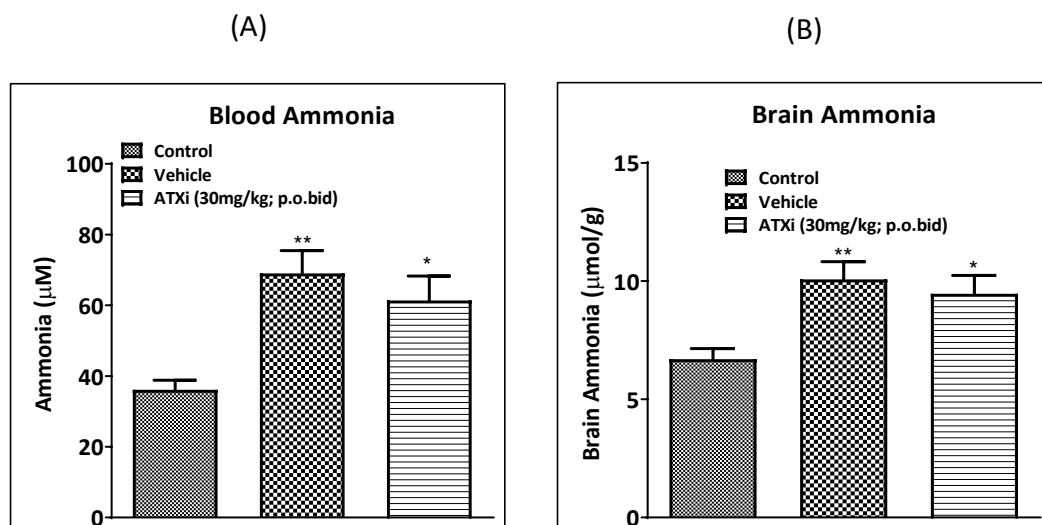

Figure 15: Effect of ATXi on ammonia induced neuroinflammation in TAA treated mice. Changes in (A) Blood ammonia and (B) brain ammonia levels. ATXi was administered b.i.d for 3 days. Data represented as

mean ± SEM, n=6-10 per group. Statistical significance is shown as ** (p<0.01) vs control mice or * (p<0.05) vs vehicle treated group.

Furthermore, patients with HE individual with several neurobehavioral symptoms, including memory impairment, locomotion abnormalities and alterations in personality and consciousness (Yuan *et al.* 2020). Interestingly, ATXi treatment exhibited significant reversal (p<0.05) of spontaneous locomotor distance travelled, rearing, active time, mobile time with respect to vehicle group (Figure 16A, 16B, 16C and 16D respectively). We also observed significant amelioration in HE related clinical grading score (Figure 16E) along with improvement in rotarod activity impairment (Figure 16F) compared with vehicle treated group. Furthermore, the novel object recognition test revealed that the discrimination index in TAA-treated mice was significantly reduced. However, ATXi, PF-8380 treatment showed significant improvement in cognitive deficit (Figure 16G).
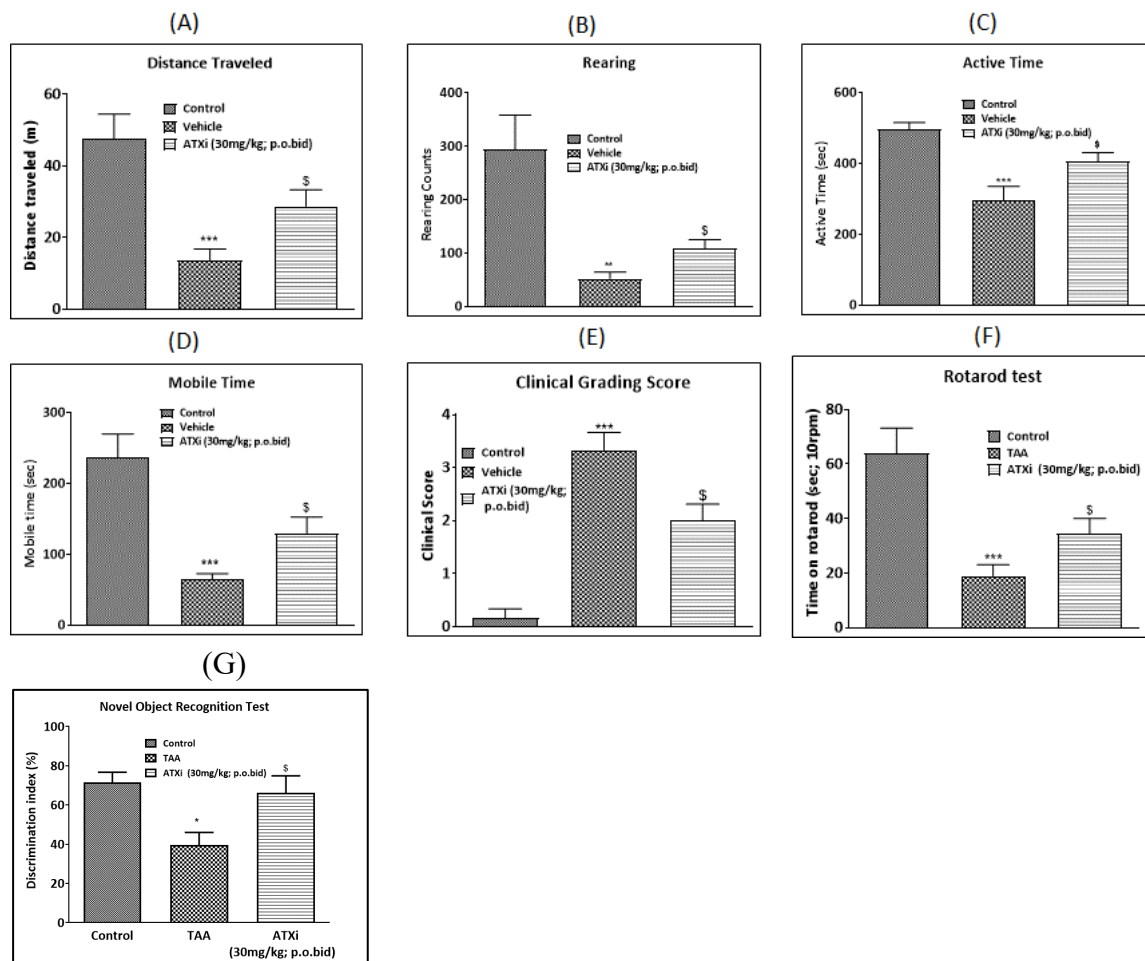
Figure 16: Effect of ATXi on spontaneous motor activity and cognition. Change in (A) Locomotor activity, (B) Rearing counts and (C) Active time, (D) Mobile time, (E) Clinical grading score, (F) Duration in rotarod (G) Recognition memory in novel object recognition test with ATXi treatment. ATXi was administered b.i.d for 3 days. Data represented as mean ± SEM, n=6-10 per group. Statistical significance is shown as ** ($p<0.01$), *** ($p<0.001$) vs control mice or $ ($p<0.05$) vs vehicle treated group.

Neuroinflammation was earlier proved to cause disrupted neurogenesis in the dentate gyrus of the hippocampus and decrease in the hippocampal level of brain derived neurotrophic factor (BDNF) (Avraham *et al.* 2009). This region is known to play a crucial role in cognition. In the current study, the BDNF level was found to be reduced in TAA group. ATXi treatment exhibited significant reversal in BDNF levels (Figure 17 A & B).
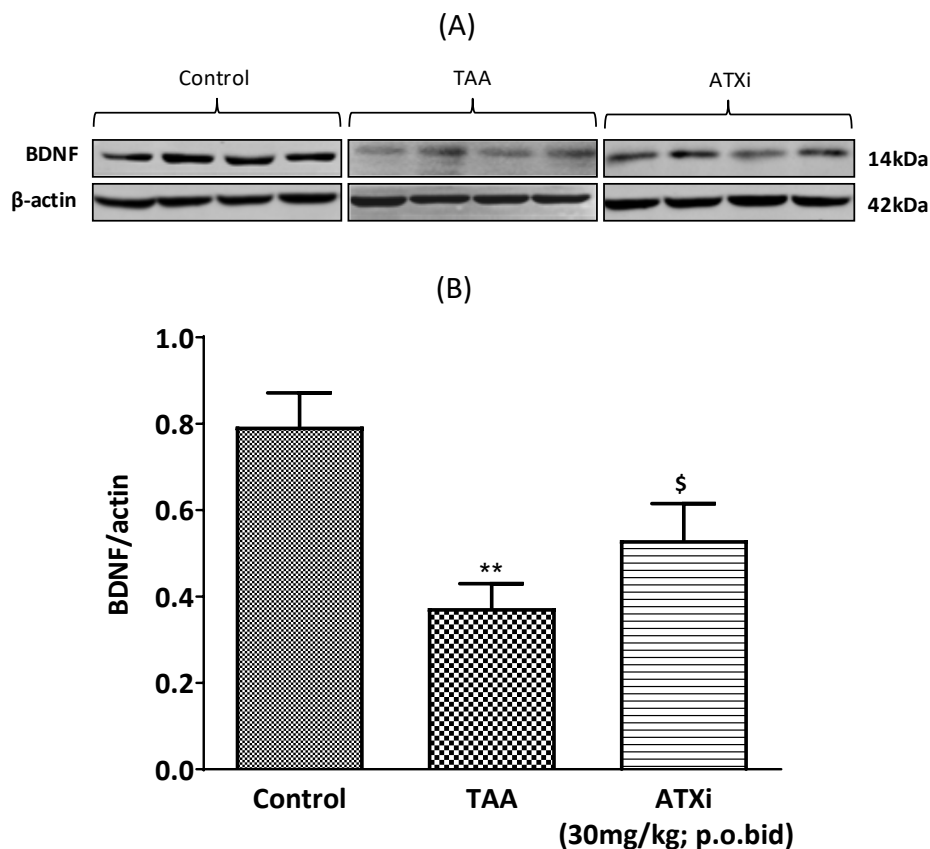

Figure 17: Western blot analysis of BDNF levels in hippocampus mice. (A) Representative Western blot; (B) Western blot analysis. ATXi was administered b.i.d. for 3 days. Data represented as mean ± SEM, n=8 per group. Statistical significance is shown as ** ($p<0.01$) vs control mice or $ ($p<0.05$) vs vehicle treated group.

Recent studies using anti-inflammatory agents such as ibuprofen and indomethacin have shown promising efficacy in mild encephalopathy in patients with cirrhosis (Butterworth, n.d.). Moreover, another study has demonstrated that TNF-α or IL-1β receptor gene deletion delays the onset of encephalopathy and attenuates brain edema in mice with acute liver failure (Rodrigo *et al.* 2010). We checked inflammatory cytokines such as TNF-α & IL-1β in the cortical and TNF-α in hippocampus homogenate. ATXi showed significant (p<0.05) reduction in inflammatory cytokines levels in cerebral cortex and hippocampus compared to vehicle treated group (Figure 18A, 18B and 18C). ATXi also reduced the increase in brain weight observed with TAA treatment (Figure 18D).
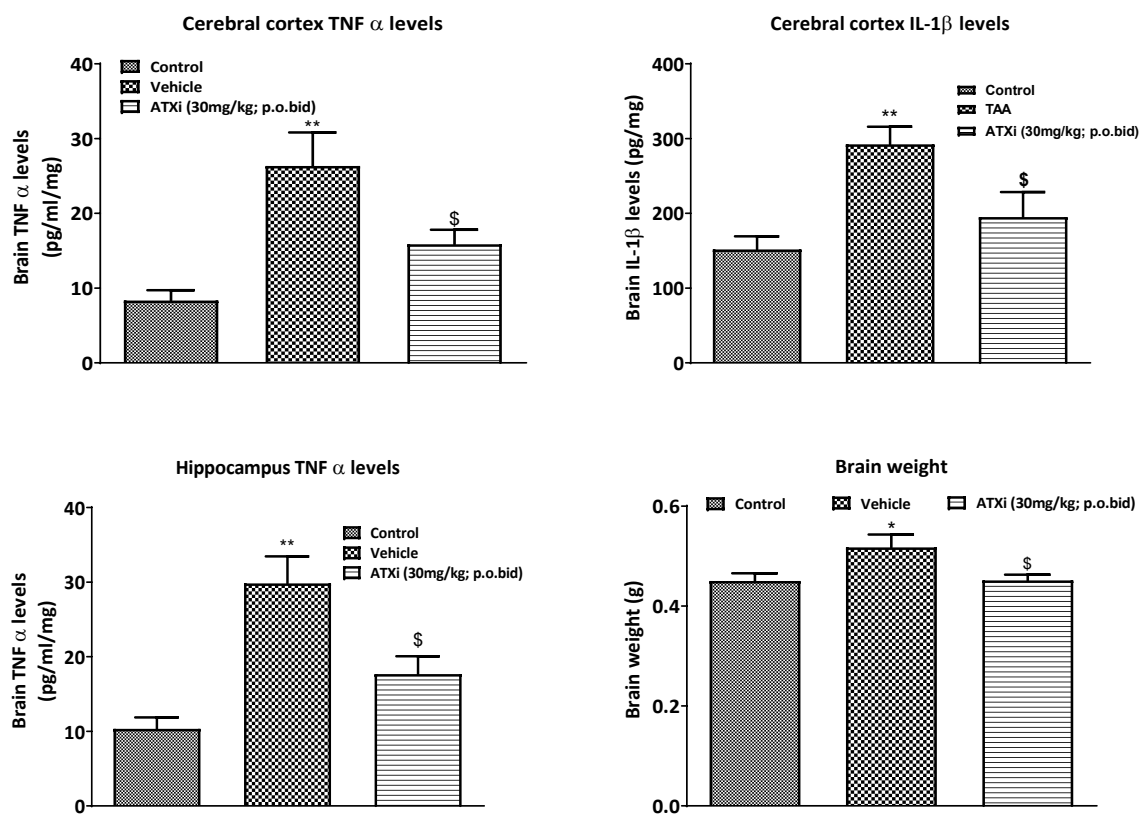

Figure 18: Effect of ATXi on ammonia induced neuroinflammation in TAA treated mice. Changes in (A) Cortex IL-1β level, (B) Cortex TNF-α level, (C) Hippocampus TNF-α level and (D) brain weight. ATXi was administered b.i.d for 3 days. Data represented as mean ± SEM, n=6-10 per group. Statistical significance is shown as ** (p<0.01) vs control mice or $ (p<0.05) vs vehicle treated group.

On analysis of terminal plasma samples, the concentration of ATXi was found to be 0.44 ± 0.05 µM (Table# 3).

Analyte	Plasma concentration
ATXi (PF-8380)	0.44 ± 0.05 µM

Table 3: Concentration of ATXi (PF-8380) in terminal plasma samples.
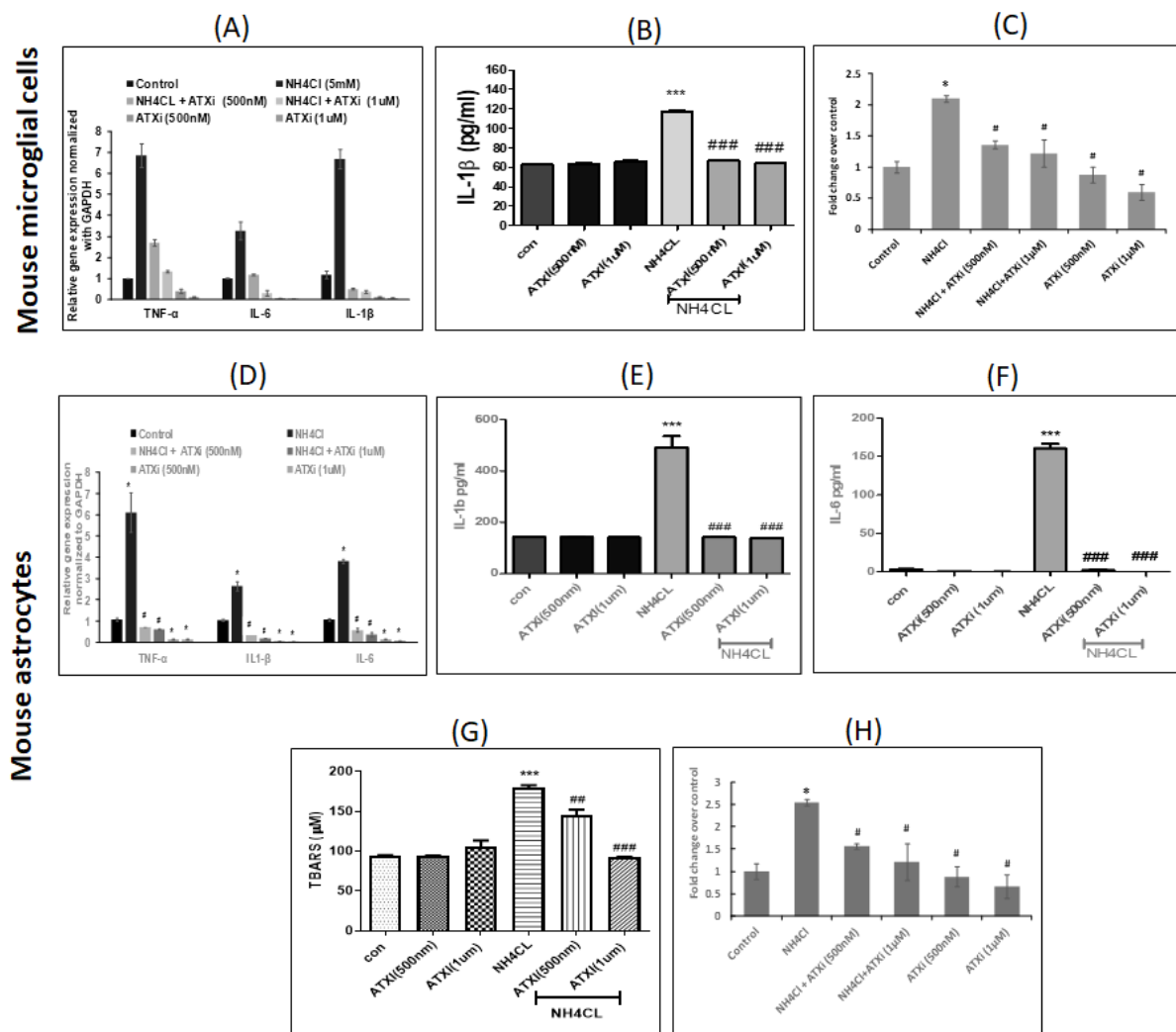
Figure 19: Effect of ATXi on ammonia induced neuroinflammation in mouse microglial cells (N9 cells) and astrocytes (C8-D1A cells). Changes in (A) mRNA expression of TNF-α IL-1β and IL-6, and (B) IL-1β measured by ELISA, (C) ROS levels in microglial cells. Changes in (D) mRNA expression of TNF-α IL-1β and IL-6, and (E) IL-1β and (F) IL-6 measured by ELISA and (G) TBARS, (H) ROS levels in astrocytes cells. Data represented as mean ± SEM. Statistical significance is shown as * ($p<0.05$) and *** ($p<0.001$) vs media control or # ($p<0.05$), ### ($p<0.01$) and ### ($p<0.001$) vs NH4CL treated cells.

Cell culture studies and animal models reveals an important role of oxidative stress in the pathogenesis of cerebral ammonia toxicity (Bosoi and Rose 2013). The nonspecific immune cells of the central nervous system, microglia are plays significant role settin in neuroinflammatory casecades. Their activation can be associated with increased synthesis or release of proinflammatory signalling molecules such as cytokines and chemokines. Additional factors that contribute to inflammation are ROS and prostanoids (Zemtsova *et al.* 2011). In our study, stimulation of mouse microglial cells with NH4CL showed significant increase in mRNA expression of proinflammatory cytokines like TNF-α, IL-1β and IL-6 (Figure 19A), IL-1β (Figure 19B) and ROS levels (Figure 19C) in supernatant which were concentration dependently reversed by ATXi. Furthermore, astrocytes are known to be the essential site for ammonia detoxification in the brain (Sepehrinezhad *et al.* 2020). It is evident from sell culture studies with astrocytes that ammonia induces oxidative stress and increases cytokines release (Guerra *et al.* 2012) (Guerra et al. 2012). In agreement, we observed significant elevation in mRNA expression of TNF-α, IL-1β and IL-6 (Figure 19D), IL-1β (Figure 19E), IL-6 (Figure 19F), TBARS (Figure 19G) and ROS levels (Figure 19H) in culture supernatant as well when mouse astrocyte cells were exposed in NH4CL. ATXi treatment significant ameliorated these inflammatory and oxidative stress markers. 19. In figure 19, apart from TBARS data and IL-1β expression, the 1μM dose of ATXi following NH4Cl treatment is showing reduced data even with compared to normal control group. Although it is hard to comment, but possibly high concentration of ATXi completely inhibits LPA release less than the basaline levels resulting in reduction in cytokines expression and oxidative stress markers even less than control group.

Furthermore, 40 % mortality was observed in TAA-treated mice as expected, which was reduced to 20% with ATXi treatment. However, the body reduction with TAA treatment

could not be reversed (Figure 20). In summary, ATXi showed significant amelioration in neuroinflammation, oxidative stress and behavioral abnormalities associated with HE and reduced mortality, although failed to protect from liver injury.
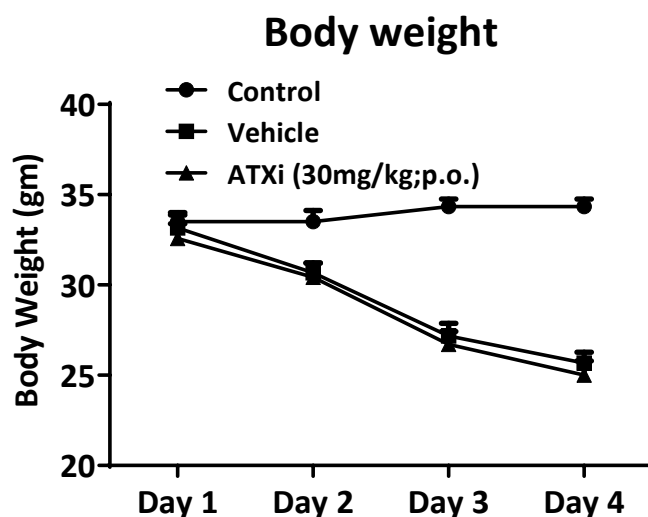
Animal survival	
Control	10
TAA	6
ATXi	8

Figure 20: Effect of ATXi on Body weight and mortality in TAA treated mice. ATXi was administered b.i.d for 3 days. Data represented as mean ± SEM, n=6-10 per group.

Conclusion

ATXi showed significant amelioration in neuroinflammation, neuropsychiatric symptoms associated with HE and reduced mortality, although failed to protect from liver injury. Therefore, autotaxin inhibition either alone or in combination with drugs targeting other mechanisms may provide an oral therapeutic option for HE.

Section: 2

Evaluation of engagement of ATX-LPA pathway in the pathogenesis of bile duct ligation induced chronic liver diseases (CLD) and hepatic encephalopathy. To investigate the therapeutic potential of an ATX inhibitor on chronic liver diseases induced hepatic encephalopathy.
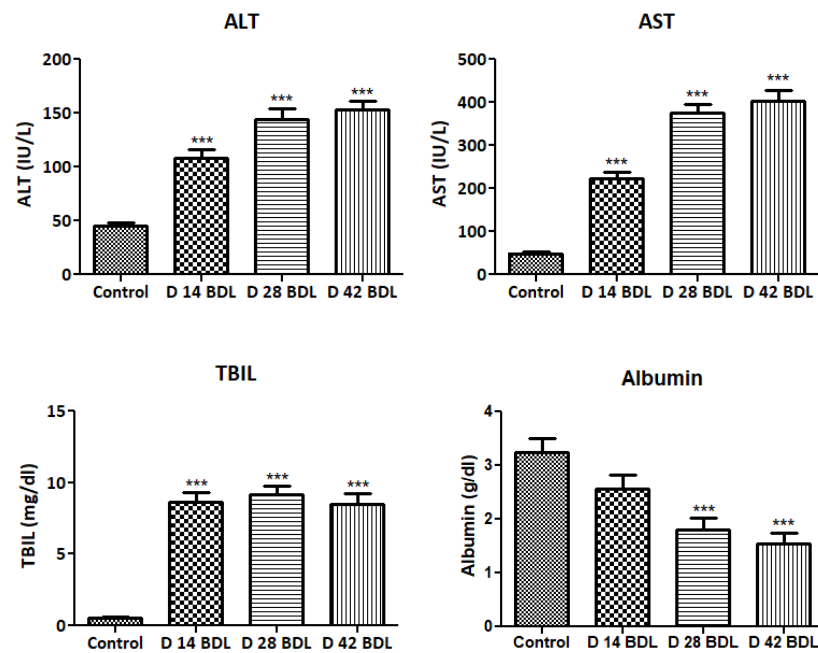
Figure 21: Effect of bile duct ligation on biochemical parameters in plasma. Data represented as mean ± SEM, n=6 per group. Statistical significance is shown as *** (p<0.001) vs control rats.

Figure 22: Effect of bile duct ligation on plasma & brain ammonia levels and brain weight. Data represented as mean ± SEM, n=6 per group. Statistical significance is shown as ** (p<0.001), ***(p<0.001) vs control rats.
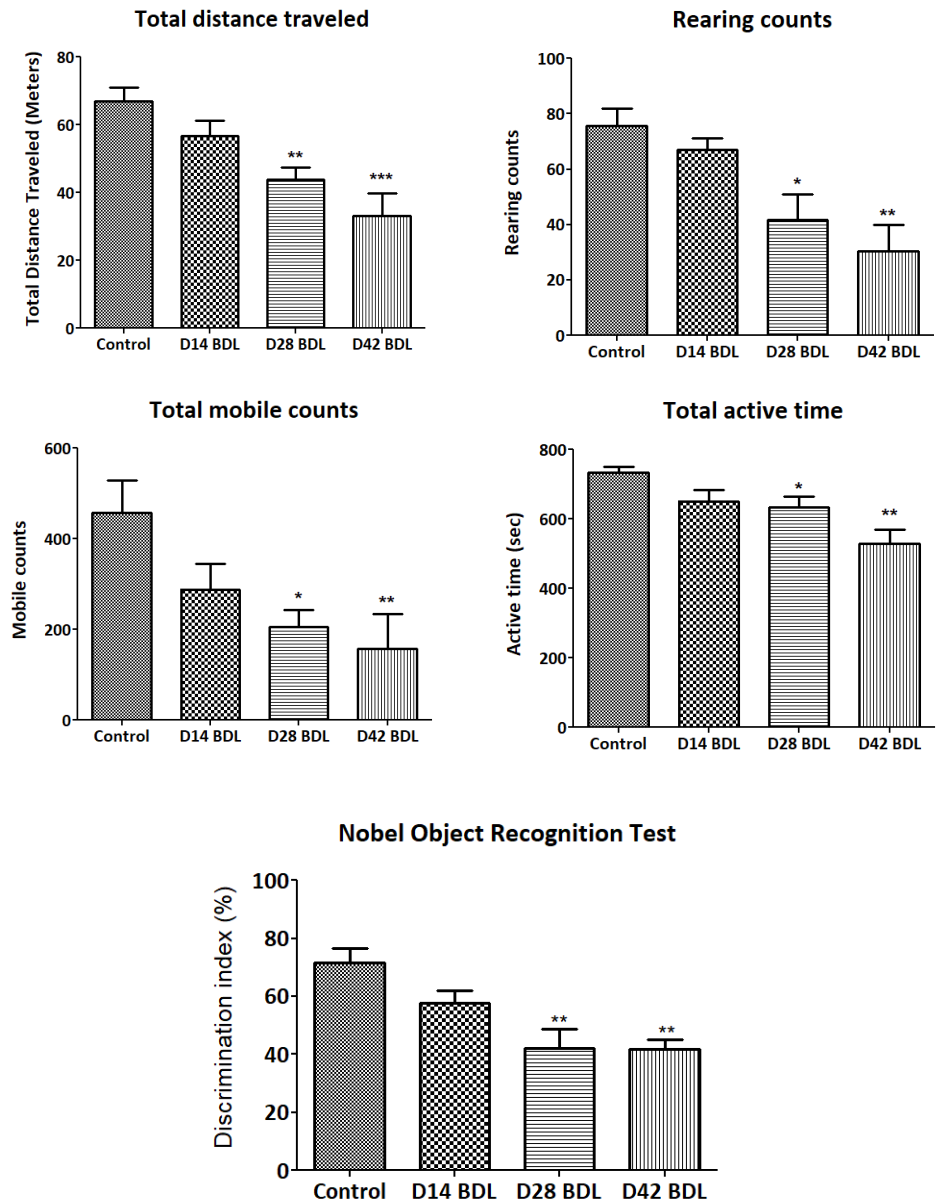
Figure 23: Effect of bile duct ligation on behavioral parameters. Data represented as mean ± SEM, n=6 per group. Statistical significance is shown as ** (p<0.001), **(p<0.01), ***(p<0.001) vs control rats.
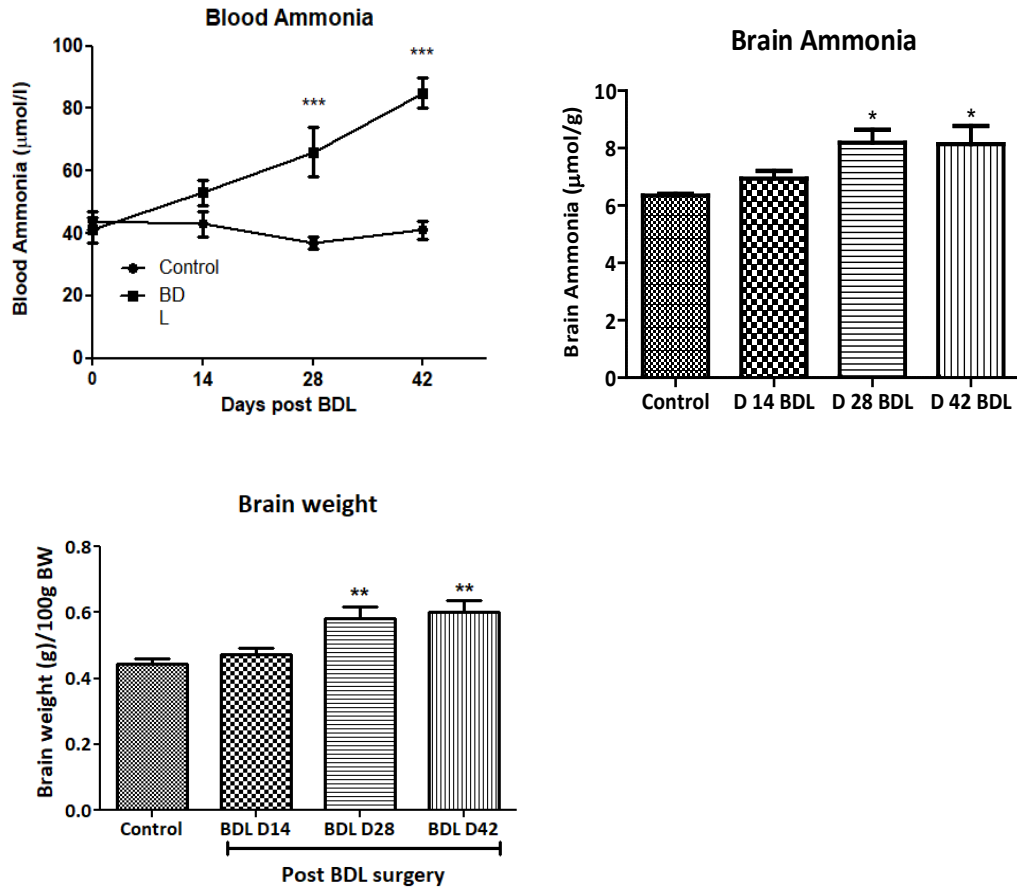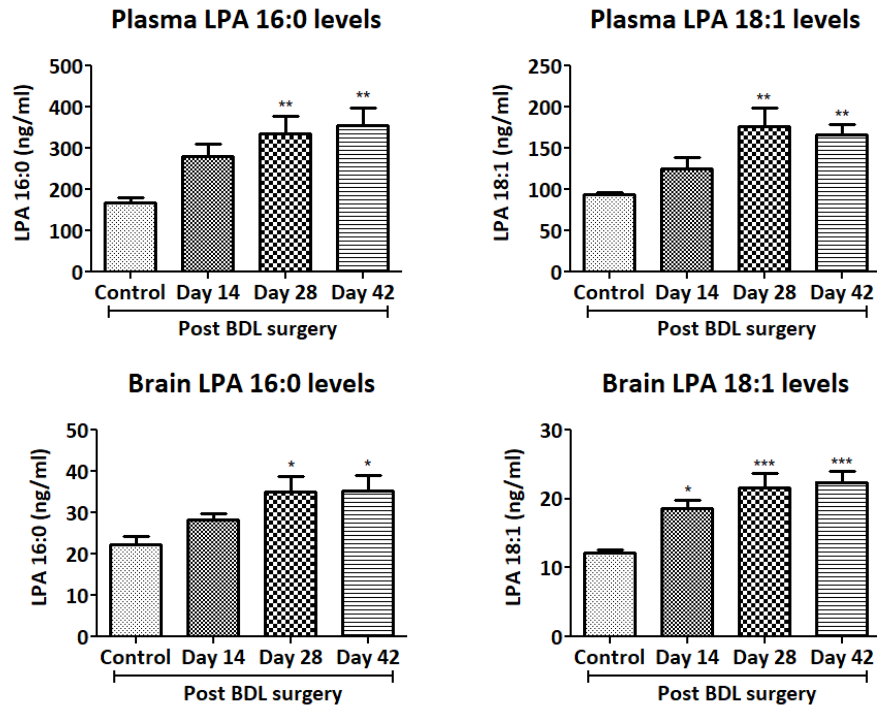
Figure 24: Effect of bile duct ligation on plasma and brain LPA levels. Data represented as mean ± SEM, n=6 per group. Statistical significance is shown as * (p<0.05), ** (p<0.01), *** (p<0.001) vs control rats.

Pilot study: Liver fibrosis, a chronic liver disease occurs because of excessive accumulation of extracellular matrices including collagen and often advances to cirrhosis if not halted (Bataller and Brenner 2005). Although there has been increasingly effort in developing new therapies for liver fibrosis, but the need is still unmet. Among new targets, Autotaxin has evolved as promising target for chronic liver disease in last decade. Although, interestingly increased ATX expression was detected in chronic liver disease (Katsifa *et al.* 2015), its role has not been reported in biliary fibrosis. In our pilot study, we considered rat as the surgical manipulation is very complicated in mouse and mortality rate is comparatively higher in mice than rats. Furthermore, bile duct ligation (BDL) in rats yields faster development and higher success rate of cirrhosis. Although, the morphological changes observed in both mice and rats following BDL are comparable to those observed in human biliary cirrhosis (Richter *et al.* 2022). We wanted to investigate

if ATX-LPA axis has any role in the pathogenesis of bile duct ligation induced liver fibrosis. We observed that BDL resulted in chronic liver disease as evident from elevated levels of liver enzymes and total bilirubin. Interestingly temporal monitoring of plasma LPA revealed significant increase in plasma LPA levels with progression of disease which agrees with (Bain *et al.* 2016). In pilot study for biomarker assessment, we first checked temporal effect of BDL various biochemical and behavioral parameters. Plasma AST, AST and TBIL levels were significantly increased time dependent manner on day 14 ($p < 0.001$), 28 ($p < 0.001$) and 42 ($p < 0.001$) post BDL compared to control rats whereas albumin level was found to be decreased gradually with time and significantly ($p < 0.001$) on day 28 and 42 post BDL (Figure 21).

Following BDL surgery, plasma ammonia level steadily built up and significant increase was observed on day 28 ($p < 0.001$) and day 42 ($p < 0.001$) compared to control rats (Figure 22). As a result, brain ammonia levels were also observed to be significant higher on day 28 ($p < 0.05$) and 42 ($p < 0.05$) (Figure 14). We also noticed significant increase in brain weight on day 28 ($p < 0.01$) and 42 ($p < 0.01$) after BDL surgery (Figure 22).

Hepatic encephalopathy (HE) is a severe neurobehavioral condition of cirrhosis, and the symptoms are extensively unpredictable, extending from mild alteration in mental state to coma. Behaviour parameters were assessed at different time points following BDL. Spontaneous motor activities such as total distance travelled, rearing counts, total mobile time and total active time were progressively decreased following BDL surgery and significant effects were observed from day 28 onwards (Figure 23). In line we observed disturbed spontaneous locomotor and exploratory activities in BDL rats (Leke *et al.* 2012). Impairment in recognition memory was also evident in BDL animals as observed in NORT which was a consequence of hepatic encephalopathy in agreement with (Collie 2005). NORT as a measure for recognition memory revealed significant decrease in discrimination index on day 28 ($p < 0.01$) and 41 ($p < 0.01$) after BDL surgery (23).

We investigated the effect of BDL induced liver injury on plasma and brain LPA and observed time dependent increase in LPA 16:0 and 18:1 in plasma as well as in brain. Both LPA 16:0 and 18:1 was found to be elevated significantly in both plasma and brain on day 28 and 42 post liver injury (Figure 24). Disease control per se at every time point was not considered at every time point. In that case we had to consider a separate cohort of animals for each timepoint because in case we would have considered one cohort, serial blood sampling from same animal at different time was not possible as blood volume required for biochemical assessment exceeds the ethical limit of blood withdrawal. In our pilot study, we just wanted to check the involvement of ATX-LPA signalling with progression of disease. Therefore, we enrolled minimum number of animals to follow 3Rs principle We considered only one control group which was terminated on day 42 of animal ethics. Regarding creatinine level, as per literature also significant renal injury is observed only at and after 4 weeks BDL surgery as evident from increase in creatinine level. Similarly, albumin level significantly changes from 4 weeks onwards as per report. (Pereira *et al.* 2008) (Mohamed *et al.* 2015). These results match with our findings.

There are several reports advocating role of ATXi inhibitor in alleviating liver fibrosis in animals. (Yamazaki *et al.* 2017), reported a strong association between autotaxin (ATX) is a secreted enzyme serum autotaxin levels with Liver Fibrosis in patients with Chronic Hepatitis C and investigated the diagnostic ability of ATX for liver fibrosis in confirmed hepatitis C virus (HCV) infection. It has been reported that liver-secreted autotaxin acts in an autocrine method to worsen NAFLD through LPA-induced inhibition of the Peroxisome proliferator activated receptor-α and may be an attractive therapeutic target for NAFLD (Qiu *et al.* 2022). Therefore, based on preclinical and clinical findings, treatment with ATX inhibitors and/or LPA receptor antagonists to reduce fibrosis in chronic liver disease patients may hold great promise for the prevention of cirrhosis and ultimately HCC (Erstad *et al.* 2017).

ATX inhibitor by Cpd17 exhibited improvement in CCl4-induced acute liver injury, and it lowered steatosis, inflammation, and fibrosis in the NASH model (Booijink *et al.* 2022). Furthermore ATXi, PF8380 showed reduction in plasma ATX activity and liver LPA levels by approximately 50% and showed some improvement in liver fibrosis in CCl4-induced liver disease models (Katsifa *et al.* 2015). But the efficacy of most of ATXi is limited. Moreover, effecacy of ATXi on hepatic encephalopathy associated with chronic liver disease has not been studied.

CBT-295
Exact Mass: 329.13
Molecular Formula: $C_{18}H_{20}ClN_3O$
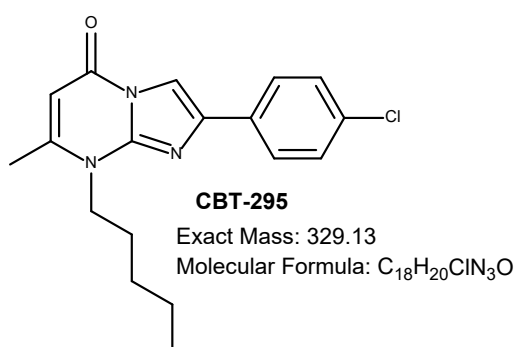
2-(4-chlorophenyl)-7-methyl-8-pentylimidazo[1,2-*a*]pyrimidin-5(8*H*)-one

Figure 25: Structure of CBT-295

Pharmacokinetic study: We initiated our investigation with pharmacokinetic assessment of an ATX inhibitor CBT-295. The pharmacokinetic parameters of CBT0295 following a single intravenous administration at 1 mg/kg and single oral administration at 10 mg/kg are shown in Table 1. The compound had a terminal half-life of 1.9 hours after intravenous administration. The total clearance and volume of distribution at steady state (Vss) were 32.4 ml/min/kg and 5.3 l/kg respectively indicating moderate to low clearance. Following oral administration of CBT-295, the plasma concentration (Cmax) reached to a maximum 882.3 ng/ml with AUC of 4529.2 ng.hr/ml and exhibited good oral bioavailability of (Foral) of 88.2%.
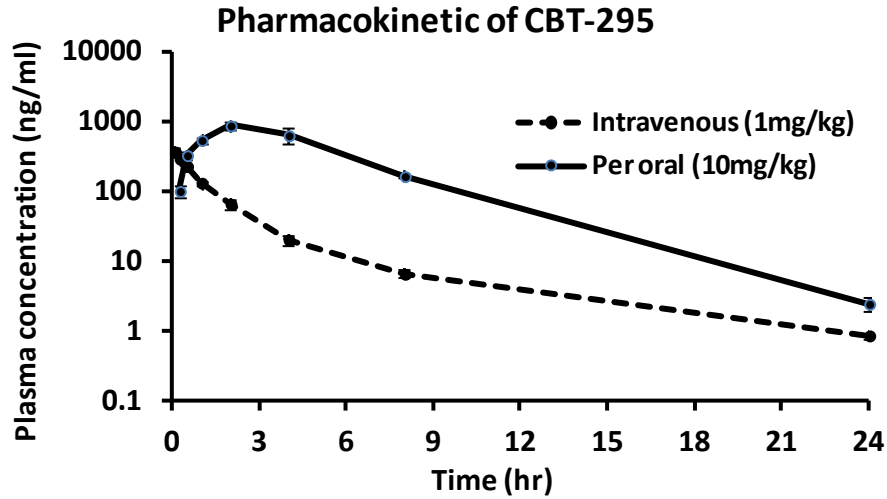
Figure 26: Plasma concentrations (nanograms per millilitre) in of CBT-295 from male Sprague-Dawley rats after a single i.v. (1mg/kg) or per oral (10mg/kg) administration (n = 3/group).

PK parameters	Intravenous	Oral
C_0 (ng/ml)	420.5±76.1	-
Cmax (ng/ml)	-	882.3±127.5
$AUC0_{last}$ (ng.hr/ml)	512.2±17.3	4529.2±696.9
$AUC0_{inf}$ (ng.hr/ml)	514.6±17.3	4538.3±698.3
Clearance (ml/min/kg)	32.4±1.1	37.3±5.5
$T_{1/2}$ (hr)	1.9±0.2	2.5±0.1
V_{ss} (L/kg)	5.3±0.04	-
MRT_{last} (hr)	2.6±0.1	4.6±0.1
MRT_{inf} (hr)	2.7±0.1	4.6±0.1
F_{oral} (%)	-	88.2±13.6

Table 5: Pharmacokinetic parameters of CBT-295 following intravenous and oral administration at 1mg/kg and 10 mg/kg respectively in Sprague Dawley rats.

It has shown favourable pharmacokinetic properties with oral bioavailability more than 88%. We selected 20mg/kg dose and twice daily administration so that the plasma concentration remains good enough to maintain ATX inhibition for prolong duration.

Tolerability study: Tolerability study with CBT-295 revealed that administration of 30mg/kg; bid; p.o. for 5 days in rats did not show adverse effect as no significant change in body weight, RBC, WBC, plasma ALT, ALT, and creatinine levels.

Figure 27: Tolerability study: Body weight, RBC, WBC, Plasma ALT, AST and creatinine levels following administration of CBT-295 (30mg/kg;bid;p.o.) for 5 days in Sprague Dawley rats (n=5/group).

Pharmacodynamic study:

Figure 28: Human ATX inhibitory activity of CBT-295.

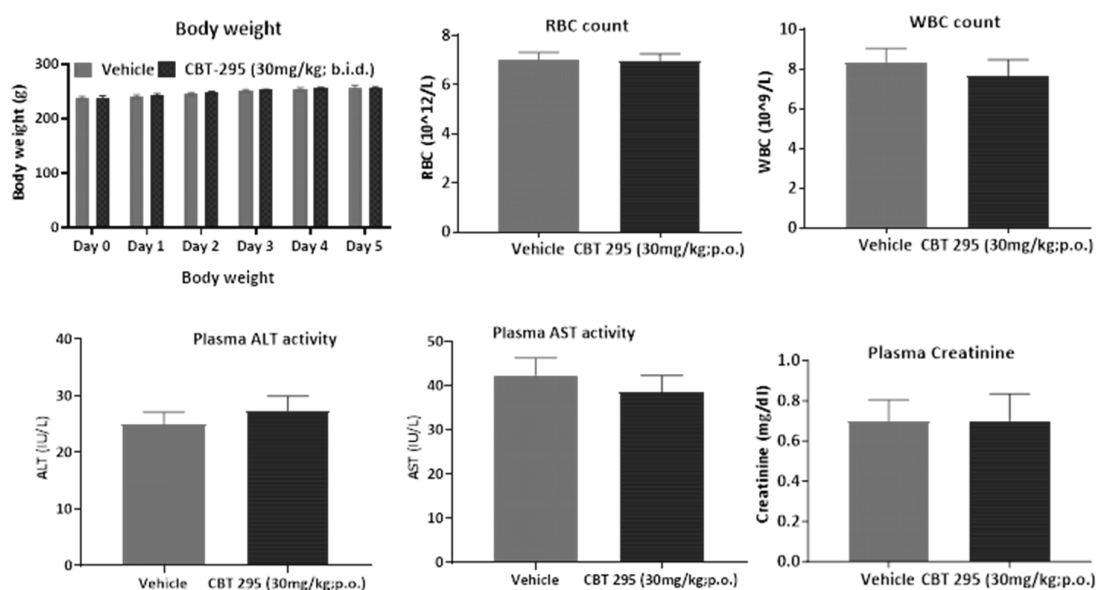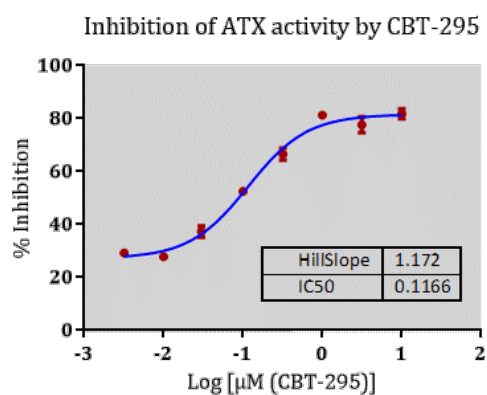CBT-295 was tested for its human ATX inhibitory activity. The IC_{50} was found to be 0.1166μM (Figure 27). We observed significant (p<0.001) increase in plasma ALT, AST and ALP levels after BDL by 3.7-fold, 3.4-fold and 2.3-fold respectively compared to sham rats. Treatment with CBT-295 at 20mg/kg for 28 days showed 21.5%, 20.4% and 21.6% reduction in ALT, AST, and ALP activity respectively, however the effect was not significant. The albumin level was found to be lowered significantly (p<0.05) in BDL group compared to sham group. CBT-295 showed marked increase (13%) in plasma albumin level (Figure 27).

Histopathological examination of liver samples revealed significant increase (9-fold) in collagen deposition in liver was found in BDL rats when compared to sham rats. CBT-295 demonstrated significant amelioration in liver collagen deposition by 26.3% with respect to BDL rats (Figure 28). CBT-295 demonstrated significant retardation in developments of bile duct ligation induced fibrosis as evident from significant reduction in collagen deposition in the liver. Although liver enzymes were reduced markedly, CBT-295 failed to show significant reversal (Figure 29).

Estimation of LPA revealed significant increase in plasma LPA 16:0 (2-fold) and LPA 18:0 (2.5-fold) compared to sham groups. Furthermore, brain LPA 16:0 (2-fold) and LPA 18:0 (2.5-fold) was also found to be elevated significantly compared to sham groups. CBT-295 treatment ameliorated both plasma and brain LPA 16:0 and LPA 18:0 significantly in comparison to BDL rats (Figure 30).

The plasma as well as the brain ammonia levels known to be major cause for hepatic encephalopathy (Farmer and Mulakkan 1990), was found to be significantly decreased with CBT-295 treatment. The blood ammonia level was markedly increased (2.2 fold) in BDL rats with respect to sham rats while CBT-295 demonstrated significant lowering (p<0.05) by 22.4%. As a result, we also observed significant elevation in brain ammonia level which got reversed significantly with CBT-295 treatment (Figure 31 A & B).

88

Recently, neuroinflammation has been reported to play a significant role in the pathogenesis of hepatic encephalopathy (Montoliu *et al.* 2015) where ammonia triggers inflammatory process in microglia and astrocytes causing brain edema (Jayakumar *et al.* 2015). In our investigation, we observed significant ($p<0.01$) increase in brain weight in BDL animals compared to sham rats. CBT-295 exhibited significant ($p<0.05$) reduction (Figure 31 C). Elevation in TNF-α levels in cortex and hippocampus were also evident which were markedly reversed by CBT-295 (Figure 31 D & E). These findings are aligned with our previous work where we had observed that ATXi reduced the neuroinflammation associated with acute liver failure and improved neurobehavioral and motor activities without altering the liver pathology (Roy *et al.* 2022). Hence, this might be possible that the improvement in impaired behavioral observed with CBT-295 possibly be a combined effect of hepato-protection and direct effect on neuroinflammation.

It is evident that hyperammonemia due to chronic liver disease namely cirrhosis induces microglial activation and neuroinflammation and these contribute to the cognitive and motor impairment during hepatic encephalopathy (Rodrigo *et al.* 2010) (Jiang *et al.* 2009). Spontaneous locomotor got deteriorated in BDL animals. Total distance travelled, rearing counts, total mobile time and total active time were significantly decreased in vehicle animals compared to sham animals. The impaired spontaneous locomotor activities were significantly reversed in CBT-295 treated animals in this study (Figure 32). We also observed impairment in recognition memory in BDL animals in NORT. Interestingly, we observed significant improvement in neuropsychiatric symptoms with CBT-295 treatment. The percent discrimination index reduced significantly ($p<0.01$) in vehicle group. CBT-295 showed significant improvement ($p<0.05$ in impaired memory with respect to vehicle group (Figure 32).

Furthermore, white blood cells (WBCs) were found elevated in chronic liver injury (Cox and Kalns 2010). In agreement, we found significant increase in WBCs in BDL rats but

CBT-295 despite of showing anti-inflammatory activity could not reverse this elevation (Table 3).

Fasting glucose levels in cirrhotic animals is expected to decrease as the glycogen get depleted to liver damage (Bai *et al.* 2021). In our study as well, the fasting glucose level is BDL rats were found to be decreased compared to sham rats. But CBT-295 failed to exhibit any effect on plasma glucose levels (Figure 33).

Conclusion

Therefore, inhibition of ATX-LPA axis might prove to be multimodal approach, where it can protect clinical decompensation of chronic liver disease by acting on several key pathogeneses.
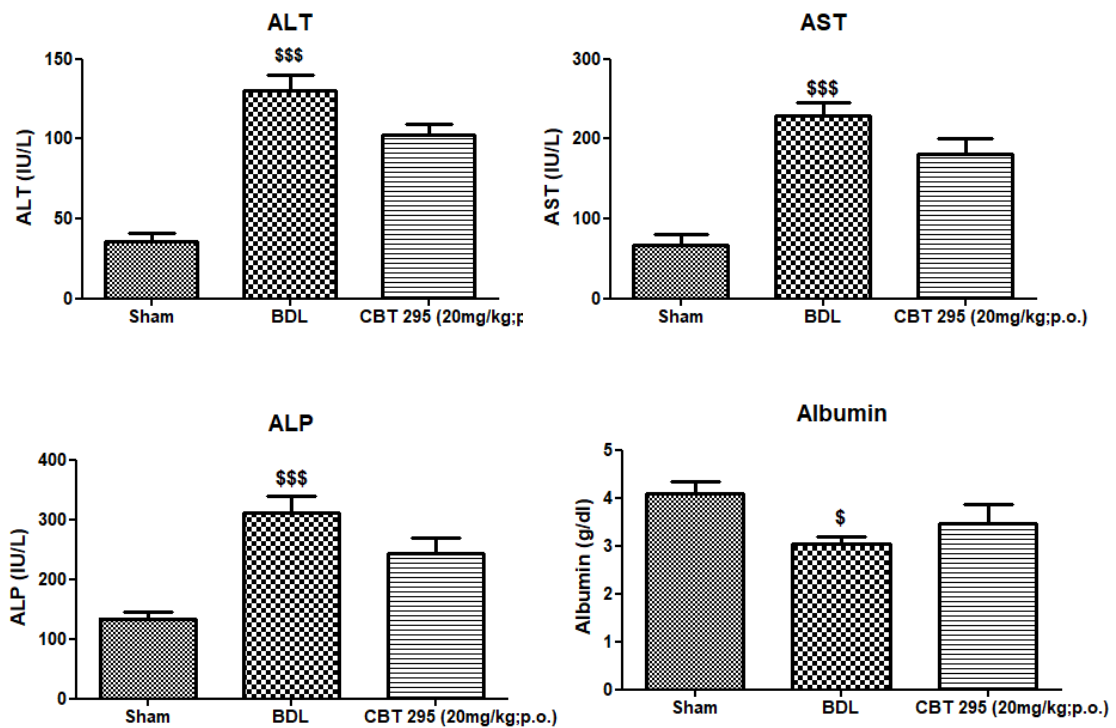
Figure 29: Effect of CBT-295 on plasma and brain LPA levels. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as $ (p<0.05), $$ (p<0.01) vs sham rats.

Figure 30: Effect of CBT-295 on plasma and brain LPA levels. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as $$$ (p<0.001) vs Sham and * (p<0.01), ** (p<0.01) vs vehicle rats.
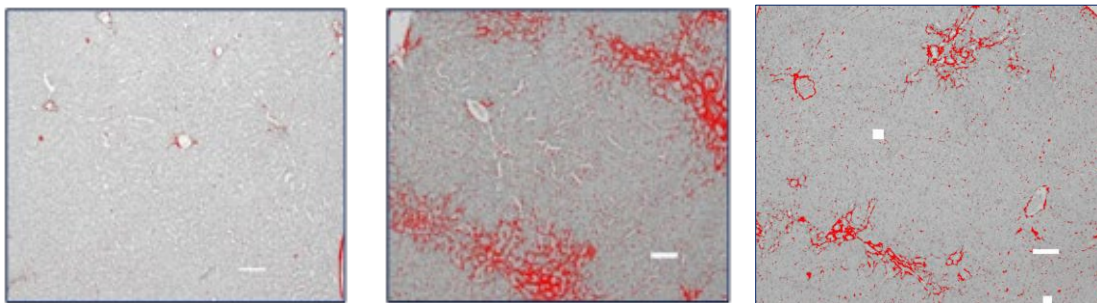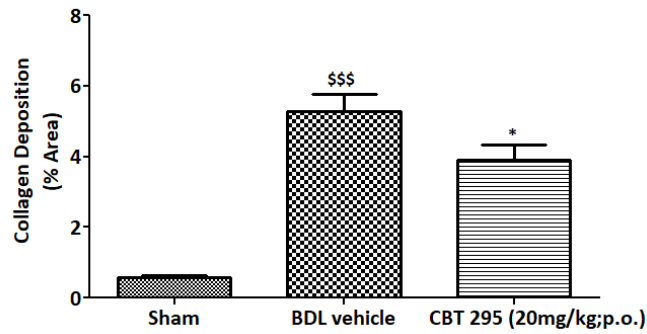
| Sham | Vehicle | CBT-295 |

Figure 31: Effect of CBT-295 on collagen deposition. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as $$$ (p<0.001) vs Sham and * (p<0.01) vs vehicle rats.

(A)   (B)

(C)   (D)

(E)

Figure 32: Effect of CBT-295 on ammonia induced neuroinflammation. (A) Blood ammonia level, (B) Brain ammonia level, (C) Brain weight, (D) Hippocampus TNF-α and (E) cortex TNF-α levels. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as $$ (p<0.01), $$$ (p<0.001) vs Sham and * (p<0.05) and (p<0.001) vs vehicle rats.

Figure 33: Effect of CBT-295 on spontaneous locomotor activity. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as $ (p<0.05), $$ (p<0.01), $$$ (p<0.001) vs Sham and * (p<0.05), ** (p<0.01), *** (p<0.001) vs vehicle rats.

Treatment group	Sham			BDl-Vehicle			CBT 295 (20mg/kg;p.o.)		
WBC (10^9/L)	8.9	±	1.9	26.7	±	5.8	26.7	±	5.0
Neutrophils (10^9/L)	1.6	±	0.8	10.2	±	1.9	12.3	±	3.3
Lymphocytes (10^9/L)	6.9	±	1.2	10.8	±	2.7	11.0	±	1.9
Monocytes (10^9/L)	0.3	±	0.1	4.9	±	2.1	2.7	±	0.8
Eosinophils (10^9/L)	0.1	±	0.0	0.4	±	0.1	0.3	±	0.1
Basophils (10^9/L)	0.1	±	0.0	0.4	±	0.1	0.5	±	0.1
%Neutrophils	14.9	±	3.4	38.6	±	3.5	41.6	±	5.1
%Lymphocytes	79.7	±	3.7	44.7	±	5.5	45.8	±	5.9
%Monocytes	3.6	±	0.6	13.8	±	4.0	10.0	±	2.7
%Eosinophils	1.1	±	0.1	1.6	±	0.3	1.0	±	0.2
%Basophils	0.7	±	0.1	1.3	±	0.2	1.6	±	0.3
RBC (10^12/L)	7.3	±	0.3	7.1	±	0.3	6.2	±	0.8
HGB (g/dL)	14.2	±	0.4	14.1	±	0.6	12.6	±	1.5
%HCT	39.5	±	1.1	39.1	±	1.5	35.4	±	4.3
MCV (fL)	54.7	±	1.2	55.2	±	0.7	58.9	±	3.1
MCH (pg)	19.7	±	0.4	19.9	±	0.4	21.5	±	1.6
MCHC (g/dL)	35.7	±	0.3	36.0	±	0.3	36.2	±	0.8
%RDW-CV	12.4	±	0.3	14.9	±	0.2	20.3	±	4.8
RDW-SD (fL)	29.0	±	0.6	34.6	±	0.5	53.0	±	16.6
PLT (10^9/L)	763.9	±	65.0	727.9	±	146.9	628.7	±	134.0
MPV (fL)	6.5	±	0.3	5.7	±	0.1	5.7	±	0.2
PDW	15.9	±	0.1	15.8	±	0.2	16.3	±	0.4
%PCT	0.5	±	0.0	0.4	±	0.1	0.4	±	0.1

Table-5: Effect of CBT-295 on haematological parameters. Data represented as mean ± SEM, n=7-10 per group.

Figure 34: Effect of CBT-295 on plasma glucose levels. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as *** (p<0.05) vs Sham.

Conclusion

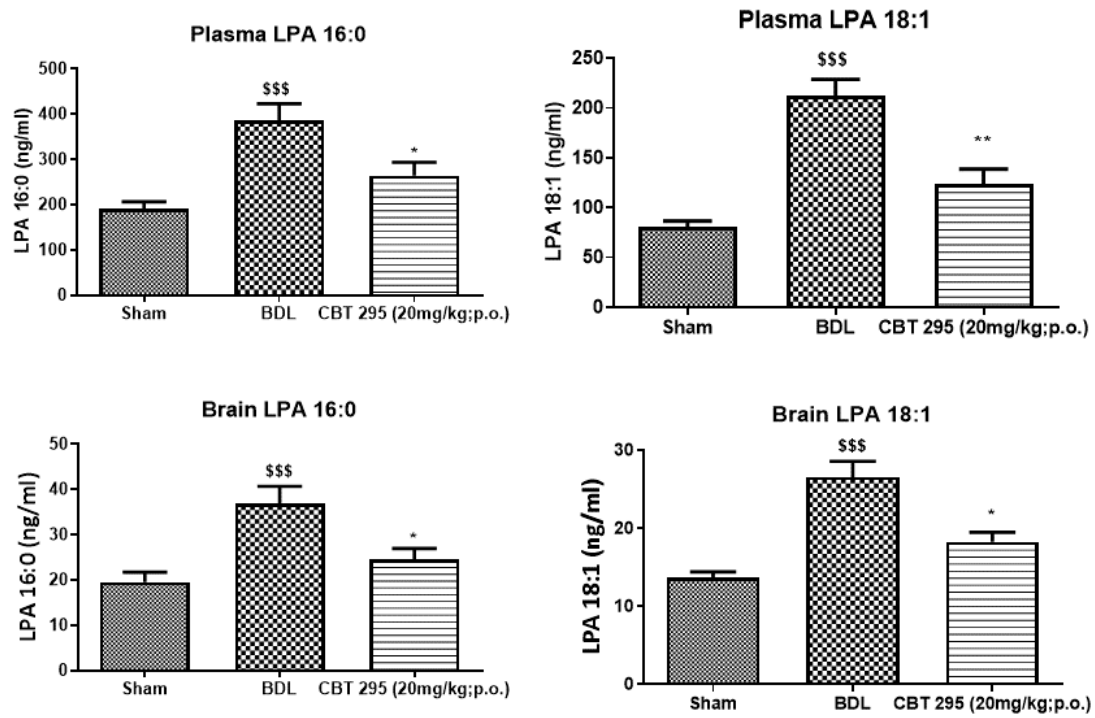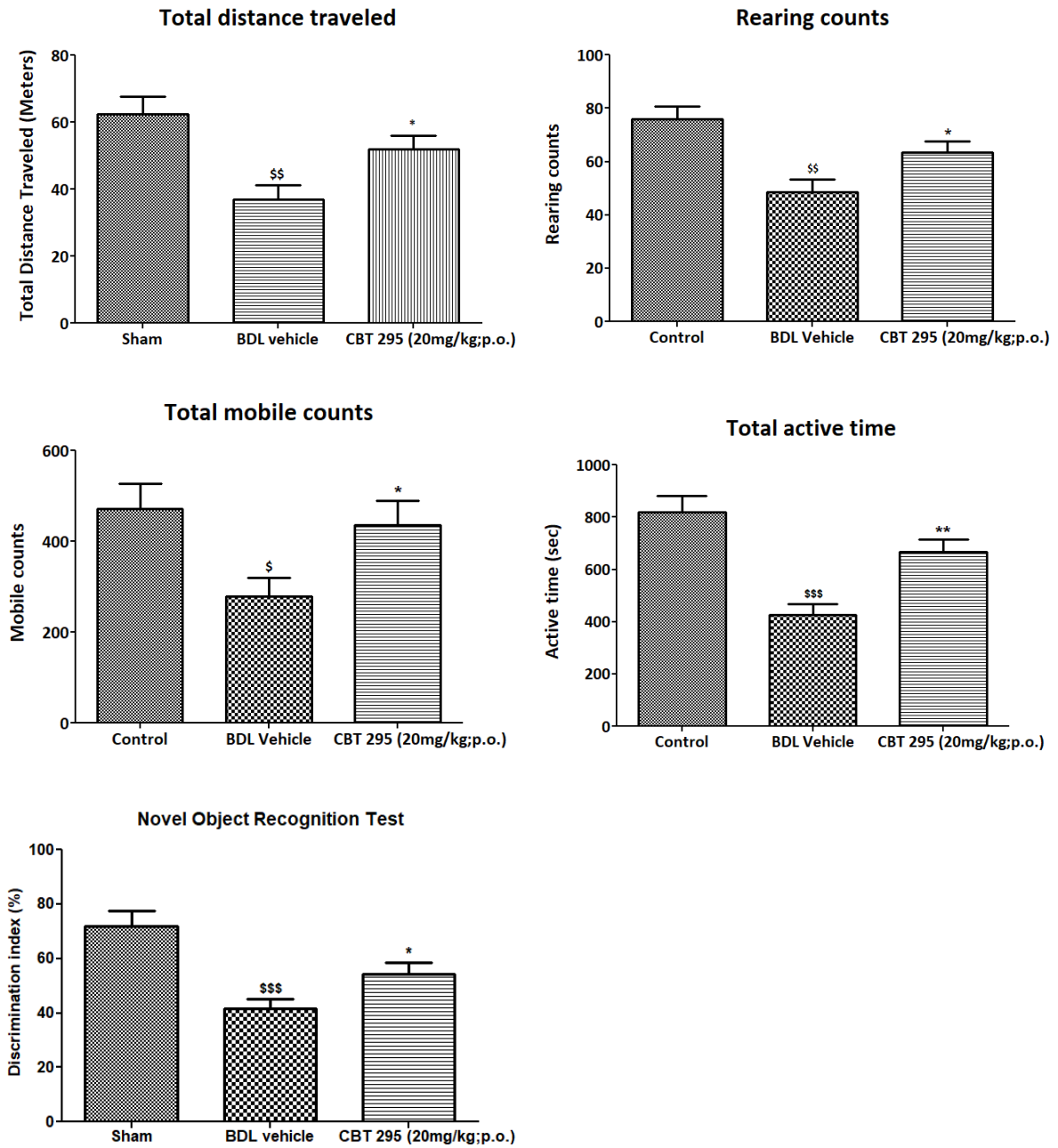Therefore, inhibition of ATX-LPA axis might prove to be multimodal approach, where it can protect clinical decompensation of chronic liver disease by acting on several key pathogeneses. Interestingly, chronic liver disease induced by bile duct ligation showed decrease in plasma glucose levels.

Section 3

Evaluation of engagement of ATX-LPA pathway in the pathogenesis of cholemic nephropathy induced by bile duct ligation induced chronic liver injury. To investigate the therapeutic potential of an ATX inhibitor on cholemic nephropathy.

Kidney damage is a usual finding in individual with liver disorders. Bile cast nephropathy also called as cholemic nephropathy is an ignored reason of kidney injury in individual with hyperbilirubinemia. It can take place because of the harmful effects of bilirubin and bile acids on the kidney tubules through various signalling pathways (El Chediak *et al.* 2020). There is no precise pharmacological treatment for cholemic nephropathy. Bile duct ligation induced liver injury leads to development of cholemic nephropathy in rats (Ommati *et al.* 2021). There is increasing evidence indicating involvement of LPA–LPAR signalling in inducing pathological alterations of cell structure and function in the kidneys. In a mouse model of renal interstitial fibrosis produced by unilateral ureteral obstruction (UUO), the concentrations of kidney autotaxin protein and activity increased with the advancement of fibrosis in injured kidneys, despite concurrent reductions in renal ATX mRNA (Sakai *et al.* 2019). Furthermore, the LPA-LPAR axis plays a vital role in the etiology of renal disease, lung fibrosis, and cancer. Furthermore, LPA level is increased in the serum and renal cortex of db/db mice, a model of diabtes with obesity. Also, ATX is increased in the fat and kidney tissues of db/db mice. A latest study showed that LPA is noticeably elevated in the urine of patients with type 2 diabetes exclusively associated with microalbumineria. There is a report which also shows that the LPA-LPAR axis can produce pathological alterations in the structure and function of renal cells by upregulation of renal inflammation and fibrosis (Lee *et al.* 2022). Autotaxin (ATX) is a key LPA-producing enzyme, and we hypothesized that ATX contributes to the development of renal interstitial fibrosis through LPA-mediated effects on fibroblast functions. However, role

of ATX-LPA signalling in cholemic nephropathy has not been explored. Therefore, we investigated the role of ATX-LPA signalling BDL induced cholemic nephropathy in rats.

Figure 35: Effect of bile duct ligation on liver function test in rats. Data represented as mean ± SEM, n=6 per group. Statistical significance is shown as *** (p<0.001) vs control rats.

Effect of bile duct ligation on liver function test: As reported, BDL rats exhibited significant elevation in plasma ALT (3-fold, figure 1) and AST (8-fold, figure 1) from Day 28 onwards compared to control animals. Total bilirubin was also found markedly higher (10-fold, figure 1) on Day 14 and persisted till day 42. Furthermore, plasma albumin level gradually decreased and found significantly decreased from Day-28 (Figure 34).

Figure 36: Effect of bile duct ligation on kidney function test. Data represented as mean ± SEM, n=6 per group. Statistical significance is shown as *** (p<0.001) vs control rats.

Effect of bile duct ligation on kidney function test. Bile duct ligation resulted in significant increase in plasma creatinine (p<0.05) and blood urea nitrogen levels (p<0.05) compared to control rats (Figure 35) which is in agreement with (J. H. Lee et al. 2022).

Figure 37: Effect of bile duct ligation on renal hydroxyproline content in rats. Data represented as mean ± SEM, n=6 per group. Statistical significance is shown as ** (p<0.001), **(p<0.01), ***(p<0.001) vs control rats.

Effect of bile duct ligation on hydroxyproline content: Renal hydroxyproline being a marker of renal fibrosis was assessed in BDL animals. It was markedly elevated in BDL rats compared to control rats at day 28 & 42 (Figure 36) as supported by (Tsai *et al.* n.d.).
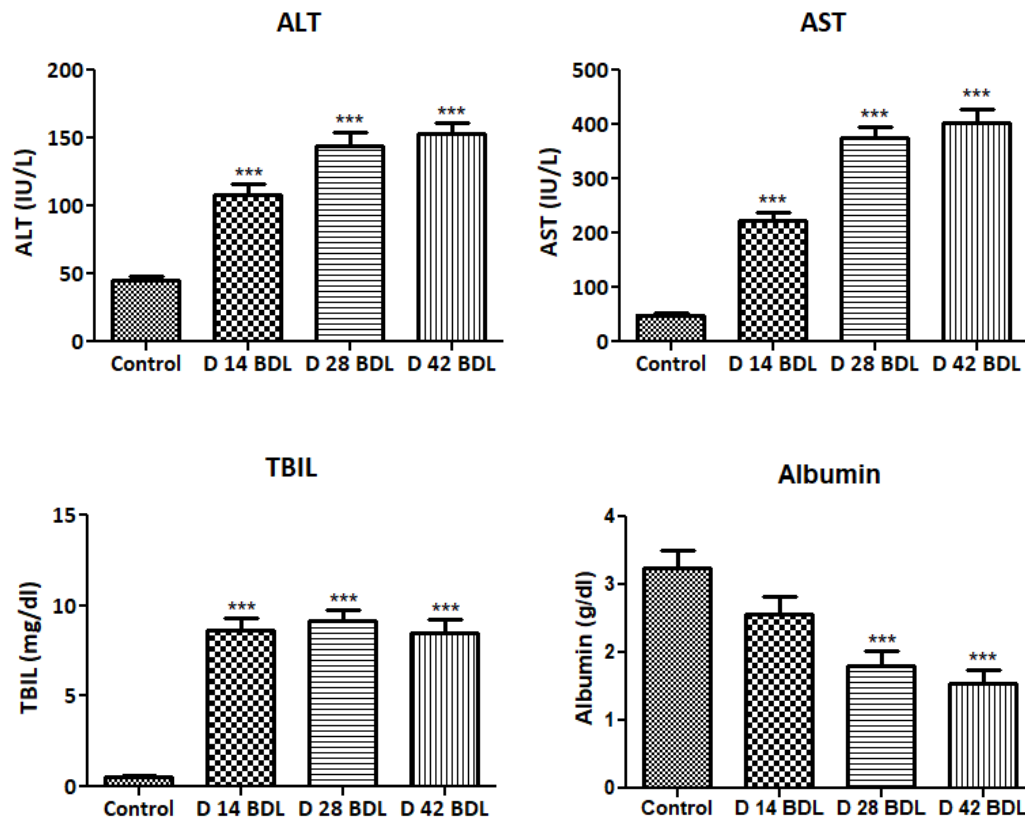

Figure 38: Effect of bile duct ligation on kidney LPA levels. Data represented as mean ± SEM, n=6 per group. Statistical significance is shown as * ($p<0.05$), ** ($p<0.01$) vs control rats.

Effect of bile duct ligation on kidney LPA levels: Interestingly, time course study revealed strong corelation with progression of BDL induced kidney injury and LPA levels as LPA levels were found significantly higher from day 28 post BDL surgery (Figure 28).

Effect of CBT 295 on kidney function test: CBT 295 at 20mg/kg; p.o.; b.i.d. failed to exhibit protection against renal injury caused by bile duct ligation. No significant reversal was in plasma creatinine and blood urea nitrogen level was observed as well with CBT 295 treatment (Figure 39).

Figure 40: Effect of CBT-295 on hydroxyproline content. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as *** (p<0.05) vs Sham.

Effect of CBT 295 on renal hydroxyproline content: CBT 295 at 20mg/kg; p.o.; b.i.d. did not show any reversal in hydroxyproline content compared to vehicle group following bile duct ligation compared to vehicle treated rats (Figure 40).

Figure 41: Effect of CBT-295 on kidney LPA levels. Data represented as mean ± SEM, n=7-10 per group. Statistical significance is shown as $\$\$\$$ p<0.001, $\$\$$ p<0.001vs sham) and * (p<0.05) vs BDL.
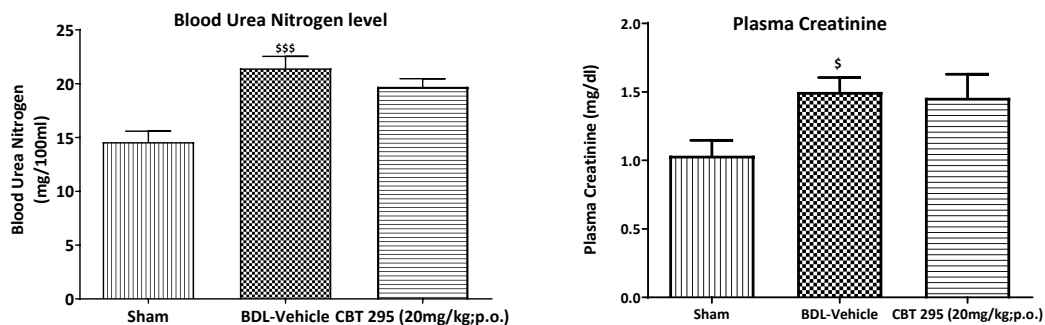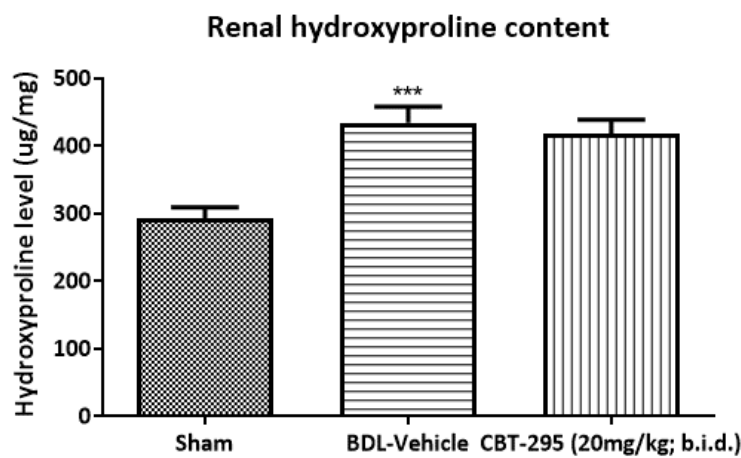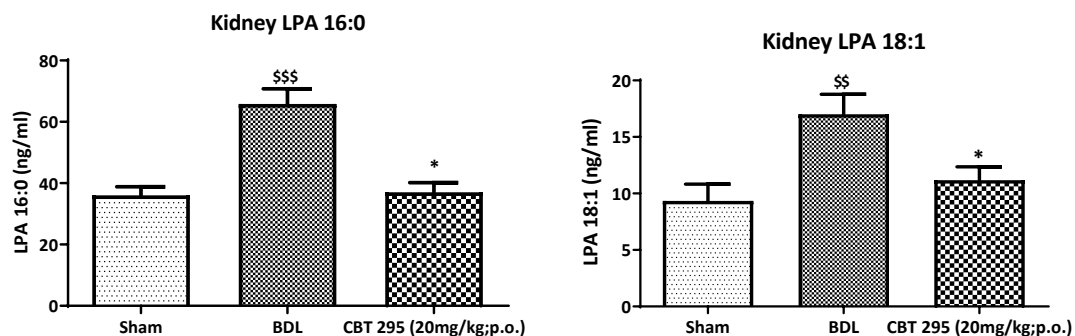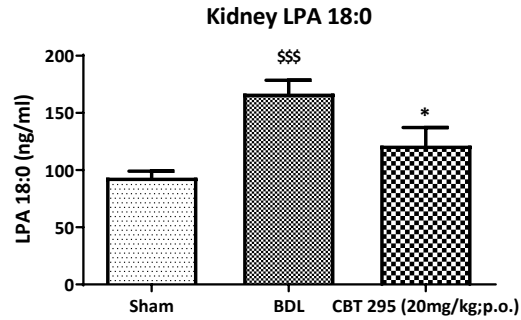
<u>Effect of CBT 295 on renal LPA levels</u>: CBT 295 at 20mg/kg; p.o.; b.i.d. demonstrated significant reduction in kidney LPA 16:0, 18:0 & 18:1 level compared to vehicle group 28 day following bile duct ligation (Figure 41).

Conclusion: Bile duct ligation in rats resulted development of cholemic nephropathy in rats as reflected by impaired renal function. A good corelation was observed between LPA levels and kidney injury as LPA were observed to be significantly higher in these animals with the progression of injury. Although CBT-295 significantly reduced LPA levels but failed to exhibit protection in renal injury.

Chapter 6. Conclusion

- ATX-LPA signaling was found to be involved in etiology of acute liver injury induced by thioacetamide in mice. LPA levels were found to be higher in plasma as well as brain samples. Elevated brain ammonia levels led to set in neuroinflammation as apparent from increased levels of inflammatory cytokines in brain samples and supported by invitro experiment in which ammonium chloride stimulated cytokine release in astrocytes and microglial cells. The mice exhibited impaired neuropsychiatric symptoms of hepatic encephalopathy.

- ATX inhibitor although failed to protect the acute liver injury in TAA model, it lessened neuroinflammation. In vitro studies revealed that ATXi decreased the release of inflammatory cytokines by astrocytes and microglial cells when stimulated by ammonium chloride. It also relieved the symptoms of hepatic encephalopathy in TAA mouse model.

- Role ATX-LPA signaling is evident in pathogenesis of liver, lung and kidney fibrosis. Autotaxin inhibitors (ATXi) have shown efficacy in non-steatohepatitis, CCl4 induced liver fibrosis, lung fibrosis and UUO induced kidney fibrosis. This is the first time we have shown the involvement of ATX-LPA signaling in bile duct ligation induced biliary fibrosis.

- Pharmacological inhibition of ATX hindered the progression of fibrosis. Furthermore, ATXi alleviated ammonia induced neuroinflammation and relieved neuropsychiatric symptoms of hepatic encephalopathy significantly.

- Chronic liver disease induced by bile duct ligation also led to development of cholemic nephropathy as evident from impaired renal function and increased hydroxyproline content. However, ATXi failed to safeguard development of cholemic nephropathy.

Chapter 7. Future perspective

We evaluated the potential of ATX inhibitor PF-8380 in TAA induced acute liver failure induced hepatic encephalopathy. Although it failed to show protection in acute liver failure, it lowered ammonia induced neuroinflammation and neuropsychiatric symptoms of hepatic encephalopathy. Since several ATX inhibitors have been reported in other liver pathological condition, more potent inhibitors can be explored for hepatoprotective effect in acute liver failure which would provide a combined effect by reducing ammonia level and neuroprotection.

We also showed first time that ATX inhibitor, CBT-295 alleviated biliary fibrosis and hepatic encephalopathy in BDL model of chronic liver disease although the effect was not very robust. A greater number of potent compounds should be tested to achieve optimal efficacy. Furthermore, toxicity studies should be performed to ensure the safety profile of ATX inhibitor as the treatment for chronic liver disease is extended over months. Combination therapy should also be evaluated targeting different signaling pathways to enhance the therapeutic benefit. In addition, CBT-295 should also be evaluated most common liver disease nonalcoholic steatohepatitis.

Being an anti-inflammatory drug, ATX inhibitors must be tested for other associated indications of liver disease such as peritonitis and ascites. The role of ATX inhibitor is evident in animal model of renal fibrosis. In our investigation, although we observed upregulation of LPA, ATX inhibitor failed to show protection in cholemic nephropathy. Therefore, other compounds should also be tested for efficacy.

Since we have seen that ATX inhibitor has a direct effect on alleviating neuroinflammation, it may be evaluated for other types of neuroinflammatory disorders such as acute disseminated encephalomyelitis, transverse myelitis, neuromyelitis optica, multiple sclerosis etc.

Chapter 8. References

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Thesis publication

- **Subhasis Roy,** Monali Chakrabarti, Trisha Mondal, Tapas Kumar Das, Tonmoy Sarkar, Sebak Datta, Mrinalkanti Kundu, Manish Banerjee, Onkar Prakash Kulkarni. Effect of an autotaxin inhibitor, CBT-295 {2-(4-chlorophenyl-7-methyl-8-pentylimidazole[1,2-a]pyrimidin-5(8H)-one} on bile duct ligation induced chronic liver disease and associated hepatic encephalopathy in rats- **Submitted (under review) in Neuropharmacology, NEUROPHARM-D-23-00828**

- **Subhasis Roy**, Monali Chakrabarti, Hemantika Dasgupta, Ashutosh Mahale, Shraddha Tripathi, Vivek Sharma, Manish Banerjee, Onkar Prakash Kulkarni. Inhibition of Autotaxin Ameliorates LPA-Mediated Neuroinflammation and Alleviates Neurological Dysfunction in Acute Hepatic Encephalopathy. ACS Chem Neurosci. 2022 Oct 5;13(19):2829-2841

Other Publications

- Ashis Roy, Tonmoy Sarkar, Sebak Datta, Arup Maiti, Monali Chakrabarti, trisha Mondal, Chaitali Mondal, Apurba Banerjee, **Subhasis Roy**, Soumen Mukherjee, Pragati Muley, Sabyasachi Chakraborty, Manish Banerjee, Mrinalkanti Kundu, Kuldeep K. Roy. Structure-based discovery of (S)-2-amino-6-(4-fluorobenzyl)-5,6,11,11a-tetrahydro-1H-imidazo[1′,5′:1,6]pyrido[3,4-b]indole-1,3(2H)-dione as low nanomolar, orally bioavailable autotaxin inhibitor. Chem Biol Drug Des. 2022 Mar;99(3):496-503.

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Brief Biography of the Supervisor:

Prof. Onkar Prakash Kulkarni is currently working as Professor in Department of Pharmacy at BITS Pilani, Hyderabad campus, Hyderabad. He was a Post-doctoral fellow at Ludwig-Maximillians University Munich (LMU), Germany (2010-2013). He obtained his PhD degree from LMU (2006-2010) under the supervision of Prof. Anders HJ, and M Pharm (Pharmacology) from College of Pharmaceutical Sciences Manipal, MAHE University (2002-2004), B-Pharm from College of Pharmacy, Solapur, Shivaji University (1998-2002). He has received many awards during his PhD and Post-doctoral research at LMU. His research group at BITS-Pilani Hyderabad campus is being funded by DST-SERB, IYBA-DBT, ICMR, DRDO as well as internal funding from BITS-Pilani. He has published around 65 articles in peer reviewed journals. At present he is guiding five PhD students in his research group.

Brief Biography of the Co-Supervisor:

Dr. Manish Banerjee has 20 years of Pharma and CRO industry experience. He started his professional journey from Ranbaxy Research Lab in the year of 2003 followed by service in TCG Lifesciences Pvt Ltd from 2008 till date. At his current role in TCGLS, he leads the "*In vivo* domain" and supports integrated discovery projects with in vivo POC generation and PK-PD studies. In his overall professional tenure, Manish got involved in preclinical discovery programs encompassing multiple therapeutic domains like neuropathic pain, inflammatory pain, respiratory and inflammatory disorder liver fibrosis, CNS disorders like anxiety, depression, cognition deficit etc. Some preclinical programs in collaboration with big Pharma and biotech partners which were actively supported by Manish led to nomination of preclinical candidate followed by further development. Manish completed his PhD from department of pharmacology in Central Drug Research Institute, Lucknow with thesis work focused in understanding cross talk between inflammatory mediators in in vitro and in vivo models. He obtained M.Sc (Molecular Biology & Biotechnology) from G. B. Pant University of Agriculture and Technology. He has published published several articles in peer reviewed journals. At present he is guiding two more PhD students.