Non-alcoholic steatohepatitis onset of mechanisms under diabetic background and treatment strategies

Thesis for the degree of Doctor of Philosophy (PhD)

by

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*My beloved parents, brother, husband, respectable teachers and specially to my supervisor*
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ABBREVIATIONS

ACCC, Acetyl-CoA Carboxylase;

ADRP, adipose differentiation related protein;

AdipoR, adiponectin receptor;

ALP, alkaline phosphatase;

ALT, alanine aminotransferase;

AMPK, Adenosine monophosphate-activated protein kinase;

ANOVA, One way analysis of variance;

ASK, apoptosis signal regulating kinase;

AST, aspartate aminotransferase;

ATF, activating transcription factor;

BCA, Bicinchoninic acid;

BCL, B-cell lymphoma;

CCL, chemokine (C-C motif) ligand

CD, Cluster of differentiation;

C/EBPβ, CCAAT/enhancer binding protein beta;

ChREBP, Carbohydrate-responsive element-binding protein;
CTGF, connective tissue growth factor;

CYP, cytochrome P450;

DAMPs, Damage-associated molecular patterns;

DCP, Des-gamma-carboxy prothrombin;

DM, Diabetes Mellitus;

eIF2a, eukaryotic initiation factor 2 alpha;

ERK, extracellular-signal-regulated kinases;

ERS, endoplasmic reticulum stress,

FAS, Fatty acid synthase;

FFA, Free fatty acid;

FC, Free cholesterol;

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase;

GLUT, Glucose transporter type;

GRP78, glucose regulated protein 78;

HCC, hepatocellular carcinoma;

H&E, hematoxylin and eosin;

HFD, high fat diet;

HFE, Human hemochromatosis protein;

HMGB, high mobility group box;
HO-1, Heme-oxygenase-1;

IFN, interferon;

IL, interleukin;

IR, Insulin Resistance;

IRE1α, inositol requiring enzyme 1 alpha;

IP10, interferon inducible protein 10;

IkB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor;

JNK, c-Jun-N-terminal kinase;

KC, Kupffer cells;

LC, Le Carbone;

MAPK, Mitogen-activated protein kinase;

MMP, Matrix metalloproteinase;

MT, Masson trichrome;

MCD, methionine- and choline-deficient;

NAFLD, non-alcoholic fatty liver disease;

NASH, non-alcoholic steatohepatitis;

NF-κB, nuclear factor-κB;
Nrf2, nuclear factor-erythroid 2-related factor-2;

Ox-LDL-R1, oxidized low-density lipoprotein receptor-1;

PAMPs, Pathogen-associated molecular patterns;

PERK, double stranded RNA depended protein kinase-like endoplasmic reticulum kinase;

PEPCK, Phosphoenolpyruvate carboxykinase;

PNPLA3, Patatin-like phospholipase domain-containing protein 3;

PGC1α, Peroxisome proliferator-activated receptor γ coactivator-α;

PPAR, Peroxisome proliferator-activated receptor;

RAGE, Receptor for advanced glycation end products;

ROS, reactive oxygen species;

SCD, Stearoyl-CoA desaturase;

SD, Sprague-Dawely;

SEM, standard error of mean;

SFA, Saturated fatty acid;

SIRT, Sirtuin;

SREBP, sterol regulatory element binding protein isoform;

STZ, Streptozotocin;

TC, total cholesterol;
TG, triglyceride;

Timp, Tissue inhibitor of metalloproteinases

TLR, toll like receptor;

TNF, tumor necrosis factor;

TRAF, TNF receptor–associated factor 2;

UPR, unfolded protein response.

VEGF, vascular endothelial growth factor

XBP, X-box binding protein
ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the buildup of extra fat in liver cells due to causes other than excessive alcohol use. Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD, characterized by necro-inflammation, lipid accumulation and fibrosis. NASH is an increasingly common chronic liver disease with worldwide distribution and a major health problem, owing to its close association with diabetes, obesity and the metabolic syndrome. NASH acts as a significant risk factor for the development of cirrhosis and hepatocellular carcinoma (HCC), a primary malignancy in liver and the third leading cause of cancer-related deaths worldwide. Little is known about the factors responsible for the transition of steatosis to steatohepatitis and its progression in NAFLD/NASH. Perpetuate liver inflammation represents a crucial aspect in NASH pathogenesis and has also been a critical factor in the progression to HCC. Understanding the molecular mechanisms linking hepatocytes necrosis to inflammation and progressive liver damage is, therefore, critical to the development of novel therapeutic strategies for NASH-HCC. Activation of High-mobility group box 1 (HMGB1), a DNA-binding nuclear protein is an inflammatory cytokine, correlates positively with disease states mediated by inflammatory stimuli, including, liver ischemia-reperfusion injury, liver transplantation, cancer and HCC. Whether HMGB1 plays any role in the novel NASH-HCC model is not well addressed. Once HMGB1 activated, binds to cell surface receptors, including toll like receptor (TLR) 2, TLR4 then enhance the nuclear factor κB (NF-κB), extracellular signal-regulated kinase (ERK) signaling, and thereby triggering a variety of inflammatory cytokines in the target cells. It is proposed that NF-κB acts as a central link between hepatic injury, fibrosis, and HCC. Since NASH is considered as a hepatic manifestation of the metabolic disorder, it is well established that the sirtuin 1 (SIRT1) ·AMP-activated protein kinase (AMPK) axis, act as a central
signaling system for controlling the lipid metabolism pathway. The activation of this signaling in several metabolic tissues, including liver has been proposed to increase the rate of fatty acid oxidation and restrain the lipogenesis mostly by modulating the activity of peroxisome proliferator-activated receptor (PPAR) γ coactivator-α (PGC-1α)/PPARα or SREBP-1c through distillation and phosphorylation, respectively. Apart from energy control and lipid metabolism, AMPK is implicated in multiple processes such as apoptosis, autophagy, and senescence, which may be promising target for the treatment of NASH-HCC.

The absence of an effective pharmacological therapy for NASH is a major incentive for research into novel therapeutic approaches for this condition. Curcumin, a natural phenolic compound, has a wide spectrum of therapeutic effects such as antitumor, anti-inflammatory, anti-cancer and so on. On the other hand, Le Carbone (LC) is a charcoal supplement, enriched with dietary fibers, having AMPK-SIRT1 enhancing activity. The study aimed to investigate the underlying mechanisms of curcumin and LC to protect liver damage and progression of NASH in a novel NASH-HCC mouse model.

In the present study, I have used two animal models: type 1 diabetic rats, and experimental NASH-HCC mice to investigate the mechanisms of hepatic complications in diabetes and in NASH-HCC along with their treatment options.

First, I investigated the role of curcumin in the liver of experimental type 1 diabetic rats. For this study, I used male Sprague-Dawley rats and experimental diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) at a dose of 55 mg/kg body weight and 3 weeks after confirmation of diabetes the rats were divided into two groups: diabetic control group (n=10), treated with vehicle and curcumin group (n=10), diabetic rats treated with curcumin (suspended in 1% gum Arabic) was at a dose of 100 mg/kg/day by oral gavage and continued for 8 weeks. The blood
glucose levels and rats weights were measured in each week during the experiment. On the day of sacrifice the blood and liver tissues were collected from the rats of each group. Plasma was separated from the blood to estimate the triglycerides (TG), total cholesterol (TC), and alanine amino transferase (ALT). The excised liver tissues were assessed by histopathology, immunohistochemistry and Western blot analysis. My findings have demonstrated that after 11 weeks of diabetes induction, diabetic rats exhibited hepatic dysfunction, as evidence by elevated plasma ALT levels and the histopathological changes which appeared fatty liver, cell hypertrophy and increased ratio of liver to body weight. Blood glucose levels and plasma TG, TC levels were also significantly elevated in diabetic rats. Hyperglycemia increased the hepatic glucose regulated protein 78, activated the sub-arm of the unfolded protein response (UPR) signaling protein such as phospho - double stranded RNA depended protein kinase – like ER kinase (PERK), inositol requiring enzyme1α, activating transcription factor (ATF)6α in the diabetic rats, which suggest there is an increased endoplasmic reticulum (ER) stress. Moreover, ER stress related apoptotic marker proteins such as C/EBP homologous protein (CHOP), tumor necrosis factor (TNF) receptor- associated factor (TRAF) 2, apoptosis signal-regulating kinase (ASK)1, phospho-p38 mitogen activated protein kinase (p-p38MAPK) and inflammatory cytokines TNFα, interleukin (IL)-1β, TLR4 liver macrophage ED1 were significantly elevated in the liver tissues of diabetic rats. Apoptotic and anti-apoptotic signaling proteins such as cleaved caspase-3 and bcl2, were significantly increased and decreased respectively in diabetic rats and curcumin treatment prevented all of these alterations. Interestingly, curcumin treatment for 8 weeks could ameliorate all of these alterations with a significant result and improved the liver function in diabetic rats.
In my next study, I have used a novel NASH-HCC mouse model under diabetic background to investigate the effect of curcumin treatment and dietary supplement, Le Carbone (LC). To induce this model neonatal C57BL/6 male mice were exposed to low-dose STZ and were fed a HFD from the age of 4 weeks to 14 or 16 weeks. The same dose of curcumin (100 mg/kg) was given daily by oral gavage, started at the age of 10 weeks and continued until 14 weeks along with HFD feeding. LC suspension was administered orally using 5 mg/mouse/day as a prevention, from the age of 6 weeks and continued until 16 weeks of age along with HFD feeding. Each week body weight and the blood glucose levels were checked in every group. At the end of the experiment, blood samples and livers were collected. Serum was separated from the blood for the estimation of TG, TC, amino transferases (ALT, AST), alkaline phosphatase (ALP) test. The liver tissues were used for histopathological and Western blot protein analysis. NASH mice developed steatohepatitis by a significant elevation of serum aminotransferases, with high NAFLD activity score and fibrosis. In the NASH mice, blood glucose levels and serum TG, TC levels were also elevated. This model has no effect on body weight changes in all the experimental groups but increased the ratio of liver weight to body weight in NASH mice significantly than normal mice. Along with these, the liver of the NASH group showed pale yellow color, swelling and granular surface with tumor protruding. In case of curcumin treatment, all of these abnormalities were significantly lesser than the NASH mice and no tumor protrusion was found. In addition, curcumin treatment markedly reduced the hepatic protein expression of oxidative stress, pro-inflammatory cytokines, including interferon (IFN) γ, IL-1β and IFN-γ-inducible protein 10, which were elevated in NASH mice. Furthermore, curcumin treatment significantly reduced the cytoplasmic translocation of HMGB1 and the protein expression of TLR4. Nuclear translocation of NF-κB was also dramatically attenuated by the curcumin in NASH liver. Curcumin
treatment effectively reduced the progression of NASH by suppressing the protein expression of glypican-3, vascular endothelial growth factor, and prothrombin, which reveal HCC in the NASH liver. Our data suggest that curcumin reduces the progression of NASH to HCC and liver damage, which may act via inhibiting HMGB1-NF-κB translocation.

Administration of LC also improved the histopathological changes in NASH liver. Furthermore, LC suspension prevented the lipogenesis and promoted fatty acid oxidation in the NASH liver by significantly increasing hepatocytes protein expression of peroxisome proliferator-activated receptor (PPAR) α in comparing with NASH mice. The reduced hepatic protein expression of phospho-AMPKα and SIRT1 were significantly elevated by the LC suspension when compared to NASH mice. In addition, fibrosis (collagen 4, MMP-9) and oxidative stress (p47phox) were also markedly reduced and the anti-oxidant HO-1, Nrf2 expression were increased in LC treated group in comparison to NASH liver.

After that, to stand the reason behind considering this novel NASH-HCC model under diabetic background for my experiment, I have compared the pathophysiology between DM rats and NASH mice. I have found that DM and NASH have shown similar pathogenic abnormalities both in liver and serum, except the hepatic protein expression of glypican-3, which was significantly elevated in NASH group only. From the other studies and my data, it is confirmed that glypican-3 is a potential liver cancer therapeutic target as it is over-expressed in HCC but not expressed or expressed at a low levels in normal and DM liver tissue.

My results suggest that the novel NASH-HCC model was associated with hyperglycemia, lipogenesis, inflammation, fibrosis, oxidative stress and tumorigenesis, which maintained a sequential progression of NASH from NAFLD to HCC under diabetic background. Curcumin treatment could
ameliorates all of these abnormalities might be through modulation of HMGB1-NF-κB translocations and protect the diabetic liver by reducing ER stress with its anti-hyperglycemic properties. Where, Le Carbone could ameliorate the oxidative stress, lipogenesis and protect the liver function through activation of the AMPK-SIRT1 pathway. Collectively, my present study provides the data to support the role of curcumin and LC in ameliorating the progression of NASH in NASH-HCC mice.

**Keywords:** AMPKα; Diabetes; Endoplasmic reticulum stress; Fibrosis; Hepatocellular carcinoma; HMGB1; NF-κB; Non-alcoholic steatohepatitis; PPARα.
INTRODUCTION
INTRODUCTION

1. Background

Steatohepatitis is a type of liver disease, characterized by inflammation of the liver with concurrent fat accumulation in the liver classically seen in alcoholics as a part of alcoholic liver disease. Frequently it is found in people with diabetes and obesity and is related to metabolic syndrome. When not associated with excessive alcohol intake, it is referred to as nonalcoholic steatohepatitis (NASH), and is the progressive form of the relatively benign non-alcoholic fatty liver disease (NAFLD) [1].

The term NASH, coined by Dr. Ludwig and his colleagues in 1980 to describe the biopsy findings in 20 middle aged patients with steatohepatitis in the absence of significant alcohol consumption, has served the field well by bringing attention to this entity and promoting further research [2]. Although the paper by Ludwig et al. is often referred to as the first report of NASH, the histopathological features seen in NASH were described earlier [3], [4]. Over the years several names have been used to describe this condition: diabetic hepatitis [5], non-alcoholic steatonecrosis, alcohol-like liver disease in the non-alcoholic [6], non-alcoholic fatty hepatitis, fatty liver hepatitis [7], bright liver syndrome [8], and non-alcoholic steatosis syndromes [9]. There is a strong association between the occurrence of fatty liver and insulin resistance (IR), one of the core features of the metabolic syndrome [10].
During the last three decades a large number of studies have challenged the nature of NASH. Some patients with this condition progress to liver cirrhosis and hepatocellular carcinoma (HCC) [11]. These observations have spurred an immense interest among scientists all over the world. In this time more than 200 articles have published investigating different aspects of this intriguing condition.

1.2. Definitions

First, **NAFLD** is the buildup of extra fat or triglyceride inside the hepatocytes that is not caused by alcohol. It is normal for the liver to contain some fat. Traditionally, if more than 5% - 10% percent of the liver’s weight is fat, then it is called a fatty liver (steatosis).

**NASH** is a severe and progressive form of NAFLD result in liver inflammation and damage, characterized by hepatocyte ballooning, necroinflammation, and often associated with fibrosis. It is a significant form of chronic liver disease both in adults and children. The natural history of NASH ranges from indolent to end-stage liver disease. NASH is a common, often “silent” liver disease. Most people with NASH feel well and are not aware that they have a liver problem. Nevertheless, it can be severe and can lead to cirrhosis, in which the liver is permanently damaged and scarred and no longer able to work properly. Where, NASH revealed as an major cause of
virus independent HCC, which may account for a large proportion of HCC in developing countries [12].

**HCC**, called malignant hepatoma, is the most common type of liver cancer. The fastest growing cause of cancer-related death is HCC, which is at least partly attributable to the rising prevalence of NASH. Most cases of HCC are secondary to either a viral infection (hepatitis B or C) or cirrhosis [11].

### 1.3. NASH and HCC

NASH can progress to cirrhosis and its related complications. Growing evidence suggests that NASH accounts for a large proportion of idiopathic or cryptogenic cirrhosis, which is associated with the typical risk factors for NASH. HCC is a rare, although important complication of NAFLD. Diabetes and obesity have been established as independent risk factors for the development of HCC [11]. New evidence also suggested that hepatic iron deposition increases the risk of HCC in NASH derived cirrhosis. Multiple case reports and reviews of HCC in the setting of NASH support the association of diabetes and obesity with the risk of HCC, as well as suggest age and advanced fibrosis as significant risks. Insulin resistance and its subsequent inflammatory cascades that is associated with the development of NASH appear to play a significant role in the pathogenesis of HCC. The complications of NASH such as cirrhosis and HCC, are expected to increase with the growing epidemic of diabetes and obesity [13].
The Liver Disease Spectrum

**Fig.1:** The spectrum of liver diseases ranging from hepatic steatosis (simple intrahepatic accumulation of lipid droplets) through steatosis with inflammation and fibrosis (i.e., non-alcoholic steatohepatitis, NASH) to cirrhosis and HCC.

**1.4. Histopathology of NASH**

NASH is a disorder that is histologically characterized by steatosis and lobular hepatitis with necrosis or ballooning degeneration and fibrosis. The different parts of this spectrum are probably best regarded as parts of histological continuum. The histopathological hallmark of NAFLD is macrovesicular steatosis, which predominantly affects the perivenular regions, and it can extend to a panacinar distribution in severe cases (Figure 2).
Figure 2: Pronounced macrovesicular steatosis (Hematoxylin and eosin).

Figure 3: Hepatocellular steatosis, hypertrophy and inflammation.
Macrovesicular steatosis (dotted line arrow): large lipid droplets are present in hepatocytes; microvesicular steatosis (bold arrow): small lipid droplets are present in hepatocytes. Hypertrophy (open arrow): the representative cell is much larger than the surrounding steatotic hepatocytes but has the same cytoplasmic characteristics. Clusters (aggregates) of inflammatory cells (within circles). Hematoxylin and eosin: magnification 200x.
Figure-4: A Masson trichrome stain shows perivenular/pericellular ("chicken wire") fibrosis (blue area) in nonalcoholic steatohepatitis (NASH) (200× magnification).

Figure-5: NASH cirrhosis (Masson trichrome staining).

When the hepatic steatosis is accompanied by features of necroinflammation, ballooning of hepatocytes, and the presence of Mallory-Denk bodies: the diagnosis of NASH can be made. Briefly, the two key features of NASH, steatosis and inflammation, were categorized as follows: steatosis was determined by
analyzing hepatocellular vesicular steatosis, i.e. macrovesicular steatosis and microvesicular steatosis separately, and by hepatocellular hypertrophy as defined in Figure 3. Inflammation was scored by analyzing the amount of inflammatory cell aggregates (Figure 3) [14].

Fibrosis is considered as a feature of steatohepatitis, the typical pattern of fibrosis of NAFLD is a perisinusoidal and/or pericellular distribution (Figure 4). Patients with NASH, mainly those with advanced fibrosis (bridging fibrosis), are at higher risk for developing decompensated cirrhosis (Figure 5), HCC.

The different histological definitions have been used by different authors [15]. The scoring system of NASH for rodent animal developed by Liang et al. [14] has been shown in Table 1. It is unifies the lesions of steatosis and necroinflammation into a “grade” and those of fibrosis into a “stage” [16].

Table 1: Grading of the histopathological lesions in NASH according to Liang et al [14].

<table>
<thead>
<tr>
<th>Histological features</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Steatosis:</td>
<td></td>
</tr>
<tr>
<td>Macrovesicular steatosis</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Microvesicular steatosis</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Hypertrophy</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Inflammation:</td>
<td></td>
</tr>
<tr>
<td>Number of inflammatory foci/field</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

Where, steatosis and hypertrophy are evaluated at 40× to 100× magnification, and inflammation is evaluated at 100× magnification.
Table 2 Staging of the fibrosis in NASH according to Brunt [16].

<table>
<thead>
<tr>
<th>Fibrosis Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
</tr>
<tr>
<td>Zone 3 perisinusoidal/pericellular fibrosis: focally or extensively present.</td>
</tr>
<tr>
<td>Stage 2</td>
</tr>
<tr>
<td>Zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis.</td>
</tr>
<tr>
<td>Stage 3</td>
</tr>
<tr>
<td>Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis.</td>
</tr>
<tr>
<td>Stage 4</td>
</tr>
<tr>
<td>Cirrhosis</td>
</tr>
</tbody>
</table>

To evaluate fibrosis 40× magnification is used.

Using multiple logistic regression the NAFLD activity score (NAS) was constructed. The NAS is the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores. NASH was defined as a NAS of ≥5, “borderline NASH” as a NAS of 3 or 4 “not NASH” as a NAS of <3.

1.5. Factors associated with NASH

As more contributing factors are continuously identified, a more complex picture of NASH pathogenesis is emerging. Obesity, diabetes mellitus (DM), hyperlipidemia, metabolic syndrome, and IR have been established as risk factors for NAFLD and its progressive form NASH.

1.5.1. Genetic factors

Recent studies have demonstrated that polymorphisms of a number of candidate genes, including those encoding for immunoregulatory proteins, proinflammatory cytokines and fibrogenic factors, could influence the
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appearance of NASH and the eventual development of liver fibrosis in patients with NAFLD [17]. Genetic variations can explain susceptibility to develop NASH in patients with obesity and/or diabetes. Moreover, it has been hypothesized that genetic factors influence fibrosis progression. The role of HFE C282Y heterozygosity and iron accumulation are unclear, since studies have yielded contradictory results [18]. A significant association with a SNP was identified in patatin-like phospholipase domain-containing 3 (PNPLA3) on chromosome 22. Variations of PNPLA3 are associated with histological severity in patients with NASH [19].

1.5.2. Epigenetics

Epigenetic changes consist in modifications at the transcriptional level affecting gene expression and phenotype. A number of epigenetic aberrations have been associated with NAFLD pathogenesis, causing alterations in lipid metabolism, IR, dysfunction of endoplasmic reticulum (ER) and mitochondria, oxidative stress and inflammation [20]. The different epigenetic pathways potentially involved in NAFLD. Aberrant DNA methylation is a major epigenetic process in NAFLD development and progression to NASH [21].

1.5.3 Dietary Factors

Lifestyle changes focusing on weight loss remain the keystone of NAFLD and NASH treatment [22]. Recent reports indicate that lifestyle modifications based on decreased energy intake and/or increased physical activity during
6–12 months cause improvement in biochemical and metabolic parameters and reduce steatosis and inflammation [23]. Conversely, increased consumption of sugar-sweetened food and beverages has been associated with NAFLD development and progression. High intake of fructose, used as food and drink sweetener, is implicated in NAFLD pathogenesis through several mechanisms. In addition, a fructose-enriched diet contributes to induce liver fibrosis in animal models of NASH [24]. Via the portal vein, dietary fructose reaches the liver in high concentrations, exerting a lipogenic action by activation of the transcription factors sterol regulatory element binding protein isoform (SREBP)1 and Carbohydrate-responsive element-binding protein (ChREBP) and subsequent induction of acetyl-CoA carboxylase (ACC) 1, fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) [25].

Dietary iron overload has been recently implicated in NASH pathogenesis. A study by Handa et al. [26] shows that dietary iron excess leads to a severe NASH phenotype in an obese, diabetogenic mouse model characterized by oxidative stress, inflammation and ballooning. Moreover, emerging evidence indicates that hepatic copper (Cu) deficiency is associated with NAFLD development and progression. In an experimental rat model, a Cu deficient diet coupled with high sucrose intake provoked NASH, even in the absence of obesity or severe steatosis.

1.5.4. Obesity and DM

The liver plays a central and crucial role in the regulation of carbohydrate
metabolism. Its normal functioning is essential for the maintenance of blood glucose levels and of a continued supply to organs that require a glucose energy source. This central role for the liver in glucose homeostasis offers a clue to the pathogenesis of glucose intolerance in liver diseases. Hepatic fat accumulation is a well-recognized complication of DM with a reported frequency of 40–70%. Hepatic fat accumulation in type 1 DM may be due to lipoprotein abnormalities, hyperglycemia-induced activation of ChREBP and SREBP 1c, with subsequent intrahepatic fat synthesis or combination of these mechanisms [27]. Fat is stored in the form of triglyceride (TG) and may be a manifestation of increased fat transport to the liver, enhanced hepatic fat synthesis, and decreased oxidation or removal of fat from the liver. In patients with DM and steatohepatitis, Mallory bodies such as those seen in alcoholic liver disease may be seen. NASH has been associated most commonly with obese women with diabetes, but the disease is certainly not limited to patients with this clinical profile [28]. There is certainly a higher prevalence in type 2 diabetic patients on insulin [7]. NASH should be considered as a cause for chronically elevated liver enzymes in asymptomatic diabetic patients particularly if they are obese and have hyperlipidemia [29]. In type 2 diabetic patients with or without obesity, up to 30% have fat with inflammation, 25% have associated fibrosis, and 1–8% have cirrhosis [30], [31], [32]. In an animal model of type 1 DM, there is a high incidence of perisinusoidal hepatic fibrosis, while in humans perisinusoidal fibrosis often
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parallels with diabetic microangiopathy [33]. There is an increased incidence of cirrhosis in diabetic patients, and, conversely, at least 80% of patients with cirrhosis have glucose intolerance [34], [35]. The reported prevalence of cirrhosis in diabetes varies widely. Diabetes increases the risk of steatohepatitis, which can progress to cirrhosis.

Large population-based cohort studies from Sweden, Denmark, and Greece demonstrated a 1.86-fold to 4-fold increase in risk of HCC among patients with diabetes, which is closely associated with obesity and NAFLD [36]. In a larger longitudinal study, using the same group of diabetic patients and nondiabetic controls over 10-15 years, it was found that the incidence of HCC was increased more than two-fold among diabetic patients with higher increase among those with longer duration of follow-up. The risk for HCC was attributable to diabetes, and could not be explained by the presence of underlying liver disease or other risk factors. Diabetes is clearly established as an independent risk factor for HCC. NAFLD was found in approximately 38% of cases and possibly contributed to a higher number of cases given the fact that all of the patients had established diabetes [13].
1.6. **Pathophysiology of NASH**

The physiopathology of NAFL and NASH and their progression to cirrhosis involve several parallel and interrelated mechanisms, such as, IR, abnormal lipid metabolism, lipotoxicity, inflammation, oxidative stress, mitochondrial dysfunction, altered production of cytokines and adipokines, gut dysbiosis and ER stress (Figure 6).

**Figure 6: Outline of the pathogenesis of NASH.** Signals generated inside the liver as a consequence of increased lipid accumulation, together with signals derived from extrahepatic organs cooperate to induce inflammation and fibrosis. FFA, free fatty acids; PAMPs, pathogen-associated molecular patterns; ER, endoplasmic reticulum; ROS, reactive oxygen species; HSC, hepatic stellate cell.

1.6.1 **Increased Lipogenesis and lipotoxicity**
Steatohepatitis develops only in a fraction of patients with NAFLD. From a pathophysiological standpoint NASH develops when the excess lipid accumulation results in hepatic lipotoxicity. A critical concept is that increased hepatic fat does not invariably result in hepatocellular damage, and understanding the mechanisms leading to lipotoxicity is of pivotal importance for the comprehension of this field. Anyhow, fat accumulation appears to be a prerequisite for the development of NASH.

Fat accumulates in the liver mainly in the form of TG, although several other lipid species are present. Human and animal studies have shown that accumulation of TG in NAFLD is the result of the expansion of the intrahepatic pool of free fatty acids (FFA). FFA influx is dependent on: a) the amount of FFA released by the adipose tissue due to IR and excessive lipolysis; b) dietary fat via chylomicron metabolism; c) de novo lipogenesis (Figure 7).
Figure 7: Central role of free fatty acids (FFA) in the pathogenesis of NAFLD.

In the presence of insulin resistance, the majority of FFA reaching the liver derive from adipose tissue lipolysis. Lipids from the diet or de novo lipogenesis also contribute to expand the FFA pool. In the hepatocyte, FFA may be oxidized at mitochondrial and extra-mitochondrial sites, or incorporated in triglycerides. These in turn may be accumulated in lipid droplets, leading to steatosis or secreted in the circulation as VLDL, leading to a proatherogenic lipid pattern.

These lipids, and in particular saturated fatty acid (SFA), can activate a variety of intracellular responses resulting in lipotoxic stress in the ER and mitochondria, respectively. As a consequence, apoptosis occurs which represents a key pathogenic feature of NASH. FFA and free cholesterol (FC) implicated in NASH have been explored experimentally, mostly in dietary studies. Such studies demonstrate the unequivocal potential of such lipid molecules to kill cells of hepatocyte lineage, by directly or indirectly activating c-Jun·N-terminal kinase (JNK) and the mitochondrial/lysosomal cell death pathway, [37] and also to stimulate pro-inflammatory signaling via nuclear factor kappa B (NF-κB) and JNK/activator protein 1 (AP-1).

Lipotoxicity drives the development of progressive hepatic inflammation and fibrosis in a subgroup of patients with NAFLD, causing NASH and even progression to cirrhosis and HCC.
1.6.2. Mitochondrial dysfunction and oxidative stress

Oxidative stress has been recognized as a major factor in the pathogenesis of NASH. Based on the evidence that a high amount of intracellular reactive oxygen species (ROS) are generated in mitochondria and ROS overproduction is elicited in the presence of respiratory chain disruption, mitochondrial impairment has been suggested as a main event in NASH development [38]. Along these lines, structural and functional defects in mitochondria have been reported in patients with NASH [38]. Several mechanisms contribute to mitochondrial impairment and subsequent hepatic cell injury during NASH, mainly associated with lipotoxicity. An adaptive mechanism dependent on the histone deacetylases sirtuin (SIRT)1 and SIRT3, aimed to enhance mitochondrial activity and hepatic β-oxidation [39].

Cytochrome P450 (CYP) 2E1 promotes oxidative stress, inflammation and protein modifications, by hydrolyzing molecules such as fatty acids and ethanol into toxic metabolites, including ROS, which cause respiratory chain disruption and mitochondrial damage, resulting in hepatocyte injury and progression to NASH [40].

1.6.3. Inflammation

Inflammation represents a crucial aspect in NASH pathogenesis. Overload of toxic lipids, mainly FFA, causes cellular stress and induces specific signals that trigger hepatocyte apoptosis, the prevailing mechanism of cell death in
NASH, correlating with the degree of liver inflammation and fibrosis [41]. Different types of immune cells are recruited and/or activated to the site of injury, contributing to NAFLD development and progression. Kupffer Cell (KC) activation is critical in NASH and precedes the recruitment of other cells [42]. Lanthier et al. [43] have shown that KC depletion increases insulin sensitivity and ameliorates inflammation and fibrosis. Differentiation of KCs towards a pro-inflammatory phenotype is principally driven by pathogen-associated molecular patterns (PAMPs) that, interacting with toll-like receptor (TLR)s, induce the secretion of various cytokines, such as interleukin (IL)-1β, IL-12, tumor necrosis factor (TNF)-α, chemokine (C-C motif) ligand (CCL)2 and CCL5, concurring to further hepatocyte damage and release of damage-associated molecular patterns (DAMPs). DAMPs, in turn, act on TLRs amplifying KCs activation and inflammation.
**Figure 8:** Pivotal role of activated Kupffer cells in the pathogenesis of nonalcoholic steatohepatitis and fibrogenesis. Increased levels of lipoipolysaccaride (LPS) and lipotoxic lipid products lead to activation of Kupffer cells, which release chemotactic factors (e.g. CCL2), and proinflammatory cytokines, and generate oxidative stress-related products including reactive oxygen species (ROS). These factors contribute to hepatocyte injury, which in turn, through danger signals, cause further activation of toll-like receptors (TLRs). In addition, inflammation and damage contribute to hepatic stellate cell activation and fibrosis.

### 1.6.4. Pattern recognition receptors and the inflammasomes

TLRs are highly conserved receptors that recognize endogenous danger signals, such as molecules released by damaged cells DAMPs or exogenous danger signals, as gut-derived PAMPs [44]. Due to the high liver exposure to danger signals via the portal system, TLR-induced pathways play a central role in activation of hepatic cells, primarily KC, but also hepatocytes and HSC. As pattern recognition receptors (PRR), TLRs act as defense mechanism, but are also implicated in the pathogenesis of NASH. Importantly, inhibition of TLR2 signaling prevents insulin resistance in HFD mice [45], whereas TLR2-deficient mice fed high fat-diet (HFD) display reduced levels of inflammatory cytokines and do not develop NASH [46].

The crucial role of TLR4 in NAFLD pathogenesis has been demonstrated in TLR4-deficient mice, that display lower levels of inflammatory mediators and
fail to develop NAFLD or IR [47]. ROS production and subsequent activation of the unfolded protein response (UPR) are also induced in TLR4-activated KCs, representing an additional mechanism triggered by TLRs in NAFLD progression. TLR4-mediated inflammatory response can also be elicited by DAMPs released by necrotic cells, such as high mobility group box 1 (HMGB1) or phospholipids. These molecules stimulate monocyte and KCs to secrete inflammatory mediators (Figure 9). HMGB1 can be regulated from activated immune cells and translocation to cytosol and extracellular mediates liver injury then also promotes HCC. It is noteworthy that, in the presence of high glucose, TLR4 activation and downstream signaling can be triggered by FFA [48], clarifying, at least in part, the mechanism by which saturated fatty acids, frequently enhanced in plasma of obese patients, have toxic effects. HMGB1 is a constitutively expressed nuclear protein that induces transcriptional activation, and is released in response to different stimuli, such as PAMPs and DAMPs. HMGB1 interacts with a broad spectrum of receptors [TLR4, TLR2, TLR9, and receptor for advanced glycation end products (RAGE)] exerting proinflammatory actions in complex with other factors, as single stranded DNA, LPS and IL-1β [49].

An important role in NASH pathogenesis has been recently ascribed to the nucleotide oligomerization domain (NOD)-like receptors (NLRs). NLR activation in response to DAMPs or PAMPs leads to the assembly of
inflammasome. Activation of inflammasome, mediated by PRRs via NF-κB, can be induced by a broad spectrum of signals, such as uric acid, ROS, ATP and mitochondrial DNA, and results in secretion of mature IL-1 and IL-18. These cytokines, acting on different cell types, elicit inflammatory signals in liver as well as in the adipose tissue and intestine, triggering steatosis, insulin resistance, inflammation and cell death [50].

Figure 9: Inflammasomes and the liver. In steatosis, hepatic damage leads to generation of damage-associated molecular pattern (DAMPs), while alterations in microbiota lead to increased availability of pathogen-associated molecular patterns (PAMPs). DAMPs and PAMPs act on receptors localized on liver cells leading to activation of different inflammasomes and release of cytokines implicated in NASH. NLRP3: NOD-like receptor family, pyrin domain containing 3; AIM2: Absent in melanoma 2.

Inflammasomes play an important role in NAFLD development and progression to NASH, found both in humans and animal models. NLRP3, an
inflammasome has been reported to activate in several diet induced steatohepatitis, as well as following methionine choline deficient (MCD), HF/HC/HS feeding [51]. Moreover, NLRP3 gain of function correlates with liver fibrosis.

1.6.5. ER stress

ER stress has been implicated in a number of liver diseases, including NASH. ER dysfunction, ATP depletion or other stimuli induce the UPR, an adaptive mechanism directed to avoid luminal accumulation of defective proteins and apoptosis initiation.

Pathways activated by cellular response to ER stress involve JNK, an activator of inflammation and apoptosis implicated in NAFLD progression to NASH [52] and SREBP-1c, which induces liver fat accumulation, worsening ER stress. In vitro studies showed that exposure of hepatic cells to a lipotoxic concentration of palmitate, a SFA, is associated with ER calcium depletion, ROS accumulation and apoptosis [53]. The pathways associated with ER stress shown in figure 10.
**Figure-10: Mammalian unfolded protein response (UPR) pathways.** The UPR is triggered by several events, including protein unfolding/misfolding, hypoxia, low adenosine triphosphate levels, ER calcium depletion, and protein/sterol over-expression, causing dissociation of 78 kDa glucose-regulated protein (GRP78) from the three UPR sensors, (A) inositol-requiring enzyme 1α (IRE1α), (B) protein kinase RNA-like endoplasmic reticulum kinase (PERK), and (C) activating transcription factor-6 (ATF6). Activated IRE1α undergoes dimerization and autophosphorylation to generate endogenous RNase activity; in turn, this is responsible for splice truncation of X-box binding protein 1 (XBP1S) mRNA. Additionally, IRE1α may also activate the extrinsic apoptosis pathway, in which tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2)-dependent downstream activation of c-Jun N-terminal kinase (JNK) and caspase-12 takes place. Once activated, PERK undergoes homodimerisation and autophosphorylation to activate eukaryotic translation initiation factor 2 (eIF2α). In turn, this induces ATF4 expression. Separately, dissociation of GRP78, allows ATF6 processing by the Golgi complex, where proteases S1P and S2P cleave an
active 50 kDa (p50) ATF6 domain that is free to translocate to the nucleus. Xbp1s, ATF4 and ATF6, as well as other unlisted factors, are responsible for three dominant cell responses to UPR. The folding pathway induces increased expression of molecular chaperones, including GRP78, assisting in compensatory ER protein folding. Alternatively, the cell may respond by increasing ER-associated protein degradation (ERAD) pathway, whereby gene products target and degrade unfolded proteins in the ER. Prolonged UPR results in the activation of the intrinsic apoptosis pathway; this ATF6 and ATF4-dependent process induces C/EBP-homologous protein (CHOP) expression. In turn, CHOP inhibits B-cell lymphoma 2 and induces apoptosis.

1.6.6. Nuclear Receptors

Nuclear receptors are ligand-dependent transcription factors that regulate glucose and lipid metabolism in the liver. Nuclear receptors are divided into seven subfamilies named as NR0-NR6 [54] and NR1 subfamily is of particular importance in NAFLD. Peroxisome proliferator-activated receptors (PPAR) α, β, γ belongs to this subfamily. PPARα regulates β-oxidation and cholesterol removal during the fasting state or when metabolism increases in adipose and/or muscle tissues [54]. Hepatic PPARα expression decreases in NAFLD leading to steatosis, but is enhanced following diet and exercise [55]. In animal models of steatosis and steatohepatitis, the use of PPARα activators improves the disease.
1.7. Epidemiology and Prevalence of NASH

With obesity reaching epidemic proportions in the 21st century, NAFLD is on the rise. NAFLD is the most common etiology of chronic liver disease in the United States and other developed countries. The annual incidence of NAFLD has been estimated to be as high as 10% with the development of NAFLD associated most directly with the metabolic syndrome and preceding weight gain. Worldwide, the prevalence of NAFLD in the general population ranges from 9%-37%. In the United States, recent estimates suggest that NAFLD affects 30% of the general population and as high as 90% of the morbidly obese [13].

There is no reliable data on the prevalence of NASH in the general population. A number of studies undertaken in obese individuals undergoing bariatric surgery. In these series the frequency of NASH varies between 14% to 56%. In hospital series of patients undergoing liver biopsy the frequency ranges from 1 to 32% [56]. These large variations on the prevalence of NASH can partly be attributed to different definitions of NASH and which histopathological findings are required for the diagnosis to be set.

In an autopsy series of 351 apparently non-alcoholic patients the frequency of NASH was 6.3%. NASH was defined as ballooning of hepatocytes with clearing of the hepatocellular cytoplasm accompanied by large droplets steatosis. NASH was found in 18.5% of obese and in 2.7% of lean patients [7].
Whereas, in a study considering 61868 patients over the period 2002-2012 found that NASH-related HCC increases from 8.3% to 13.5%, an increase of near 63%. Furthermore, they found that the number of NASH-HCC patients undergoing liver transplantation increased nearly 4-fold during this period [57].

1.8. Treatment for NASH

The standard of care for patients with NAFLD/NASH is life style modification with weight loss as the mainstay therapy. As overall weight loss is difficult to achieve and maintain for most patients, pharmacological therapy is often needed. Treatment of NAFLD includes aggressive cardiovascular risk factor management, including obesity, dyslipidemia, and diabetes. At present there are no FDA-approved agents with an indication for the treatment of NASH. Multiple pharmacological intervention have been attempted for NASH, including thiazolidinediones, Dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 receptor agonist, sodium/glucose cotransporter 2 inhibitors, antioxidants/supplements, lipid lowering drug, nonselective phosphodiesterase inhibitors, farnesoid X receptor agonist. There are no good large randomized controlled trials in humans for many of these compounds, except vitamin E [58]. Vitamin (800 IU/day) is an option for patients biopsy proven NASH without diabetes [59].
Treatment strategies for NASH aim to improve insulin sensitivity, modify underlying metabolic risk factors, or to protect the liver from further insult by oxidative stress and inflammation.

1.9. CURCUMIN

Curcumin \([1,7\text{-bis (4\text{-hydroxy \cdot3\text{-methoxyphenyl})\cdot1,6\text{-heptadiene\cdot3,5\text{-dione}}]}\) or \([\text{diferuloylmethane}]\) is a natural polyphenol compound found in the turmeric, *Curcuma longa* plant & possesses potent anti-oxidant, anti-inflammatory properties, modulate the activity of protein kinases, membrane ATPases, and transcription factors.

![Diferuloylmethane](image)

To date several clinical trials have been completed; most of these trials have suggested that curcumin is safe and effective in a number of human diseases. The most promising effects of curcumin have been observed with cancer, inflammatory conditions, skin, liver and neurological disorders and pain.
Accumulating evidences suggest that curcumin has a diverse range of molecular targets, including complete inhibition of the activity of protein kinase C. It can enhance the pancreatic β cell function by inhibiting phosphodiesterase activity and regulated insulin secretion under glucose stimulated condition. Curcumin treatment can protect the liver from drug – induced toxicity.

Curcumin protects liver injury against tetra chloromethane, aflatoxin B₁, alcohol and paracetamol toxicity. A previous study has pointed to the
protective effect of curcumin on acute liver injury by inhibiting NF-κB and oxidative stress. It is also reported that curcumin possesses the activity against ER stress. Several studies suggested that curcumin is safe and well tolerated treatment [60].

1.10. Le Carbone

Le Carbone (LC) is a charcoal supplement, enriched with dietary fibers. Today, beyond use in hospitals as an antidote for drugs and poisons, activated charcoal is a global remedy for general detoxification, digestion issues, gas bloating, heart health, and anti-aging [61]. Toxicology studies show activated charcoal to be harmless to human health.

In the early 20th century, the charcoal activation process development sparked many medical journal to publish research revealing activated charcoal as an antidote for poisons and way to improve intestinal disorder. In patients with high cholesterol, activated charcoal able to reduce the total cholesterol levels. Some study proved that co-treatment of activated charcoal with anti-cancer drug increase the survival rate after stomach cancer surgery [62]. Experiment evidence suggest that LC has the adenosine monophosphate-activated protein kinase (AMPK) enhancing and anti-inflammatory activities [63]. By enhancing AMPK it may contribute in lipid metabolism. Several reports suggested that that activated charcoal also has the anti-atherosclerosis and lipid lowering activity [64].
Based on the above reports and for the strong biological activities against different diseases along with inflammation, oxidative stress and lipid lowering activity, for my study I selected the curcumin and LC as treatment option for my experiment.
SCOPE OF STUDY
The Present studies were aimed:

1. To study the effect of type 1 DM on liver and its treatment option.

2. To investigate the relationship between NASH and HCC under diabetic condition and their mechanisms.

3. To investigate the treatment and prevention options of NASH-HCC under diabetic background.
CHAPTER ONE

Curcumin Ameliorates Streptozotocin Induced Liver Damage through Modulation of Endoplasmic Reticulum Stress Mediated Apoptosis in Diabetic Rats
1. Introduction

DM is one of the most common endocrine metabolic disorders. Studies have shown that hepatobiliary disorders, such as the inflammation and necrosis or fibrosis of non-alcoholic fatty liver disease can follow diabetes [65], [66]. It has been reported that the standardized mortality rate from end-stage liver disease (i.e. cirrhosis) in diabetic patients is higher than those with cardiovascular disease [67]. Several mechanisms can cause pancreatic β-cell dysfunction, including chronic inflammation, oxidative stress, excessive hyperglycemia and nutrient levels, lipotoxicity, ER stress (ERS), etc. [68], [69], [70]. A previous study has shown that hepatic fat accumulation and oxidative stress play a critical role in the development of diabetic liver injury [71] and a number of reports have shown that antioxidants could attenuate the complications of diabetes, including fatty changes in patients and in experimental models [72], [73]. Nowadays, ERS has attracted significant attention and has been proposed to play a crucial role in the development of insulin resistance [69]. It is also reported that insulin resistance, a common underlying reason for the β cell failure that occurs in type 2 diabetes, is associated with higher levels of ERS in β cells in animal models of disease [74], [75] and also in humans [76], [77]. It is proposed that oxidative damage that is caused by ERS may be the fundamental in the etiology of the β cell failure associated with both type 1 and 2 diabetes [78]. Liver, as the major
target organ of insulin, plays important roles in the development of insulin resistance and diabetes mellitus.

The ER is a complex intracellular membranous network that regulates protein synthesis and folding. Alterations in ER homeostasis due to increased protein synthesis, accumulation of misfolded proteins or alterations in the calcium or the redox balance of the ER, lead to a condition called ERS [79]. To overcome the deleterious effects of ERS induction, cells have evolved various protective strategies, which are known as the UPR [80]. Furthermore, the results from these reports have suggested that induction of ERS is closely associated with the energy metabolism, especially the lipid metabolism with the involvement of UPR signaling pathways. Rutkowski and Cols examined the contribution of each arm of the UPR pathway to the regulation of metabolic genes and development of hepatic dyslipidemia and concluded that all three arms of UPR signaling pathway double stranded RNA depended protein kinase – like ER kinase (PERK), inositol requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6) are activated, leading to metabolic dysregulation [81].

When ERS is prolonged in the presence of hyperglycemia condition, the ER triggers the apoptotic pathway by activating the CCAAT/ enhancer – binding protein homologous protein (CHOP) [82], the IRE1-TRAF2-ASK1-MAP kinase pathway [83], and/or the ER-localized cysteine protease caspase-12 [84]. Moreover, cell death signaling cascades including p38 mitogen activated
protein kinase (p38MAPK) and apoptosis signal regulating kinase 1 (ASK1) are also activated by pro-inflammatory cytokine tumor necrosis factor α (TNFα), IL-1β, TLR4 and by the activation of the UPR signaling marker IRE1α in diabetic liver. In diabetes involving both inflammation and ERS lead to hepatic apoptosis and liver damage.

Curcumin is the active ingredient of the traditional herbal remedy and dietary spice turmeric (Curcuma longa) and is the subject of clinical trials for various diseases such as cancer, Alzheimer’s disease, and ulcerative colitis [85]. The polyphenol curcumin (diferuloylmethane) comprises 2-8% of most turmeric preparations and is generally regarded as its most active component, having potent antioxidant, anti-inflammatory and anti-carcinogenic properties. Curcumin has been shown to modulate the activity of protein kinases [86], membrane ATPases [87], and transcription factors [88], [60]. It is also reported that curcumin plays important role to diminish myocardial ERS signaling proteins and to decrease the key regulator or inducer of apoptosis in experimental autoimmune myocarditis rats [89]. A previous study has pointed to the protective effect of curcumin on acute liver injury by inhibiting NF-κB and oxidative stress [90]. However, to the best of my knowledge, studies have not been revealed the effect of curcumin on ERS in diabetic liver. Although many aspects of curcumin-induced cytoprotection are studied, the molecular mechanism by which curcumin protects liver
tissues against streptozotocin (STZ) - induced liver injury is not clear. The present study was designed to shed light on this issue.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were of analytical grade and purchased from Sigma (Tokyo, Japan).

2.2. Animals and Experimental design

All animals were treated in accordance with the guidelines for animal experimentation of our institute [91]. Male Sprague-Dawley rats (weight 250-300 g) were obtained from Charles River Japan Inc. (Kanagawa, Japan). Animals were housed in a temperature of 23 ± 2 °C and humidity of 55 ± 15%, and were submitted to a 12 hour light/dark cycle, and allowed free access to standard laboratory chow and tap water. Animals were allowed to fast for 4 hours and then induced diabetes by a single intraperitoneal (i.p.) injection of freshly prepared solution of STZ (Sigma-Aldrich, Inc. Saint Louis, MO, USA) at a dose of 55 mg/kg, diluted in citrate buffer 20 mM (pH 4.5). Forty eight hours later, blood glucose was measured by tail-vein sampling using Medi-safe chips (Terumo Inc., Tokyo, Japan). Diabetes was defined as a morning blood glucose reading of ≥ 300 mg/dL. Thirty rats were randomly divided into three groups (n = 10/group): nondiabetic normal control group (Normal), diabetic rats treated with vehicle, 1% gum Arabic (Control) and diabetic rats
treated with curcumin 100 mg/kg/day [92] diluted in vehicle, 1% gum Arabic (Curcumin) (curcumin was purchased from Sigma-Aldrich, Tokyo, Japan). Curcumin treatment was started at 3 weeks after STZ injection and was administered via oral gavage for 8 weeks. All rats were sacrificed at 11 weeks after the induction of diabetes for analysis of liver tissues.

2.3. Biochemical analysis

Each week, rats were weighed and their blood glucose levels were measured. Urine samples were collected over a 24 h period in individual metabolic cages for the measurement of protein in urine at 1, 3, 7, and 11 weeks and were determined by the Bradford method. At the end of experimentation, heparinized whole blood was collected from anesthetized rats via heart puncture. EDTA-blood was centrifuged at 3000 g, 15 min at 4°C for the separation of plasma. The plasma was used for the estimation of triglyceride (TG) and total cholesterol (TC), alanine aminotransferase (ALT).

2.4. Histopathological analysis

Formalin-fixed liver sections (4 μm) were stained with hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS). Morphological analysis was done by computerized image analysis system on ten microscopic fields per section examined in a 400-fold magnification (CIA-102; Olympus), with the observer blind to the study group [93], [94].

2.5. Western blotting analysis
The liver tissue protein lysate was prepared using a method similar to that
described by Soetikno [95]. The total protein concentration in the samples
was measured by the bicinchoninic acid (BCA) method. For the determination
of protein levels, equal amounts of protein extracts (50 µg) were separated by
7.5–15% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis
(Bio-Rad, CA, USA) and transferred electrophoretically to nitrocellulose
membranes. Membranes were blocked with 5% non-fat dry milk or bovine
serum albumin (BSA) in Tris buffered saline Tween (20 mM Tris, pH 7.6, 137
mM NaCl, and 0.1% Tween 20). Primary antibodies against GRP78, IRE1α,
p-IRE1α, TRAF2, ATF6, PERK, p-PERK, CHOP/GADD153, TNFα, IL-1β,
TLR4 and β Tubulin were obtained from Santa Cruz Biotechnology, Santa
Cruz, CA, USA. Primary antibodies against bcl2, cleaved caspase-3, p-
p38MAPK and ASK1 were obtained from Cell Signaling Technology Inc.,
Beverly, MA, USA. And primary antibody cleaved caspase-12 was obtained
from Bio Vision Inc., Milpitas, CA, USA. All the antibodies were used at a
dilution of 1:1000. The membrane was incubated overnight at 4°C with the
primary antibody, and the bound antibody was visualized using the
respective horseradish peroxidase (HRP) conjugated secondary antibodies
(Santa Cruz Biotechnology Inc.) and chemiluminescence developing agents
(Amersham Biosciences, Buckinghamshire, UK). The levels of β Tubulin,
PERK (for p-PERK) and IRE1α (for p-IRE1α) were estimated in every
sample to check for equal loading of samples. Films were scanned, and band
densities were quantified by densitometric analysis using the Scion Image program (Epson GT-X700, Tokyo, Japan).

2.6. **Immunohistochemistry**

Formalin-fixed, paraffin-embedded liver tissue sections were used for immunohistochemical staining. After deparaffinization and hydration, the slides were washed in Tris-buffered saline (TBS: 10 mM/L Tris HCl, 0.85% NaCl, pH 7.2). Endogenous peroxidase activity was quenched by incubating the slides in methanol and 0.3% H$_2$O$_2$ in methanol. After overnight incubation with the primary antibody, that is, mouse monoclonal anti- ED1 antibody (diluted 1:50) (sc-59103; Santa Cruz Biotechnology Inc. CA, USA) at 4°C, the slides were washed in TBS and (HRP) -conjugated goat anti-mouse secondary antibody was then added and the slides were further incubated at room temperature for 1 h. The slides were washed in TBS and incubated with diaminobenzidine tetra hydrochloride as the substrate, and counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to verify the antibody specificity. ED1 positive hepatocytes were counted in 100 hepatocytes/ group under 200-fold magnification and expressed as cells/hepatocyte cross section [96].

2.7. **Statistical analysis**

All values are expressed as means ± standard error of mean (SEM) and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s methods for post hoc analysis or two-tailed t-test when appropriate. A value
of $p<0.05$ was considered statistically significant. For statistical analysis, Graph Pad Prism 5 software (San Diego, CA, USA) was used.

3. Results

3.1. Biochemical parameters in experimental animals

For the confirmation of diabetic model and the effect of treatment periodically the blood glucose level was checked, which is shown in Figure 1, before treatment the blood glucose level was significantly higher than that of normal group but during the treatment period from 6 weeks curcumin treatment significantly decreased this blood glucose level. Moreover, it is also reported that curcumin has the capability to improve the pancreatic islets [97] and has been demonstrated in prevention of isolated β cells death and dysfunction induced by STZ [97], [98].

![Graph showing blood glucose level over weeks with significant decreases during treatment.](image-url)
Fig 1: Time-course changes in blood glucose. Blood glucose increased progressively in the untreated diabetic rats following induction of diabetes. Curcumin treatment significantly reduced blood glucose in the beginning of treatment and these were maintained throughout the study period until sacrifice. Values are means ± SEM. **p < 0.01, ***p < 0.001 vs Normal, ###p < 0.001 vs Control.

As shown in Table 1, body weight was significantly decreased in diabetic rats, but curcumin treatment could not prevent this declined body weight. The ratio of liver weight and body weight (g/kg) were significantly increased in untreated diabetic rats compared to normal rats and curcumin treatment reduced this ratio. Compared with the normal group, diabetic rats developed elevated mean plasma glucose. Plasma TG, TC and ALT were also elevated in the diabetic rats in comparison to the normal rats (p<0.01, p<0.001). All of these abnormalities significantly attenuated by curcumin treatment (p< 0.05, p< 0.001). To evaluate the effect of curcumin on preventing hyperfiltration induced by STZ, we measured 24-h urine volume and urinary protein excretion. Although the control group showed a marked elevation of 24-h urine and urinary protein excretion, curcumin treatment could not reduce this elevated urinary excretion but significantly reduced the urinary protein excretion (p< 0.05).
### Table 1: Changes in Biochemical Parameters after 8 Weeks of Treatment with Curcumin in Diabetic Rats Induced by Streptozotocin

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=10)</th>
<th>Control (n=10)</th>
<th>Curcumin (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BW) (g)</td>
<td>539.5 ± 38.48</td>
<td>316.33 ± 15.7***</td>
<td>368.75 ± 24.62***</td>
</tr>
<tr>
<td>Liver weight (LW) /BW (g/kg)</td>
<td>28.57 ± 1.47</td>
<td>43.23 ± 1.35***</td>
<td>37.92 ± 4.64#*</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>116.25 ± 22.1</td>
<td>761.8 ± 50.8***</td>
<td>287.66 ± 78.4###**</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>94.75 ± 5.72</td>
<td>431.25 ± 118.92###</td>
<td>109 ± 11.94###</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>58.50 ± 3.8</td>
<td>105.5 ± 10.9***</td>
<td>81.25 ± 13.72#</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>39.75 ± 2.36</td>
<td>124 ± 28.82**</td>
<td>97 ± 30.26*</td>
</tr>
<tr>
<td>Urine Volume (mL/Kg/24h)</td>
<td>19 ± 1</td>
<td>618 ± 137***</td>
<td>592 ± 109***</td>
</tr>
<tr>
<td>Protein in urine/24h (g)</td>
<td>13 ± 2</td>
<td>38 ± 15.63*</td>
<td>15 ± 10#</td>
</tr>
</tbody>
</table>

Normal, age matched normal rats; Control, untreated diabetic rats; Curcumin, diabetic rats treated with curcumin 100 mg/kg/day. TG: triglyceride; TC: total cholesterol, ALT: alanine aminotransferase. Values are expressed as means ± SEM.

*p <0.05, **p <0.01, ***p <0.001 vs normal, #p< 0.05, ###p< 0.001 vs control.

### 3.2. Effect of curcumin on ERS marker protein

Activation of GRP78 protein expression was used to demonstrate the ERS induction, whose expression is reported to be increased in the diabetic rats. ERS leads to the activation of caspase-12 and other markers involved in the hepatic changes associated with DM. My study with diabetic rats also revealed the involvement of ERS as evidenced by a significant increase in the
hepatic GRP78 expression but cleaved caspase-12 expression was not so significant. Prevention of ERS is one of the strategies involved in the reduction of liver complications of diabetes. In the present study curcumin treated animals had shown significant attenuation of GRP78 and cleaved caspase-12 was slightly attenuated when compared with the control group (Figure 2A and 2B).

**Fig 2: Expression of hepatic protein involved in ERS.** Representative western blots (lower panel) show specific bands for GRP78 (A) and Caspase12 (B) and the representative histograms (upper panel) show the band densities with relative β Tubulin. The blots are representatives of five independent experiments. Each bar represents mean ± SE. Normal, age matched normal rats; Control, untreated diabetic rats; Curcumin, diabetic rats treated with curcumin 100 mg/kg/day. **p<0.01 vs Normal, #p<0.05 vs Control based on one way ANOVA followed by Tukey’s test.
3.3. **Effect of curcumin on expression of UPR signaling proteins in liver**

The signals from activated UPR molecules to the relevant effector proteins are conveyed by three different proteins, PERK, IRE1α, and ATF6α. As expected, here I have found that the phosphorylation of PERK protein was significantly increased in the diabetic rats compared with the normal SD rats (Figure 3A). Diabetic rats have displayed a significant up-regulation in the hepatocyte expression levels of p-IRE1α protein compared with the normal SD rats (Figure 3B). And curcumin treatment significantly attenuated the increased liver p-PERK and p-IRE1α levels. But interestingly, I did not find any significant difference in the level of ATF6α protein expression in the diabetic rats compared with the normal SD rats (Figure 3C).
Fig 3: Expression of hepatic proteins involved in UPR signaling pathway. Representative western blots (lower panel) show specific bands for p-PERK (A), p-IRE1α (B) and ATF6α (C) and the representative histograms (upper panel) show the band densities with relative β Tubulin, PERK (for p-PERK) and IRE1α (p-IRE1α). The blots are representatives of five independent experiments. Each bar represents mean ± SE. Normal, age matched normal rats; Control, untreated diabetic rats; Curcumin, diabetic rats treated with curcumin 100 mg/kg/day. *p<0.05, ***p<0.001 vs Normal, #p<0.05 vs Control based on one way ANOVA followed by Tukey’s test.

3.4. Effect of curcumin on hepatocyte protein expression levels of CHOP/GADD153 in the diabetic rats

It has been proposed that enhancement of the hepatocyte protein expression of CHOP/GADD153 gene could eventually induce apoptosis. Immunoblot analysis revealed that the diabetic rats have demonstrated significant
elevation of CHOP/GADD153 protein expression compared with the normal rats (Figure 4A) and curcumin treatment significantly decreased the increased liver CHOP expression.

3.5. Effect of curcumin on liver expression of TRAF2, ASK1 and p-p38MAPK

The TRAF2 protein expression was significantly higher in the diabetic rats than the normal rats, which was significantly attenuated by curcumin treatment (Figure 4B). There was a significant increase in the expression of p-p38MAPK and ASK1 in the diabetic control rats. This result suggests activation of IRE1α recruit TRAF2, then ASK1 directly binds to TRAF2 and activated. Curcumin treatment modified these changes in the liver of diabetic rats. Protein expression of these markers were attenuated significantly in the curcumin treated rats (Figure 4B, 4C and 4D).
Non-alcoholic steatohepatitis onset of mechanisms under diabetic background and treatment strategies

Fig 4: Hepatic expressions of CHOP, TRAF2, ASK1 and p-p38MAPK.
Representative western blots (lower panel) show specific bands for CHOP/GADD153 (A), TRAF2 (B), ASK1 (C) and p-p38MAPK (D) and the representative histograms (upper panel) show the band densities with relative β Tubulin. The blots are representatives of five independent experiments. Each bar represents mean ± SE. Normal, age matched normal rats; Control, untreated diabetic rats; Curcumin, diabetic rats treated with curcumin 100 mg/kg/day. **p<0.01, ***p<0.001 vs Normal, ##p<0.01, ###p<0.001 vs Control.

3.6. Effect of curcumin on hepatocyte protein expression levels of cleaved caspase-3 and bcl2 in diabetic rats
Immunoblot analysis revealed that the diabetic rats have displayed an increase in the hepatocyte protein expression of cleaved caspase-3 compared with the normal rats (Figure 5A) and decreased protein expression level of
bcl2. This increase in liver of cleaved caspase-3 protein expression was markedly suppressed and significantly increased the bcl2 protein expression by curcumin treatment in the liver of diabetic rats. This clearly indicates that curcumin attenuated the hepatocytes apoptosis.

**Fig 5**: Hepatic expressions of cleaved caspase-3 and bcl2. Representative western blots (lower panel) show specific bands for cleaved caspase-3 (A) and bcl2 (B) and the representative histograms (upper panel) show the band densities with relative β Tubulin. The blots are representatives of five independent experiments. Each bar represents mean ± SE. Normal, age matched normal rats; Control, untreated diabetic rats; Curcumin, diabetic rats treated with curcumin 100 mg/kg/day. *p < 0.05, **p < 0.01 vs Normal, #p < 0.05, ##p < 0.01 vs Control.
3.7. **Effect of curcumin on inflammatory marker proteins in the diabetic liver**

Inflammation is involved in the mechanism of metabolism-related diseases such as diabetes mellitus and obesity [99], [100]. Inflammatory cytokines produced by liver infiltrated autoreactive immune cells are the major factors causing cell death in type 1 diabetes [101]. It has been proposed that ERS pathways play crucial roles in the inflammatory response. Therefore, I performed western blot analysis to measure the hepatocyte protein expression levels of TNFα, IL-1β and TLR4 in diabetic rats. The diabetic rats displayed significantly upregulated protein expression levels of TNFα, IL-1β and TLR4 compared with those in normal rats (p < 0.05 and p < 0.001), and these increases were significantly attenuated by curcumin treatment. (Figure 6A, 6B and 6C).

![Graph A: Comparison of TNFα and β Tubulin levels with Normal, Control, and Curcumin treatments](image)

![Graph B: Comparison of IL-1β and β Tubulin levels with Normal, Control, and Curcumin treatments](image)
Fig 6: Hepatic expressions of TNFα, IL-1β and TLR4. Representative western blots (lower panel) show specific bands for TNFα (A), IL-1β (B) and TLR4 (C) and the representative histograms (upper panel) show the band densities with relative β Tubulin. The blots are representatives of five independent experiments. Each bar represents mean ± SE. Normal, age matched normal rats; Control, untreated diabetic rats; Curcumin, diabetic rats treated with curcumin 100 mg/kg/day. *p<0.05, ***p<0.001 vs Normal, #p<0.05, ##p<0.01 and ###p<0.001 vs Control.

3.8. Histopathological findings

Normal histology was seen in the normal control rats (Figure 7). The normal liver contains a large amount of glycogen. This therefore leads to the staining of hepatocytes intensely pink with a PAS stain. In the PAS staining, examined liver sections of normal control rats showed the normal pattern distribution of glycogen granules, a liver section of diabetic rats showed marked depletion in the glycogen granules, meanwhile this glycogen level
became increased in curcumin treated animals (Figure 7A). Fatty liver was shown by H&E staining as an unstained area in liver parenchymal cells (Figure 7B). In the untreated diabetic rats, microvacuolar vacuolization, focal necrosis and inflammation in the portal area were significantly apparent compared with the normal rats and curcumin treated diabetic rats (Figure 7B) improved these findings.

3.9. Effect of curcumin on macrophage (kupffer cells) infiltration and fibronectin accumulation

Macrophages are a heterogeneous population of myeloid –derived mononuclear cells that are a critical component of innate immune response [102], [103]. I investigated that livers from normal rats did not show any significant macrophage infiltration (Figure 7C). On the other hand, diabetic rats demonstrated prominent macrophage (ED1 positive cells) recruitment in the liver (Figure 7C and 7C1), whereas diabetic rats treated with curcumin showed marked reduction of macrophage activation ($p < 0.01$) (Figure 7C and 7C1). Fibronectin accumulation in diabetic rat liver was also significantly higher compared to that of normal control group (Figure 7D). When curcumin was given to STZ diabetic groups, the sections of liver showed reduced fibronectin accumulation (Figure 7D).
Non-alcoholic steatohepatitis onset of mechanisms under diabetic background and treatment strategies

**A**

PAS

**B**

H&E

**C**

ED1

**D**

Fibronectin

**C1**

Number of ED1 positive cells/100 fields

<table>
<thead>
<tr>
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<th>Control</th>
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Number of ED1 positive cells/100 fields

- Normal
- Control
- Curcumin

**Non-alcoholic steatohepatitis onset of mechanisms under diabetic background and treatment strategies**

**A**

PAS

**B**

H&E

**C**

ED1

**D**

Fibronectin

**C1**

Number of ED1 positive cells/100 fields

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Number of ED1 positive cells/100 fields

- Normal
- Control
- Curcumin
**Fig 7: Effect of curcumin on histopathological changes.** Histological staining with Periodic acid-Schiff (PAS) in liver (A) shows that glycogen contents of rat liver decreased in diabetic animals when compared to normal control animals but these levels increased to near normal after treatment with curcumin. In hematoxylin and eosin (H&E), (B) light microscopic photographs of livers of experimental animal showed the liver of normal control group, lipid accumulation indicated by the unstained area in liver tissues, microvascular fattening and focal necrosis, portal inflammation in the untreated diabetic group, in curcumin treated diabetic group, the severity of these changes was less than those in the untreated diabetic group. (C and C1) Immunohistochemical staining for macrophage (ED1 positive cells) and its quantification graph in each group. (D) Immunohistochemical staining for fibronectin in liver section. Each bar represents mean ± SE. Normal, age matched normal rats; Control, untreated diabetic rats; Curcumin, diabetic rats treated with curcumin 100 mg/kg/day. **p<0.01 vs Normal, ##p<0.01 vs Control.

4. Discussion

The ER is a membranous network that provides a specialized environment for processing and folding newly synthesized proteins. The ER regulates protein folding, calcium storage and the biosynthesis of macromolecules such as steroids, lipids and carbohydrates. Disruption of ER homeostasis, often termed ERS, has been observed in liver and adipose tissue of humans with nonalcoholic fatty liver disease and/or obesity [65], [104], [105], [106], [107]. As the metabolic demands increase, which can perturb the protein folding in the ER, and then does the work load of this protein factory collectively called
ERS [108]. Song and Scheuner indicated that nutrient fluctuations and insulin resistance increase proinsulin synthesis in β cells beyond the capacity for folding of nascent polypeptide within the ER lumen, thereby disrupting ER homeostasis and triggering the UPR and chronic ERS promoted apoptosis [78]. Since hepatocytes have a well-developed ER structure, ERS is involved in liver-related disease [109]. As curcumin also has the ERS inhibitory effect [89], in light of the important role of the liver in glucose homeostasis and the pathogenesis of diabetes, I sought to examine the potential effect of curcumin on the ERS response signal in hepatocytes of diabetic animals. So, I question whether the beneficial function of curcumin on suppressing the ERS in the liver.

The ER lumen provides a specialized environment for protein folding and maturation and a unique complement of molecular chaperones and folding enzymes [110]. Recent studies have suggested that ERS and UPR signaling are tightly associated with hepatic lipid metabolism [111], [112]. In mammalian cells, UPR activation involves three ER-localized proteins IRE1α, PERK, and ATF6α [113]. It is currently thought that in unstressed cells all three proteins are maintained in an inactive state via their association with the ER protein chaperone GRP78. GRP78 acts as a master regulator of the activation of UPR signaling pathways. Upon ERS, GRP78 is released and sequestered on unfolded proteins, allowing activation of PERK, IRE1α and ATF6α [114]. In the present study, I investigated ERS signaling in diabetic
liver and observed the elevated protein expression of GRP78, p-PERK and IRE1α in the liver of diabetic rats, which were attenuated by curcumin treatment (Figure 2A, 3A and 3B). But the expression of ATF6α was not significant in the liver of diabetic rats compared with normal rats (Figure 3C).

Considering all these findings, the increase of GRP78 expression and activation of the UPR is often used as indicators of ERS due to the complexity of directly measuring the ER integrity or protein aggregates and I hypothesize that the increase in the protein expression of GRP78 and UPR signaling proteins might actually indicate the induction of ERS in the diabetic rats. Curcumin treatment attenuated the ERS in the liver of diabetic rats by reducing these marker proteins.

Prolonged or insufficient ERS may turn physiological mechanisms into pathological consequences. When ERS inducing stresses is too severe or prolonged to allow for recovery of ER function, the apoptosis pathway is activated to remove damaged cells [115], [116], [117],[118], [119]. It is proposed that at least three pathways are involved in the ER stress-mediated apoptosis [115],[116], [117], [118],[119],[120]. The first is transcriptional activation of the gene for CHOP. The second is activation of the IRE1-TRAF2-ASK1-MAP kinase pathway. The third is activation of ER-associated caspase-12.
CHOP is expressed at low levels under physiological conditions, but it is strongly induced at the transcriptional level in response to ER stress [78], [115], [116], [121], [122]. The transcription of the chop gene is activated by all three ER stress sensors (IRE1α, ATF6 and PERK) signaling pathways. The activation of PERK plays a dominant role in the induction of transcription factor CHOP/GADD153 [123] over that of ATF6α and IRE1α signaling pathways, although the presence of all three signaling pathways is required to achieve the maximal induction of CHOP [116], [122]. Moreover, the failure of the UPR to ameliorate ERS, can lead to cell death via several mechanisms and CHOP is among the best characterized of the UPR-regulated proapoptotic proteins [116].

Correlates with these results, the phosphorylation of hepatic PERK and CHOP gene expression were shown to be significantly increased in the diabetic rats when compared with normal rats. Treatment with curcumin significantly attenuated the changes in the hepatic expression of p-PERK and CHOP/GADD153 and provided evidence for the prevention of ERS, one of the possible mechanisms of hepatic apoptosis in diabetic rats (Figure 3A and 4A).

Whereas, IRE1α also appears to mediate rapid degradation of specific mRNAs, presumably in an effort to reduce production of proteins that require folding in the ER lumen [124], [125]. Under ERS conditions, activated IRE1α, one of the ERS sensors, recruits TRAF2; then ASK1 directly binds to TRAF2 and is activated. It is thought that the mitochondria pathway is involved in
ASK1 mediated apoptosis. In this study, the liver of diabetic rats has displayed a significant increase of TRAF2 and ASK1 protein expression (Figure 4B and 4C). Treatment with curcumin significantly down regulated the protein expression of TRAF2 and ASK1 in the liver of diabetic rats. IRE1α activation has also been linked to the activation of p38MAPK and JNK [126], [127], [128]. The p38MAPK and JNK are subfamily of the MAPK superfamily and are classified as a stress response. In the present study, the hepatic levels of phosphorylated forms of p38MAPK are significantly lesser in the curcumin treated rats when compared with the control group (Figure 4D) suggesting that curcumin treatment avoided the activation of MAPK signaling cascade with the prevention of stress in the liver of diabetic rats through which it has prevented the liver injury involved in STZ-induced diabetic rats. Interestingly, in this experiment I did not observe any significant difference in the phosphorylation of JNK among the three groups (data is not shown here).

Pro-caspase-12 is localized to the cytosolic side of ER membrane and is activated by ERS but the mechanism has not been confirmed. It is reported that caspase-12 knockout cells are partially resistant to ERS induced apoptosis [117]. Interestingly, in this study, I did not observe any significant difference in the expression of cleaved caspase-12 among the normal, control and treatment group (Figure 2B). For further confirmation of apoptosis in the liver of diabetic rats and the effect of curcumin treatment on diabetic liver, I
checked the apoptotic marker protein cleaved caspase-3 and anti-apoptotic protein bcl2. In this study, I found that the expression levels of activated caspase-3 were significantly increased and the anti-apoptotic proteins bcl2 were reduced in the diabetic liver. Curcumin treatment prevented all of these alterations (Figure 5A and 5B).

Furthermore, it is reported that the ERS pathway plays crucial roles in the inflammatory response [127], [129], [130]. IRE1α.TRAF2 complex also involved in the transcriptional induction of inflammation related genes. Nanji et al. (2003) have demonstrated the protective effect of curcumin in rat liver injury induced by alcohol, where curcumin administration prevented ALT increases, blocked the activation of NF-κB, the expression of proinflammatory cytokines (TNFα) and iNOS [131]. In this experiment, I found that activation of TLR4 as well as proinflammatory cytokine expression; TNFα and IL-1β were increased in diabetic rats compared with the levels in normal rats. Curcumin prevented all of these alterations (Figure 6A, 6B and 6C). I showed that the significant elevation level of ALT in diabetic rats was reduced slightly by curcumin treatment.

Stimuli that injure the liver can activate multiple intracellular stress responses, such as the inflammatory response. Macrophage or kupffer cells have been implicated in the pathogenesis of various liver diseases [132]. In the present study, using the accumulation of ED1 as a marker of macrophage activation, I have demonstrated increased activation of macrophage/ kupffer
cells in the hepatic tissue of diabetic animals (Figure 7C and 7C1) and increased fibronectin accumulation in diabetic liver (Figure 7D). In this study, I demonstrated that macrophage recruitment in diabetic liver is ameliorated by the administration of curcumin. Curcumin is a representative polyphenolic compound found in the dietary spice turmeric. From the morphological study, I found that, long-term curcumin treatment improved many pathological changes, degenerated hepatocytes with polymorphic nuclei, dilated sinusoids and mononuclear cell infiltrate extending through hepatic tissue in diabetic rats. In the present study, curcumin significantly reduced the blood glucose level. It is also reported that it has the capability to improve the activity of pancreas thus curcumin able to increase the insulin secretion [97]. Pancreatic islet cell death is the cause of deficient insulin production in diabetes. Approaches towards prevention of cell death are of prophylactic importance in control and management of hyperglycemia. Meghana et al investigated and reported that islet viability and insulin secretion in curcumin pretreated islets are significantly higher than islets exposed to STZ alone [98]. Recently it is reported that curcumin enhances pancreatic β cell function by inhibiting phosphodiesterase activity and regulated insulin secretion under glucose stimulated condition [133]. From this experiment, I can conclude that curcumin may indirectly reduce the ERS by reducing hyperglycemia or it may directly reduce the ERS in diabetic liver and protect the liver damage (Figure 8).
Figure 8: Schematic summary of the experiment. Diabetes mellitus (Type 1 diabetes) causes hyperglycemia and increases advanced glycation end products (AGE). Hyperglycemia enhances ER malfunction, which causes dissociation of GRP78 and activation of UPR proteins (IRE1a, PERK) and caspase pathway. Then leads to hepatic apoptosis, which may cause cell death. Ultimately caused the liver damage. Curcumin treatment significantly reduced the hyperglycemia in diabetic rats and protected ER function and reduced liver damage.
5. Conclusion

The ERS related inflammation and apoptosis are involved in the pathogenesis of various diseases, including hepatopathy. Therefore, the ERS pathway can be a new target for the treatment of those diseases. The result presented here show that the administration of curcumin inhibits ERS, ERS related apoptosis and inflammation in the liver of STZ-induced diabetic rats. All the above results suggest that the beneficial effect of curcumin occurs, at least in part through modulation of the UPR signaling pathway. Given these promising preclinical findings, I believe that the curcumin, might be considered as a potential adjuvant entity for preventing diabetic liver damage.
CHAPTER TWO

Curcumin ameliorates liver damage and progression of NASH in NASH-HCC mouse model possibly by modulating HMGB1-NF-κB translocation
1. Introduction:

HCC is the fifth most common form of cancer and the third common cause of cancer-related deaths [134]. Recently, NAFLD is the well-known reason for persistent liver disease in well-off countries. There is a range of liver diseases, extending from hepatic steatosis (simple intrahepatic accumulation of lipid droplets) and steatosis with inflammation and fibrosis (NASH) to cirrhosis and HCC [135]. Since NASH is considered as a hepatic manifestation of the metabolic disorder and exceedingly connected with diabetes, it is speculated that the relationship amongst diabetes and HCC is identified with the progression of NASH [136]. Fujii et al., established a novel NASH-HCC model in mice under diabetic background [11]. Inflammation promotes the progression of NASH [137] and has also been a critical factor in the progression of HCC [138]. Understanding the molecular mechanisms linking hepatocytes necrosis to inflammation and progressive liver damage is, therefore, critical to the development of novel therapeutic strategies for NASH-HCC.

HMGB1, a DNA-binding nuclear protein is a leader-less pro-inflammatory cytokine and is an important member of inflammatory cascade [139]. HMGB1 can be either actively regulated from activated immune cells or passively from injured cells [140] and signals tissue damage [141]. More importantly, the role of HMGB1 as a DAMP has led to its implication in a variety of
disease states mediated by inflammatory stimuli, including, sepsis, arthritis, liver ischemia-reperfusion injury, liver transplantation, cancer, HCC [142,143] and atopic dermatitis [144]. Extracellular HMGB1 can bind to different cell surface receptors, including TLR2, TLR4 and the RAGE act on target cells and promotes inflammation [145]. Activation of these receptors results in the activation of NF-κB, extracellular signal-regulated kinase (ERK) signaling, thereby triggering a variety of inflammatory and pro-inflammatory cytokines. Furthermore, the elevated levels of these cytokines can induce NF-κB activation and thereby worsen the disease condition. The role of pro-inflammatory transcription factor like NF-κB [146], and HMGB1 [147] in the development and progression of HCC is also well established.

Curcumin (diferuloylmethane) is a polyphenol comprising 2-8% of most turmeric preparations. It acts as an active ingredient and exerts potent lipid regulatory, anti-inflammatory, anti-oxidative and anti-carcinogenic properties that are important for protecting against NASH. One of the main properties of curcumin is its hepatoprotective activity. It has been reported that curcumin protects several drugs induced liver injury [148,149]. In our previous study, we reported that curcumin protects the liver damage of streptozotocin (STZ) induced diabetic rats by inhibiting ERS and ERS-related apoptosis and inflammation [150]. It has also been reported that curcumin inhibits NF-κB, an oncogenic signaling molecule in liver cancer [151].
Although some studies reported that curcumin reduces Propionibacterium acnes [152], carbon tetra chloride [153] and concanavalin A [154] induced acute liver injury through inhibiting the HMGB1 release, to the best of our knowledge, no other experimental studies revealed the effect of either curcumin or other treatments on this novel NASH-HCC model. Thus, in the present study, I explored the possible protective mechanisms of curcumin on the liver damage and progression of NASH in NASH-HCC mouse model.

2. Materials and methods

2.1. Animals and experimental designs

All animals were treated in accordance with the guidelines for animal experimentation of our institute (Approve No. H270310) [150]. C57BL/6J mice were bred in my laboratory. Animals were housed in a temperature of 23 ± 2°C and humidity of 55 ± 15% and were submitted to a light/dark cycle, allowed free access to standard laboratory chow and tap water. NASH-HCC was induced in male mice by a single subcutaneous injection of 200 µg STZ (Sigma, MO, USA) at 2 days after birth. They were being started feeding with high-fat diet (HFD) 32 (CELA Japan) ad libitum at the age of 4 weeks, and continued up to 14 weeks age of mice. The normal diet provided 404 Kcal/100 g (water 10%, protein 25.0%, fat 4.5%, ash 6.7%, carbohydrate 49.3% and fiber 4.5%), whereas, the HFD 32 provided 507.6 Kcal/100 g (water 6.2%, protein 25.5%, fat 32.0%, ash 4.0%, carbohydrate 29.4%, and fiber 2.9%).
Mice were randomly selected into three groups (n=6/group): the normal group (Normal) were normal mice subjected to the normal diet; the NASH group (NASH) were STZ injected mice subjected to the HFD32 treated with 1% gum Arabic (the vehicle of curcumin); the NASH and Curcumin group (NASH + Curcumin) were STZ injected mice subjected to the HFD32 (NASH mice) treated with curcumin (100 mg/kg/day, Sigma-Aldrich, Tokyo, Japan) suspended in 1% gum Arabic. Curcumin treatment was started at the age of 10 weeks and administered via oral gavage continued up to 14 weeks of age along with HFD. All mice were sacrificed at the age of 14 weeks, and serum was separated from blood. Liver tissue was isolated for histological, biochemical, and molecular biological analysis [11].

2.2. **Biochemical analysis**

Fasting blood glucose was measured using the G-checker (Sanko Junyaku, Tokyo, Japan). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglyceride (TG) and total cholesterol (TC) were measured by FUJI DRI-CHEM 7000 (Fujifilm, Tokyo, Japan) as previously described [150].

2.3. **Histological examination**

Liver sections (4 µm) of different groups of mice were immediately fixed in 10% formaldehyde solution, embedded in paraffin, cut into several transversal sections and mounted on glass slides. Then the liver tissue
sections were deparaffinized and stained with hematoxylin and eosin (H&E). Morphological analysis was done by computerized image analysis system on ten microscopic fields per section examined in a 40-fold magnification (AmScope, USA), with the observer blind to the study group [150]. Here, macrovesicular steatosis, microvesicular steatosis and hepatocellular hypertrophy were separately scored and the severity was graded, based on the percentage of the total area affected, into the following categories: 0 (<5%), 1 (5-33%), 2 (34-66%) and 3 (>66%). Inflammation was evaluated by counting the number of inflammatory foci per field, a focus has defined a cluster of ≥5 inflammatory cells. Different fields were counted and scored as per field: 0 (<0.5), 1 (0.5-1.0), 2 (1.0-2.0) and 3 (>2). By adding the score of the above four parameters were used to calculate the NAFLD activity score, resulting in a total clinical score ranging from 0 (healthy) to 12 (maximal severity of NASH) [14]. NAFLD score ≥ 5 was identified as definite NASH.

2.4. Determination of liver collagen content

Formalin-fixed, paraffin-embedded, liver sections (4 µm) were deparaffinized and stained with Masson trichrome (MT) [155]. MT staining was performed following the manufacturer’s instructions (Accustain HT15, Sigma-Aldrich, St. Louis, MO).

2.5. Liver homogenates and nuclear extract preparation

Briefly, 100 mg of liver tissue was homogenized in 0.5 mL of buffer A containing 10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1
mM EDTA, 0.1 mM PMSF, 1 µM pepstatin, and 1 mM $p$-amino benzamidine using a tissue homogenizer for 20 s. Homogenates were kept on ice for 15 min, and then 125 µL of a 10% Triton X-100 solution was added and mixed for 15 s and the mixture was centrifuged for 2 min at 12 000 rpm. The supernatant containing cytosolic proteins was collected. The pelleted nuclei were washed once with 200 µL of buffer A plus 25 µL of 10% Triton X-100, centrifuged, then suspended in 50 µL of buffer B containing 50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% v/v glycerol, mixed for 20 min, and centrifuged for 5 min at 12 000 rpm. The supernatant containing nuclear proteins was stored at –80°C [156].

2.6. **Western blot analysis**

The frozen liver tissues were weighed and homogenized in an ice-cold buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 20 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM 2-mercaptoethanol, 0.01 mg/mL leupeptin, 0.01 mg/mL aprotinin). Homogenates were then centrifuged (3000 × g, 10 min, 4°C) and the supernatants were collected and stored at –80°C. The total protein concentration in samples was measured by the BCA method [150]. Samples with equal amounts of protein were separated in polyacrylamide gel, transferred and identified on nitrocellulose membrane with specific antibodies followed by HRP-labelled secondary antibodies. The antibodies applied: connective tissue growth factor (CTGF), interferon gamma (IFNγ)
inducible protein (IP) 10, IL-1β, IFN γ, TLR4, p-NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) α, nuclear factor-erythroid 2-related factor (Nrf)-2, HMGB1, cluster of differentiation (CD) 68, CYP2E1, p67phox, vascular endothelial growth factor (VEGF), SREBP-1c, peroxisome proliferator-activated receptor (PPAR) α, PPARγ, oxidized low-density lipoprotein receptor (Ox-LDL R) 1, adipose differentiation related protein (ADRP), CCAAT/enhancer binding protein (C/EBP)β, glypican-3, β tubulin, and LaminA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κB, p-ERK1/2, ERK1/2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology Inc., Beverly, MA, USA), prothrombin (Abcam, Cambridge, UK). The levels of GAPDH, NF-κB (for p-NF-κB), ERK1/2 (for p-ERK1/2), LaminA (for HMGB1 and C/EBPβ) and β tubulin (for TLR4, PPARγ and Nrf2) were estimated in every sample to check for equal loading of samples.

2.7. Statistical analysis

Data are shown as means ± SEM and were analyzed using ANOVA followed by Tukey’s method or Kruskal-Wallis test followed by Dunn’s multiple comparison when appropriate. A value of $p<0.05$ was considered statistically significant. GraphPad Prism 5 software (San Diego, CA, USA) was used.
3. Results

3.1. Effect of curcumin on clinicopathology and biochemical parameters in NASH-HCC mice

There was no huge difference in energy consumption and body weight among the three groups (Table 1). The relative liver weight (%) was significantly increased in NASH group compared to normal, but in the curcumin treated group this increased proportion was significantly less. Fasting blood glucose level and serum TG, TC, ALT, AST and ALP levels were also significantly increased in NASH group compared to the normal group. These biomarkers were of lower concentration in the NASH+ Curcumin group compared to NASH group (Table 1). The liver of the NASH group also showed pale yellow color, swelling and granular surface with tumor protruding whereas the liver of the NASH + Curcumin group showed moderate fatty liver with a smooth surface (Fig. 1A). H&E staining showed extreme steatosis, enlarged hepatocytes, scattered lobular inflammatory cells in NASH mice. However, these changes were lesser in NASH + Curcumin group than the NASH group (Fig. 1B, Table 2).
Table 1: Changes in biochemical parameters after 4 weeks of treatment with curcumin in NASH-HCC mice

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<th>NASH group (n=6)</th>
<th>NASH + Curcumin group (n=6)</th>
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<td>Food intake /day/mouse (g)</td>
<td>4.0 ± 0.6</td>
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<td>Energy consumption/day/mouse (Kcal)</td>
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<td>Body weight (BW) (g)</td>
<td>24.8 ± 2.7</td>
<td>20.7 ± 5.9</td>
<td>24.6 ± 2.2</td>
</tr>
<tr>
<td>% of Relative liver weight (LW) /BW</td>
<td>4.01 ± 0.21</td>
<td>8.91 ± 1.5***</td>
<td>7.2 ± 0.43#***</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>101.3 ± 23.4</td>
<td>488.0 ± 19.9***</td>
<td>363.0 ± 23.9###***</td>
</tr>
<tr>
<td>Serum TG (mg/dL)</td>
<td>17.5 ± 1.3</td>
<td>46.3 ± 12.1*</td>
<td>40.3 ± 11.7*</td>
</tr>
<tr>
<td>Serum TC (mg/dL)</td>
<td>81.8 ± 3.8</td>
<td>253.8 ± 18.9***</td>
<td>189.5 ± 10.6###***</td>
</tr>
<tr>
<td>Serum ALT (IU/L)</td>
<td>38.0 ± 9.0</td>
<td>304.3 ± 218.7*</td>
<td>61.3 ± 15.9#</td>
</tr>
<tr>
<td>Serum AST (IU/L)</td>
<td>72.4 ± 5.4</td>
<td>270.5 ± 84.4***</td>
<td>108.3 ± 25.0##</td>
</tr>
<tr>
<td>Serum ALP (IU/L)</td>
<td>322.8 ± 49.3</td>
<td>653.8 ± 141.9***</td>
<td>408.4 ± 47.2##</td>
</tr>
</tbody>
</table>

Normal, age matched normal mice; NASH, untreated NASH-HCC mice; NASH + Curcumin: NASH-HCC mice treated with curcumin 100 mg/kg/day. TG: triglyceride; TC: total cholesterol, ALT: alanine aminotransferase, ALP: alkaline phosphatase, AST: aspartate aminotransferase, NAFLD: non-alcoholic fatty liver disease. Values are expressed as means ± SEM. Statistical analysis was carried out using One way ANOVA followed by Tukey’s method where, *p <0.05, ***p <0.001 vs normal, #p < 0.05, ##p <0.01, ###p < 0.001 vs NASH.
Fig. 1. Curcumin attenuates the clinicopathology in NASH-HCC mice. (A), Representative macroscopic appearance of livers (Black arrow: liver tumors). (B), H&E staining (Black arrow: macrovesicular steatosis, red arrow: microvesicular steatosis, yellow arrow: hypertrophy, circles: inflammatory cells). (C) Fibrosis deposition by Masson trichrome staining (blue area). (D), the hepatic protein level of CTGF. Data are mean ± SE, (n= 6/ group).
Normal, age matched normal mice; NASH, untreated NASH-HCC mice; NASH + Curcumin; NASH-HCC mice treated with curcumin 100 mg/kg/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s method where, \( *p<0.05 \) vs Normal, \( \#\#p<0.01 \) vs NASH.

3.2. **Effect of curcumin on hepatic fibrosis in NASH-HCC mice**

To examine the effect of curcumin in hepatic nutritional fibrosis, I performed MT staining of hepatic tissues of three groups. MT staining demonstrated pericellular fibrosis around central veins, and its positive area (%) was (median (interquartile range)): in normal group (0.26 (0.075-0.5)); in NASH group (1.755 (1.34-2.375)); while in NASH + Curcumin group (0.6075 (0.131-1.470)) (\( p=0.0291 \)) (Fig. 1C and Table 2). Consistent with the reduced fibrosis, the hepatic protein expression of fibrosis promoter, CTGF was significantly lower in NASH + Curcumin mice (\( p<0.01 \) vs. NASH) (Fig. 1D).
Table 2: Histopathological analysis scores after 4 weeks of treatment with curcumin in NASH·HCC mice.

<table>
<thead>
<tr>
<th>Item</th>
<th>Definition</th>
<th>Score</th>
<th>Normal group (n=6)</th>
<th>NASH group (n=6)</th>
<th>NASH + Curcumin group (n=6)</th>
<th>Kruskal-Wallis P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Macrovesicular steatosis</td>
<td>&lt;5%</td>
<td>0</td>
<td>0 (0-0)</td>
<td>2 (2-2)**</td>
<td>1 (1-1)</td>
<td>0.0183</td>
</tr>
<tr>
<td></td>
<td>5-33%</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33-66%</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;66%</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Microvesicular steatosis</td>
<td>&lt;5%</td>
<td>0</td>
<td>0 (0-0)</td>
<td>2 (1-2)**</td>
<td>1 (1-1)</td>
<td>0.0289</td>
</tr>
<tr>
<td></td>
<td>5-33%</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33-66%</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;66%</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Hepatocellular hypertrophy</td>
<td>&lt;5%</td>
<td>0</td>
<td>0 (0-0)</td>
<td>2 (2-2)**</td>
<td>1 (1-1)</td>
<td>0.0183</td>
</tr>
<tr>
<td></td>
<td>5-33%</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33-66%</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;66%</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. Inflammation:</td>
<td>Number of inflammatory foci</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.5</td>
<td>0</td>
<td>0 (0-0)</td>
<td>2 (2-3)*</td>
<td>2 (1-2)</td>
<td>0.0344</td>
</tr>
<tr>
<td></td>
<td>0.5-1.0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0-2.0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;2.0</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAFLD activity score</td>
<td>Score of A+ B + C + D</td>
<td>0-12</td>
<td>0 (0-0)</td>
<td>8 (7-9)*</td>
<td>5 (4-5)</td>
<td>0.0234</td>
</tr>
<tr>
<td>MT score (Fibrosis Score)</td>
<td>(Blue stained areas / non-stained areas)×100</td>
<td>0-4</td>
<td>0.26</td>
<td>1.755</td>
<td>0.6075</td>
<td>0.0291</td>
</tr>
</tbody>
</table>

Normal, age matched normal mice; NASH, untreated NASH·HCC mice; NASH + Curcumin: NASH·HCC mice treated with curcumin 100 mg/Kg/day. NAFLD: non-alcoholic fatty liver disease, MT: Masson trichrome. Values are
expressed as medians (interquartile ranges). Statistical analysis was carried out using Kruskal-Wallis test followed by Dunn’s multiple comparison where, *$p<0.05$, **$p<0.01$ vs normal.

3.3. **Effect of curcumin on the cytoplasmic HMGB1 level in liver**

To find the altered distribution of HMGB1 in NASH liver of mice, Western blot was performed to investigate the HMGB1 expression in the cytoplasm and nuclear fraction from the liver of NASH and normal mice. The cytosolic expression of HMGB1 was higher than nuclear expression in NASH group. In contrast, the translocation of HMGB1 from the nucleus to cytoplasm was significantly lower in the NASH + Curcumin group compared to NASH group (Fig. 2A).
**Fig. 2.** Curcumin attenuates hepatic inflammatory cytokines and macrophage infiltration in NASH-HCC mice.

Representative Western blots show specific bands for nuclear HMGB1 (for LaminA) and cytosolic HMGB1 (A), TLR4 (for β tubulin) (B), IP10 (C), IFNγ (D), IL-1β (E), and CD68 (F) and the representative histograms show the band densities with relative to that GAPDH. The ratio of HMGB1 expression in the cytoplasmic fraction to nuclear fraction was measured by densitometric quantification (A). Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; NASH + Curcumin; NASH-HCC mice treated with curcumin 100 mg/kg/day. Statistical analysis was
carried out using One way ANOVA followed by Tukey’s method where, $^* p<0.05$, $^{**} p<0.01$, $^{***} p<0.001$ vs Normal, $^# p<0.05$, $^{##} p<0.01$, $^{###} p<0.001$ vs NASH.

3.4. **Effect of curcumin on hepatic chemokines, cytokines, and other pro-inflammatory molecules**

The hepatic IP10, IFNγ and IL-1β protein expression were significantly increased in NASH mice. I observed that the liver protein level of TLR4 and the expression of KCs, CD68, a marker of macrophages [157], were significantly increased in NASH group compared to the normal group. Curcumin treatment essentially suppressed these protein levels in the liver of the NASH + Curcumin group compared to NASH group (Fig. 2B-F).

3.5. **Effect of curcumin on NF-κB pathway**

I noticed that the increased hepatic nuclear translocation of NF-κB subunits p65 and diminished cytosolic IκBα protein level (Fig. 3A & B) in NASH group compared to the normal group. The expression of Ox-LDL-R1 was also significantly increased in NASH liver, which activates the NF-κB signal transduction pathway [158] (Fig. 3C). But curcumin treatment markedly prevented all of these alterations as shown in the NASH + Curcumin group compared to NASH group.
**Fig. 3. Curcumin attenuates NF-κB pathway in NASH-HCC mice**

Representative Western blots show specific bands for nuclear and cytosolic p-NF-κB (for NF-κB) (A), IκBα (B) and Ox-LDL-R1 (C), and the representative histograms show the band densities with relative to that GAPDH. The ratio of p-NF-κB expression in the nuclear fraction to cytosolic fraction was measured by densitometric quantification (A). Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; NASH + Curcumin; NASH-HCC mice treated with curcumin 100 mg/kg/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s method where, *p<0.05, **p<0.01 vs Normal, #p<0.05, ##p<0.01 vs NASH.
3.6. Effect of curcumin on hepatic lipogenesis

The hepatic protein expression of lipogenic controllers that include SREBP-1c, ADRP and genes which regulate adipogenesis, such as PPARγ were significantly increased in NASH group compared to normal mice. On the other hand, these increased hepatic protein expressions were significantly lesser in the NASH + Curcumin group compared to NASH group (Fig. 4A-C). The protein expression of PPARα was reduced in the NASH group while it was increased in the liver of NASH + Curcumin group but non-significantly (Fig. 4D).

![Graph showing the effect of curcumin on hepatic lipogenesis](image)
Fig. 4. Curcumin attenuates hepatic lipogenesis in NASH-HCC mice.

Western blots show specific bands for hepatic SREBP1c (A), ADPR (B), PPARγ (for β tubulin) (C) and PPARα (D) and the representative histograms show the band densities with relative to that GAPDH. Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; NASH + Curcumin; NASH-HCC mice treated with curcumin 100 mg/kg/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s method where, *p<0.05, **p<0.01 vs Normal, ###p<0.01, ####p<0.001 vs NASH.

3.6. **Curcumin attenuated oxidative stress in NASH-HCC liver**

I evaluated the hepatic levels of C/EBPβ and CYP2E1 using Western blot analysis. Both nuclear C/EBPβ and cytosolic CYP2E1 protein expression were significantly increased in the NASH group compared to the normal group, whereas, in the NASH + Curcumin group these were significantly less corresponding to NASH mice (Fig. 5A and B). The expression of cytosolic NADPH oxidase component p67phox and p-ERK1/2 were markedly increased but the expression of Nrf2 was significantly decreased in the liver of NASH-HCC mice compared to normal mice. However, curcumin treatment markedly reversed all of these expressions in the liver of the NASH + Curcumin group (Fig. 5C-E).
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A

B

C

D

E

C/EBPβ/Lamin A

CYP2E1/GAPDH

p-ERK1/2/ERK1/2

p67phox/GAPDH

Nrf2/β tubulin

Normal NASH NASH + Curcumin

0.000 0.005 0.010 0.015 0.020 0.025

** #

Normal NASH NASH + Curcumin

0.00 0.05 0.10 0.15 0.20

** ###

Normal NASH NASH + Curcumin

0.0 0.1 0.2 0.3 0.4

** ###

Normal NASH NASH + Curcumin

0.00 0.01 0.02 0.03 0.04

** #

Normal NASH NASH + Curcumin

0.0 0.5 1.0 1.5

* ##

Normal NASH NASH + Curcumin

0.00 0.015 0.03 0.045

**

Normal NASH NASH + Curcumin

0.00 0.005 0.01 0.015 0.02

**

C/EBPβ/Lamin A

CYP2E1/GAPDH

p-ERK1/2/ERK1/2

p67phox/GAPDH

Nrf2/β tubulin
Fig. 5. Curcumin attenuates hepatic oxidative stress in NASH-HCC mice.

Western blots show specific bands for hepatic C/EBPβ (for Lamin A) (A), CYP2E1 (B), p-ERK1/2 (for ERK1/2) (C) and p67phox (D) and Nrf2 (for β tubulin) (E) and the representative histograms show the band densities with relative to that GAPDH. Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; NASH + Curcumin; NASH-HCC mice treated with curcumin 100 mg/kg/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s method where, \( p<0.05 \), \( **p<0.01 \) vs Normal, \( #p<0.05 \), \( ##p<0.01 \), \( ###p<0.001 \) vs NASH.

3.7. Effect of curcumin on hepatic VEGF, glypican-3 and prothrombin expression

I investigated the hepatic protein expression level of VEGF, glypican-3, and prothrombin in this NASH-HCC mouse model, I found that these were significantly increased in NASH group compared to the normal group, but in curcumin treated group the expression of these proteins were significantly less when compared to NASH group (Fig. 6A-C).
**Fig. 6. Curcumin attenuates hepatic VEGF, Glypican-3 and prothrombin expression in NASH-HCC mice.** Western blots show specific bands for hepatic VEGF (A), Glypican-3 (B) and prothrombin (C) and the representative histograms show the band densities with relative to that GAPDH. Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; NASH + Curcumin: NASH-HCC mice treated with curcumin 100 mg/kg/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s method where, *p<0.05, **p<0.01 vs Normal, #p<0.05, ###p<0.01 vs NASH.
4. Discussion

Animal models are key to translate human diseases and testing the effectiveness of various available drugs for treatment. There are several models of NASH and HCC, but they neither show high reproducibility nor meet the clinicopathological process from fatty liver, NASH, fibrosis to HCC under diabetic background [159]. Although, diabetes is a known danger element for HCC, it is also reported that neither diabetes nor obesity alone causes of HCC [160]. In this experiment, I considered a novel NASH-HCC model, which was developed by injecting a low-dose STZ and feeding a HFD both in mice. This model shows 100% reproducibility for HCC, with quick results for the study of the disease progression and maintains a sequence from fatty liver, NASH, fibrosis to HCC under diabetic background [11]. To date, there is no effective treatment to manage NASH. In this study, I investigated the possible hepatoprotective mechanism of curcumin at a dosage that translates to a curcumin intake of around of 6 g/day in adult humans [161].

The replication of human NASH histopathology in animal model remains the only mean of accurately assessing NASH [159]. Briefly, the two key features of NASH, steatosis, and inflammation; categorized by Liang et al. as micro/macrosteatosis, hepatocellular hypertrophy and inflammatory cell aggregation [14]. In our experiment, based on this scoring the mice in NASH-
HCC group reached a higher NAFLD activity score along with fibrosis (Table 2) and increased serum aminotransferases and blood glucose levels (Table 1). Increase in aminotransferase levels leads to focal inflammation, hepatic necrosis and fibrosis without developing resistance to insulin [137]. The CTGF also plays a key role in the progression of liver fibrosis in mice. High level of CTGF protein expression was found in NASH liver, with pericellular fibrosis (Fig. 1D); which represents human NASH “chicken wire fibrosis”. Interestingly, these biomarkers were of lower concentration in the NASH + Curcumin group compared with the NASH group along with reduced NAFLD score and fibrosis marker.

It is well established the importance of liver inflammation in the initiation and progression of cancer. Several study groups worked on characterizing the role of DAMPs, including HMGB1, in liver inflammation [142]. It is proved that the release of nuclear HMGB1 to the extracellular, not only mediates acute phase of liver injury in ischemia reperfusion, but also promotes HCC invasion and provides a link between inflammations to HCC [147]. The HMGB1 release can be correlated with its ability to signal through receptors TLR2, TLR4 and RAGE [145]. As it is reported that the TLR4-deficient mice exhibited reduced hepatic ischemia-reperfusion injury, partially protects from diet-induced steatohepatitis and downregulated the inflammatory cytokines [162], brought an attention to TLR4 in NASH. In line with other
inflammation-associated HCC, in this study, I identified the significantly increased levels of cytosolic translocation of HMGB1 in the liver of NASH mice with upregulation of TLR4 protein levels. Interestingly, both the protein expression levels of TLR4 and cytosolic translocation of HMGB1 were significantly less in the liver of curcumin treated mice compared to NASH group (Fig. 2A-B).

In similarity, glycyrrhizin, a direct HMGB1 inhibitor [163], has been used clinically [164] and experimentally [165] to prevent liver cirrhosis and HCC for decades in Japan. Interaction of HMGB1 with TLR4 induces nuclear translocation of activated NF-κB, and activates ERK signaling and ultimately resulting the release of pro-inflammatory cytokines [144]. The secreted stimuli like IFNγ [166], and IL-1β [167] can also stimulate the release of HMGB1 from macrophages. Accordingly, I found the elevated hepatic protein expression levels of pro-inflammatory cytokines IP10, IFNγ, IL-1β and macrophage infiltration (CD68) in NASH group, whereas, in the NASH + Curcumin group these levels were significantly lesser (Fig. 2C-F).

It is already proved that NF-κB is a key regulator of early hepatic inflammatory recruitment and liver injury in NASH [146] and HCC [168]. While KCs CD68 display powerful NF-κB activation in NASH liver, resulting in the production and secretion of pro-inflammatory cytokines that strongly implicated as a promoter of fibrosis and HCC [169]. Therefore, curcumin treatment also protects the nuclear translocation of NF-κB
pathway and its upstream and downstream inflammatory effectors, which induce liver injury and progression of steatohepatitis (Fig. 3).

My data also suggest that curcumin treatment protects the STZ-HFD-induced liver from steatosis and liver injury. Where, steatosis sensitizes the liver to injury by cytokines, endotoxin, adipokines, mitochondrial dysfunction and oxidative stress, which result in de novo lipogenesis and increased release of free fatty acids and abnormal lipid deposition in the liver. The protein expression levels of SREBP-1c and ADRP were significantly lower in the curcumin treated group when compared with the NASH group (Fig. 4A and B), which are involved in de novo fatty acid synthesis. Hepatic expression of PPARγ is augmented in various models of fatty liver disease in which liver regeneration has been reported to be impaired [170]. In contrast, Gazit et al. suggested that increased hepatic PPARγ activity might improve regeneration of fatty liver [171], but, in my study, I found that the elevated level of PPARγ in NASH liver was significantly downregulated by curcumin treatment (Fig. 4C). Curcumin treatment increased the protein expression of fatty acid oxidation regulatory gene PPARα in NASH liver (Fig. 4D).

Although, HMGB1 does not contain a leader sequence, the mechanisms for regulating HMGB1 release and activity vary depending on context. Many experimental studies reveal that oxidative stress is likely a common
mechanism regulating HMGB1 translocation, release and activity [172]. In another study, Wang et al., reported that high blood glucose level induced HMGB1 translocation through NADPH oxidase and PKC dependent pathway [173]. Consistent with these reports, I found that the hepatic protein expression of CYP2E1 and its upstream C/EBPβ, downstream p-ERK1/2 and NADPH oxidase subunit p67phox were significantly lesser in curcumin treated group along with reduced blood glucose level compared to NASH group (Fig. 5A-D, Table 1). Activation of CYP2E1, is involved in the metabolism of fatty acid and produce ROS directly or indirectly. Redox systems, including antioxidants and phase II detoxifying enzymes provide protection against ROS actuated tissue damage. Nrf2 is a vital cytoprotective transcriptional variable that induces the expression of several antioxidants and phase II detoxifying enzymes [156] and acts as a defense system in the development of NASH [174]. Actually, besides Nrf2’s part in directing cellular antioxidant guard, it likewise has anti-inflammatory capacities [156]. Here, I found that the significantly higher expression of Nrf2 in the liver of the NASH + Curcumin group when compared to NASH group (Fig. 5E).

Oxidative stress and inflammatory pathways lead to the transformation of a normal cell to a tumor cell, its survival, and proliferation [175]. In my experiment, I noticed the tumor protruding in the liver of NASH group, in contrast, it was absent in the NASH + Curcumin group (Fig.1A). It is
reported that VEGF may act as a major regulator of hepatic tumorigenesis in a mouse model of experimental liver metastasis [176]. This tumor protruding may be malignant or progress to HCC, and, as it has been reported that the hepatic expression of glypican-3 [177] and DCP or prothrombin [178] are significantly increased in most HCCs compared with benign liver lesions and normal liver, they act as a useful prognostic tumor marker for HCC. In my study, I observed that VEGF, glypican-3 and prothrombin, markers for HCC were significantly higher in the NASH group compared to the normal group (Fig. 6A-C). But, these expressions were significantly lower in the curcumin treated group compared to NASH group.

In summary, my data indicate that curcumin has a beneficial effect in NASH, it might be partly by reducing the cytosolic and nuclear translocation of HMGB1 and NF-κB, either directly or indirectly by reducing oxidative stress and blood glucose level in NASH liver. Furthermore, by inhibiting the progression of NASH to HCC curcumin might be useful in the management of NASH and may constitute a powerful approach for the novel NASH-HCC model.
CHAPTER THREE

Le Carbone modulates liver damage in non-alcoholic steatohepatitis-hepatocellular carcinoma mouse model through activation of AMPKα-SIRT1 signaling
1. **Introduction**

NAFLD has become increasingly emergent due to changes in lifestyle and resultant over-nutrition [179]. NASH is an extended form of NAFLD, characterized by necro-inflammation, lipid accumulation and fibrosis, act as a significant risk factor for the development of cirrhosis and HCC [180]. Although still obscure, the transition from benign steatosis to steatohepatitis in NASH is regarded as the increased levels of toxic lipids, induced by dysregulated lipid metabolism and triggered by the oxidative stress and pro-inflammatory cytokines [181,182].

Several animal model of NAFLD is associated with the impairment of the hepatic SIRT1-AMPK axis, a central signaling system for controlling the lipid metabolism pathway [183]. SIRTs belong to the silent information regulator-2 family. SIRT1 deacetylation has been recognized as a regulatory mechanism for several proteins involved in NAFLD pathogenesis [184] and low SIRT1 expression has been noticed in different NAFLD models [185]. Similarly, the AMPK signaling system plays a vital role in cellular and organismal survival during stress by maintaining metabolic homeostasis [186].

The activation of SIRT1-AMPK signaling in several metabolic tissues, including liver has been proposed to increase the rate of fatty acid oxidation and restrain the lipogenesis mostly by modulating activity of PPARγ.
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coactivator-α (PGC-1α) /PPARα or SREBP-1 through deacetylation and phosphorylation, respectively [187], [188].

PPARγ is a transcription factor, thought to be the master regulator of fat storage and energy homeostasis [189], and although the function of PPARγ is controversial, the increased expression of PPARγ is found in obese mice and which is associated to increase the lipogenesis. PPARα acts as a regulator of β-oxidation and removes cholesterol at the time of necessity. It is also found that hepatic PPARα expression is low in NAFLD due to steatosis, but enhanced following diet and exercise [190].

Dietary supplementation along with vitamin E found to improve the NAFLD activity score in non-diabetics with biopsy proven NASH patients [191]. LC, a charcoal supplement, comprises with a large amount of dietary fibers. In my previous study, I found that LC supplementation able to enhance the AMPK-SIRT1 expression in colon tissue of experimental colitis in a mouse model and reduced the inflammation [63]. It is also suggested that, activated charcoal possesses wound healing, anti-aging properties [61]. Some study proved that chemotherapy with mitomycin C adsorbed onto activated charcoal (MMC·CH) may increase the survival rates after stomach cancer surgery, when anticancer drugs fail to reduce the secondary cancer development [62]. Furthermore, in patients with trimethylaminuria (a metabolic disorder), activated charcoal supplement improve the life of individual suffering [192].
Activated charcoal also used to prevent abnormal hardening (sclerosis) in the heart and coronary blood vessels by improving lipid profile [64]. For the above-mentioned beneficial effects of charcoal supplement, in this experiment I hypothesized that LC may prevent the liver damage in a novel mouse model of NASH-HCC by activating AMPK-SIRT1 expression.

2. Materials and Methods

2.1. Drugs and chemicals

HFD32 was purchased from CLEA, Japan. LC was obtained from mcprot., Biotechnology- EJC, Japan. All other chemicals used were purchased from Sigma, Japan unless mentioned otherwise.

2.2. Composition of normal diet, HFD32 and LC

The normal diet provided 404 Kcal/100 g (water 10%, protein 25.0%, fat 4.5%, ash 6.7%, carbohydrate 49.3% and fiber 4.5%), whereas, the HFD 32 provided 507.6 Kcal/100 g (water 6.2%, protein 25.5%, fat 32.0%, ash 4.0%, carbohydrate 29.4%, and fiber 2.9%). LC was formulated as capsule; entirely with the activated Carbone and contained a minor quantity of minerals (in 100 g of LC) such as sodium 2.2 mg, potassium 77.6 mg and manganese 0.4 mg. Where other minerals like non-phosphorous, iron, calcium, magnesium, copper, zinc and heavy metals (cadmium, lead, arsenic and total mercury) were not present. According to the Corporation Vision Bio-test results, the
nutritional value of LC (in 100 g): energy 4 Kcal, moisture 0.6 g, protein minimum, lipid minimum, carbohydrate 1.1 g, ash 0.2 g, water soluble dietary fiber 0.6 g, insoluble dietary fiber 97.5 g and the dietary fiber total amount 98.1 g.

2.3. Animals and experimental designs

All animals were treated in accordance with the guidelines for animal experimentation of our institute (Approve No. H270313) [150]. Animals were housed in a temperature of 23 ± 2°C and humidity of 55 ± 15% and were submitted to a light/dark cycle, allowed free access to standard laboratory chow and tap water. C57BL/6J mice were bred in my laboratory. NASH-HCC was induced in male mice by a single subcutaneous injection of 200 µg STZ (Sigma, MO, USA) at 2 days after birth and after then they were being started feeding with HFD32 ad libitum at the age of 4 weeks, and continued up to 16 weeks age of mice. Mice were randomly selected into three groups (n=6/group): group 1 (Normal) were normal mice subjected to the normal diet; group 2 (NASH) were STZ injected mice subjected to the HFD32 treated with 1% methyl cellulose (MC) (the vehicle of LC); group 3 (LC) were STZ injected mice subjected to HFD32 treated with LC suspension at (5 mg/mouse/day, suspended in 1% MC). LC treatment was started at the age of 6 weeks and administered via oral gavage continued up to 16 weeks of age along with HFD. All mice were sacrificed at the age of 16 weeks, and serum was
separated from blood. Liver tissue was isolated for histological, biochemical, and molecular biological analysis [11].

2.4. **Biochemical analysis**

Fasting blood glucose level was measured using the G-checker (Sanko Junyaku, Tokyo, Japan). Serum ALT, AST, ALP, TG and TC were measured by FUJI DRI-CHEM 7000 (Fujifilm, Tokyo, Japan) as previously described [150].

2.5. **Histological examination**

Formalin-fixed liver sections (4 μm) were stained with H&E. Morphological analysis was done by computerized image analysis system on ten microscopic fields per section examined in a 20-fold magnification (CIA-102; Olympus, Tokyo, Japan), with the observer blind to the study group [150]. Here, macrovesicular steatosis, microvesicular steatosis and hepatocellular hypertrophy were separately scored and the severity was graded, based on the percentage of the total area affected, into the following categories: 0 (<5%), 1 (5-33%), 2 (34-66%) and 3 (>66%). Inflammation was evaluated by counting the number of inflammatory foci per field, a focus has defined a cluster of ≥5 inflammatory cells. Different fields were counted and scored as per field: 0 (<0.5), 1 (0.5-1.0), 2 (1.0-2.0) and 3 (>2). By adding the scores of the above four parameters the NAFLD activity score was calculated, which
result in a total clinical score ranging from 0 (healthy) to 12 (maximal severity of NASH) [14]. NAFLD score ≥ 5 was identified as definite NASH.

2.6. Determination of liver fibrosis content

Formalin-fixed, paraffin-embedded, liver sections (4 µm) were stained with MT [155]. MT staining was performed following the manufacturer’s instructions (Accustain HT15, Sigma-Aldrich, St. Louis, MO).

2.7. Western blot analysis

The liver tissue protein lysate was prepared using a method similar to that described previously [193]. The total protein concentration in the samples was measured by the BCA method. For the determination of protein levels, equal amounts of protein extracts (50 µg) were separated by 7.5–15% SDS polyacrylamide gel electrophoresis (Bio-Rad, CA, USA) and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk or BSA in Tris buffered saline Tween (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20). Primary antibodies against SIRT1, Heme-oxygenase-1 (HO-1), p47phox, nuclear factor-erythroid 2-related factor (Nrf)-2, glucose transporter type (GLUT) 4, phosphoenolpyruvate carboxykinase (PEPCK)-C, PPARα, PPARγ, ADRP, adiponectin receptor (AdipoR)1, IL-10, IL-1β, IL-6, tissue inhibitor of metalloproteinases (Timp) 4, matrix metalloproteinase (MMP)-9, p53, p-p53, and glypican-3 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Phospho-AMPKα, AMPKα, PGC1α, JNK, p-JNK, ERK1/2, p-
ERK1/2, β-actin and GAPDH antibodies were obtained from Cell Signaling Technology Inc., Beverly, MA, USA. Collagen IV and prothrombin were obtained from Abcam, Cambridge, UK. The levels of AMPKα (for p-AMPKα), JNK (for p-JNK), ERK1/2 (for p-ERK1/2), p53 (for p-p53) and GAPDH or β-actin were estimated in every sample to check for equal loading of samples.

2.8. Immunohistochemistry

Formalin-fixed, paraffin-embedded liver tissue sections were used for immunohistochemical staining. After deparaffinization and hydration, the slides were washed in Tris-buffered saline (TBS: 10 mM/L Tris HCl, 0.85% NaCl, pH 7.2). Endogenous peroxidase activity was quenched by incubating the slides in methanol and 0.3% H₂O₂ in methanol. After overnight incubation with the primary antibody, that is, mouse monoclonal anti-cluster of differentiation (CD)36 antibody (diluted 1:100) (sc-59103; Santa Cruz Biotechnology Inc. CA, USA) at 4°C, the slides were washed in TBS and (HRP) -conjugated goat anti-rabbit secondary antibody was then added and the slides were further incubated at room temperature for 1 h. The slides were washed in TBS and incubated with diaminobenzidine tetrahydrochloride as the substrate, and counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to verify the antibody specificity. CD36 positive hepatocytes were counted in 100 fields/ group under 20 fold magnification and expressed as cells/field [63].
2.9. **Statistical analysis**

Data are shown as means ± SEM and were analyzed using ANOVA followed by Tukey’s method or Kruskal-Wallis test followed by Dunn’s multiple comparison when appropriate. A value of $p<0.05$ was considered statistically significant. GraphPad Prism 5 software (San Diego, CA, USA) was used.

3. **Results**

3.1. **Effect of LC on clinicopathology and biochemical parameters in NASH-HCC mice**

There was no huge difference in energy consumption and body weight among the three groups during the experimental period (Table 1). The ratio of liver weight and body weight (g/Kg) was significantly increased in NASH group compared to normal ($p<0.01$), but in LC treated group this increased proportion were significantly lesser than the NASH group ($p<0.05$) (Fig. 1B). Fasting blood glucose level and serum TG, TC, ALT, AST and ALP levels were also significantly elevated in NASH group compared to the normal group. The serum ALT and AST levels, indicators of liver damage, were significantly prevented in LC treated group ($p<0.05$), and other lipid profile markers were also noticeably lesser in LC group when compared to NASH group (Table 1). But the administration of LC did not show any effect on the increased blood glucose level (Table 1). Macroscopically, the liver of the NASH showed the abnormal shape and swelling with tumor protruding.
whereas the liver of LC group showed moderate fatty liver with a smooth surface (Fig. 1A). H&E staining showed extreme steatosis, enlarged hepatocytes, and scattered lobular, inflammatory cells with an increased NAFLD activity score in NASH mice. However, these changes were lesser in LC group than the NASH group (Fig. 1C-D).

**Table 1: Changes in biochemical parameters after treatment with Le Carbone in NASH-HCC mice**

<table>
<thead>
<tr>
<th></th>
<th>Normal group (n=6)</th>
<th>NASH group (n=6)</th>
<th>LC (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake /day/mouse (g)</td>
<td>3.133 ± 0.151</td>
<td>2.575 ± 0.053</td>
<td>2.505 ± 0.154</td>
</tr>
<tr>
<td>Energy consumption/day/mouse (Kcal)</td>
<td>12.66 ± 0.611</td>
<td>13.1 ± 0.27</td>
<td>12.72 ± 0.783</td>
</tr>
<tr>
<td>Body weight (BW) (g)</td>
<td>24.23 ± 0.75</td>
<td>22.70 ± 4.66</td>
<td>22.33 ± 1.62</td>
</tr>
<tr>
<td>Liver weight (LW) /BW (g/Kg)</td>
<td>53.48 ± 2.61</td>
<td>86.85 ± 14.32**</td>
<td>63.33 ± 11.29#</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>110.75 ± 13.67</td>
<td>469.60 ± 65.00***</td>
<td>490.50 ± 14.18***</td>
</tr>
<tr>
<td>Serum TG (mg/dL)</td>
<td>47.33 ± 13.8</td>
<td>59.00 ± 18.33</td>
<td>30.50 ± 13.53</td>
</tr>
<tr>
<td>Serum TC (mg/dL)</td>
<td>90.00 ± 7.81</td>
<td>202.00 ± 29.02***</td>
<td>148.50 ± 19.94#</td>
</tr>
<tr>
<td>Serum ALT (IU/L)</td>
<td>62.00 ± 3.46</td>
<td>367.00 ± 173.53*</td>
<td>65.75 ± 27.29#</td>
</tr>
<tr>
<td>Serum AST (IU/L)</td>
<td>145.67 ± 46.29</td>
<td>799.25 ± 353.26*</td>
<td>186.00 ± 66.39#</td>
</tr>
<tr>
<td>Serum ALP (IU/L)</td>
<td>358.33 ± 35.84</td>
<td>646.25 ± 215.13</td>
<td>334.25 ± 89.20#</td>
</tr>
</tbody>
</table>

Normal, age matched normal mice; NASH, untreated NASH-HCC mice; LC, NASH-HCC mice treated with Le Carbone suspension at 5 mg/mouse/day. TG: triglyceride; TC: total cholesterol, ALT: alanine aminotransferase, ALP:
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alkaline phosphatase, AST: aspartate aminotransferase. Values are expressed as means ± SEM. \*_{p}<0.05, \**_{p}<0.01, \***_{p}<0.001 vs normal, \#_{p}< 0.05 vs NASH.

**Fig. 1.** Le Carbone attenuates the clinicopathology in NASH-HCC mice. (A), Representative macroscopic appearance of livers (circles: liver tumors). (B), Histogram for the ratio of liver weight (LW) and body weight (BW) in gm/kg.
(C), H&E staining (yellow arrow: macrovesicular steatosis, black arrow: microvesicular steatosis, red arrow: hypertrophy, circles: inflammatory cells).

(D), Histogram of non-alcoholic fatty liver disease (NAFLD) activity score.

(E), Fibrosis deposition by Masson trichrome staining (blue area).

(F), percentage of fibrosis in each group. Data are mean ± SE, (n= 6/group).

Normal, age matched normal mice; NASH, untreated NASH·HCC mice; LC, NASH·HCC mice treated with Le Carbone suspension at 5 mg/mouse/day.

Statistical analysis was carried out using One way ANOVA followed by Tukey’s test where \( **p<0.01 \) vs Normal, and \( #p<0.05 \) vs NASH, except \( *p<0.05 \) vs Normal analyzed by Kruskal-Wallis test.

### 3.2. Effect of LC on hepatic fibrosis in NASH·HCC mice

To examine the effect of LC in hepatic fibrosis, I performed MT staining of hepatic tissues of three groups. MT staining demonstrated pericellular fibrosis around central veins, and its percentage of positive area was [median (interquartile range)]: in normal group [0.03 (0.023-0.0456)]; in NASH group [0.95 (0.6131-1.23)]; whilst in LC group [0.135 (0.065-0.228)] (p=0.0273), (Fig. 1E-F).

### 3.3. LC administration stimulated hepatic p-AMPK\(\alpha\) and SIRT1 expression in NASH·HCC mice

Since AMPK and SIRT1 play an important role in liver lipid metabolism I investigated the protein expression levels in NASH livers using Western blot. The protein expression of p-AMPK\(\alpha\) (\(p<0.01\)) and SIRT1 (\(p<0.05\)) were significantly reduced in the NASH group compared to that of normal group.

The phosphorylation of AMPK\(\alpha\) was increased in the liver of LC treated
group by 1.5-fold, compared to the NASH group but did not reach a significant level (Fig. 2A). The hepatic protein expression of SIRT1 was significantly increased in LC group by 1.7-fold when compared to NASH group (p<0.01) (Fig. 2B).

Fig. 2. Effect of Le Carbone on hepatic AMPKα, SIRT1, GLUT4, PGC1α and PEPCK-C expression in NASH-HCC mice.
Representative Western blots show specific bands for hepatic (A), p-AMPKα (for AMPKα); (B), SIRT1 (for β-actin); (C), GLUT4; (D), PGC1α; and (E), PEPCK-C and the representative histograms show the band densities with relative GAPDH. Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; LC, NASH-HCC mice treated with Le Carbone suspension at 5 mg/mouse/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s test where, *p<0.05, **p<0.01 vs Normal, and ##p<0.01 vs NASH.

3.4. Effect of LC on hepatic GLUT4, PGC1α, and PEPCK-C expression in NASH-HCC mice

The upregulation of AMPK and SIRT1, reduce the hepatic lipid accumulation [183], [194]. To check this I detected the downstream proteins of AMPK using Western blot, including GLUT4, PGC1α and PEPCK-C, which are all associated with lipid metabolism. The hepatic protein expression of GLUT4 and PEPCK-C were trended to lesser in the NASH group by 0.84-fold and 0.612-fold respectively, while, PGC1α (p<0.01) expression was significantly lesser by 0.42-fold when compared to the normal mice. But, in LC group, hepatic expression of GLUT4 were trended to increase by 1.1-fold compared to NASH mice (Fig. 2C). The protein levels of PGC1α and PEPCK-C were about 1.6-fold higher in LC treated liver than the NASH liver (Fig. 2D-E).

3.5. Effect of LC on hepatic expression of PPARα, PPARγ, ADRP, and AdipoR1 protein levels in NASH-HCC mice
I examined the effect of LC on the central regulator of lipid metabolism, PPARα, and steatosis-related protein expression such as PPARγ, ADRP and AdipoR1 in the liver of NASH mice. I observed that the hepatic expression of PPARα was modestly lesser by 0.72-fold in NASH mice than the normal, whereas, in the mouse of LC treated group, the reduced PPARα level was significantly increased by 2.1-fold than the NASH mice ($p<0.01$).

Interestingly, I found that the hepatic expression of PPARα in LC group was also significantly higher than the normal mice by 1.4-fold ($p<0.05$) (Fig. 3A). The expression of PPARγ and ADRP ($p<0.01$) protein were increased in the NASH liver by 1.9-fold and 3.2-fold respectively than the normal mice. In LC treated mice, the increased levels of PPARγ was significantly prevented by ($p<0.01$) and the level of ADRP was 0.77-fold lesser but did not reach a significant level when compared to the NASH mice (Fig. 3B–C). On the other hand, the expression of AdipoR1 was modestly decreased in the NASH group by 0.72-fold than the normal liver, but in the mice of LC group it was slightly higher (by 1.1-fold) than the NASH mice (Fig. 3D).
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Fig. 3. Le Carbone attenuates hepatic lipogenesis in NASH-HCC mice.

Western blots show specific bands for hepatic (A), PPARα (for β-actin); (B), PPARγ; (C), ADRP; and (D), AdipoR1 and the representative histograms show the band densities with relative to GAPDH. Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; LC, NASH-HCC mice treated with Le Carbone suspension at 5 mg/mouse/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s test where, *p<0.05, **p<0.01 vs Normal, ###p<0.01 vs NASH.

3.6. Effect of LC on hepatic oxidative and anti-oxidative marker expression in NASH-HCC mice

Since, oxidative stress plays important role in progression of NASH and AMPK-SIRT1 signaling can repress ROS production [195], I checked some protein expression levels by Western blot including anti-oxidative marker proteins HO-1, Nrf2 and NAD(P)H oxidative subunit p47phox in all groups. The hepatic protein expression of HO-1 (p<0.05) and Nrf2 were about 0.70-
fold lower and p47phox was 1.9-fold higher \((p<0.01)\) in NASH mice compared to normal mice. But the protein expression level of HO-1 and Nrf2 were significantly higher in LC group by 1.4-fold and 1.85-fold respectively than the NASH group \((p<0.05)\) (Fig. 4A & C). Interestingly, the increased p47phox level was significantly prevented in LC group \((p<0.01)\) than the NASH liver (Fig. 4B).
3.7. Effect of LC on the protein expression levels of p-JNK and p-ERK1/2 in NASH-HCC liver

The phosphorylation of JNK, and ERK1/2 were markedly elevated in the liver of NASH group by 1.5-fold, and 1.4-fold \(p<0.01, p<0.05\) respectively than the
normal group. While both of these protein expressions were markedly lesser in LC group than that of the NASH group (p<0.05, p<0.001) (Fig. 4D-E).
**Fig. 5. Effect of LC on the expression of inflammatory and fibrogenic proteins in NASH-HCC liver**

Western blots show specific bands for hepatic (A), IL-10; (B), IL-1β; (C), IL-6; (D), Timp4; (E), collagen IV; and (F), MMP-9 and the representative histograms show the band densities with relative GAPDH. Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; LC, NASH-HCC mice treated with Le Carbone suspension at 5 mg/mouse/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s test where, *p<0.05 vs Normal, #p<0.05 vs NASH.

3.8. **Effect of LC on the expression of inflammatory and fibrogenic proteins in NASH-HCC liver**

I next investigated the protein expression of IL-10, IL-1β, IL-6, Timp4, collagen IV and MMP-9 in NASH liver. The hepatic expression of IL-10 was significantly lower where Timp4 was significantly and IL-1β, IL-6, collagen IV, and MMP-9 were trended to increase in NASH group than the normal group. Interestingly, the expression of IL-10, IL-6 and collagen IV were trended to reverse and the protein expression of IL-1β, Timp4 and MMP-9 were significantly reduced in LC treated group (Fig. 5A·F). Along with these data immunohistochemistry data showed that the CD36 positive cells were significantly lower in the hepatic tissue of NASH group than the normal group (p<0.01), but in LC treated group CD36 positive cells were trended to higher than in the NASH hepatic tissue (Fig. 6A&B).
Fig. 6. Effect of LC on the expression of CD36 positive cells in NASH-HCC liver

(A-B), Immunohistochemical staining of CD36 positive cells and their quantitative data. Data are mean ± SE, (n= 6/group). Normal, age matched normal mice; NASH, untreated NASH-HCC mice; LC, NASH-HCC mice treated with Le Carbone suspension at 5 mg/mouse/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s test where, **p<0.01 vs Normal.
3.9. Effect of LC on p-p53, p53, glypican-3 and prothrombin in NASH-HCC liver

The hepatic protein expression of p53, glypican-3 and prothrombin were elevated in NASH group (Fig. 7B-D) but the phosphorylation of p53 was trended to decrease in NASH liver (Fig. 7A) when compared to that of normal liver. The protein expression of p53, glypican-3 and prothrombin were prevented in LC treated group, whereas the phosphorylation of p53 was trended to increase in this group when compared to that of NASH group (Fig. 7A-D).
**Fig. 7. Effect of LC on p-p53, p53, glypican-3 and prothrombin in NASH-HCC liver**

Western blots show specific bands for hepatic (A), p-p53 (for p53); (B), p53; (C), Glypican-3; and (D), prothrombin (for β-actin); (E), and the representative histograms show the band densities with relative GAPDH. Each bar represents mean ± SE. Normal, age matched normal mice; NASH, untreated NASH-HCC mice; LC, NASH-HCC mice treated with Le Carbone suspension at 5 mg/mouse/day. Statistical analysis was carried out using One way ANOVA followed by Tukey’s test where, *p*<0.05, **p*<0.01 vs Normal, #p<0.05 vs NASH.

**4. Discussion**

In the present study, I have demonstrated that LC supplement plays an important role to prevent or protect the liver damage by attenuating steatohepatitis in NASH-HCC mice. Accordingly, in the liver of NASH mice, LC suspension stimulated the AMPK-SIRT1 signaling system, activated PPARα signaling, suppressed PPARγ expression, increased the rate of fatty acid oxidation, reduced lipid synthesis, and attenuated liver fat deposition. Furthermore, LC treatment reduced the oxidative stress, inflammation, fibrosis, and the progression of NASH to HCC.

To gain insight into the pathogenesis and evaluate the treatment options, mouse models of NASH-HCC are of utmost importance. There is a high phenotypical variety in the available mouse models, however models that truly display the full spectrum of histopathological and metabolic features
associated with human NASH are rare. Since NASH is considered as a hepatic manifestation of the metabolic disorder and exceedingly connected with diabetes, it is also suspected that there is a relationship amongst diabetes and HCC with the progression of NASH [196]. In the present study, I considered a novel NASH-HCC mouse model, which was developed by injecting a low-dose STZ and feeding a HFD in mice, meet the clinicopathological process from fatty liver, NASH, fibrosis to HCC under diabetic background [11]. Some reports suggested that charcoal has the ability to detoxify the fatty liver in obese and alcoholic subjects and can also lower the concentration of total lipids, cholesterol and triglycerides in the blood serum, liver, heart and brain [197], [64]. In this study, I investigated the possible hepatoprotective mechanism of LC, a charcoal supplement in this NASH-HCC mouse model.

It is well established that AMPK is the master regulator of energy homeostasis, plays a concerning role in diabetes, metabolic syndrome, cardiovascular diseases and cancers [198], [199]. Reduced AMPK activity contributes to lipogenesis, thereby influencing steatosis and possibly lipotoxicity, as well as hepatocellular injury and prolongation of liver inflammation in steatohepatitis, thoroughly in the NASH-HCC mouse model. AMPK and SIRT1 are evolutionary conserved partners, which have similar functions in metabolism and cellular survival [200]. As energy metabolism is critical in cancer development, AMPK/SIRT1 might be a promising target for
HCC treatment. It is also reported that, in clinical samples, the phosphorylation of AMPK is lower in HCC tissues than the normal tissues [201]. Along with these data, in my experiment, phosphorylation of AMPK and the expression of SIRT1 were significantly lower in NASH liver than the normal liver. LC treatment enhanced the p-AMPK and SIRT1 expressions in LC group (Fig. 2A–B). Emerging evidence indicating that activation of the AMPK pathway reduces the intracellular ROS to prevent cellular oxidative stress damage triggered by different insult: hyperglycemia, fatty acids [195], [202]. ROS also downregulate SIRT1 [203]. Many target proteins of AMPK are so called longevity factors such as, SIRT1, PGC1α, HO-1, and FOXOs, which not only increase the stress resistance but also inhibit inflammatory response [204]. The nutrient sensor SIRT1, also exerts the critical control of gluconeogenic gene expression via deacetylation of PGC-1α [205]. It is shown that the activation of PGC-1α in liver diminished triglyceride production and secretion [206] and the rates of fatty acid oxidation is also diminished in hepatocytes isolated from PGC-1α deficient mice [207]. Similarly, in my study, I found that the protein expression of PGC-1α was significantly diminished in NASH liver, but the LC treatment enhanced this expression (Fig. 2D). In addition, the expression GLUT4 and PEPCK-C were trended to reduce in this NASH group where trended to restore in LC treated group and play a role in glucose utilization (Fig. 2C&E).
Moreover repression of AMPK induces the lipid accumulation in the hepatocytes, decreases the ability of mitochondria to oxidize free fatty acids, and subsequently increases the production of ROS ultimately results in the progression of NAFLD to NASH [208]. Here, I found that the LC treatment markedly enhanced the expression of PPARα in NASH liver, which is a powerful fatty acid oxidation inducer involved in lipid breakdown (Fig. 3A). Activation of PPARγ is associated with hepatic lipid accumulation in both alcoholic and nonalcoholic fatty liver [209], [210]. There are some reports that heterozygous PPARγ-deficient mice are protected from HFD or aging induced adipocyte hypertrophy, obesity and insulin resistance [211], [212].

Interestingly, my present study showed that the hepatic protein expression of PPARγ and ADRP were elevated in NASH mice, but significantly reduced in the liver of LC treated mice (Fig. 3B-C). The expression of AdipoR1 was reduced in NASH liver, but LC treatment did not show any effective role to restore this expression (Fig. 3D). These data, might suggest that LC protects the liver from steatosis other than non-adiponectin –mediated mechanisms. These findings were corroborated by improved liver histology through reduced macro-and micro-steatosis, and serum ALT, AST levels in LC treated group (Fig. 1C, Table 1).

A large body of evidence has proved an undeniable contribution of oxidative stress and proinflammatory cytokines to the advancement of steatosis to steatohepatitis. In my study, I found the expression of NAD(P)H oxidase
subunit p47phox was significantly lesser in LC treated mice (Fig. 4B). Redox systems, including antioxidants and phase II detoxifying enzymes provide protection against ROS actuated tissue damage. Nrf2 is a vital cytoprotective transcriptional variable that induces the expression of several antioxidants and phase II detoxifying enzymes [156] and acts as a defense system in the development of NASH [174]. Actually, besides Nrf2’s part in directing cellular antioxidant guard, it likewise has anti-inflammatory capacities [156]. Moreover, HO-1 acts as an anti-oxidant marker and can also inhibit the inflammatory response; some study reported that the expression of HO-1 is upregulated in NASH [213]. In contrast, it is reported that the overexpression of HO-1 suppresses HCC progression by inhibiting the proliferation and metastasis of HepG2 [214]. Here, I found that the markedly higher expression of HO-1 and Nrf2 in the liver of the LC treated mice than the liver of NASH-HCC mice (Fig. 4A, C). Oxidative stress and JNK activation progress, ERK1/2 activation become evident and enhance inflammatory cytokine accumulation [215]. It is reported that the JNK, ERK also act as oncogenic mediators in HCC [216]. Phosphorylation of JNK, ERK were enhanced by HFD feeding in HCC progenitor cells derived tumors [216]. Consistent with these reports, in my study, I found the tumors protrusion in NASH liver (Fig. 1A) and the expression of p-JNK, and p-ERK1/2 were also elevated in NASH groups (Fig. 4D-E). Where the expressions of these
increased protein levels were markedly lower in LC treated group and no tumor protrusion was found.

Inflammation seems to play a leading role in NASH progression as recruitment of inflammatory macrophages occurs to create inflammatory foci and to phagocyte lipid droplets [217]. Phagocytosis of macrophage decreased later as seen in human NASH [218], indicating the altered function of macrophages at a later phase of inflammation. Macrophages residing by activated fibroblast and both participate in fibrotic foci in liver. Increased hepatic IL-6 is suggested to contribute to accelerate macrophage fibroblast interaction within Disse’s space in the liver [219]. Furthermore, in a pilot study, increased levels of plasma IL-6 were found in NASH patients and it is also reported that circulating IL-6 is strongly correlated with adverse prognosis in HCC [220]. On macrophages CD36 involves in phagocytosis. Studies showed that deletion of CD36 in mice increased MCP-1 in hepatocytes, promotes macrophage migration to liver and aggravates hepatic inflammatory response and fibrosis [221]. Where, IL-10 is the protective cytokine against diet-induced liver inflammation. In my study, I observed that liver inflammation and fibrosis were substantially increased in NASH group. Here, I found that administration of LC in NASH-HCC mice displayed attenuation of inflammation and fibrosis in liver by H&E and MT staining (Fig. 1C-F), and enhanced the anti-inflammatory cytokine IL-10 (Fig. 5A) and CD36 positive cells in the hepatic tissue (Fig. 6A-B) and suppressed the
hepatic protein expression of IL-1β, IL-6, Timp4, collagen IV and MMP-9 (Fig. 5B-F).

Oxidative stress and inflammatory pathways lead to the transformation of a normal cell to a tumor cell, its survival, and proliferation. The p53 tumor suppressor is a crucial regulator in response to various stress signals, such as DNA damage, hypoxia and abnormal oncogenic events. Phosphorylation of p53 is a key mechanism responsible for the activation of its tumor suppressor functions in response to DNA damage [222], and p-p53 could trigger the AMPK-dependent cell cycle arrest [223]. The tumor protruding may be malignant or progress to HCC, and, as it has been reported that the hepatic expression of glypican-3 [177] and DCP or prothrombin [178] are significantly increased in most HCCs compared with benign liver lesions and normal liver, they act as a useful prognostic tumor marker for HCC. In my study, I observed that p53, glypican-3 and prothrombin, markers for HCC were higher, but the phosphorylation of tumor suppressor p53 lower in the NASH group compared to the normal group (Fig. 7A-D). But, all of these expressions were altered in the LC treated group compared to NASH group.

In summary, my present study demonstrates that the preventive action of LC against NASH-HCC in mice is mediated, at least in part, through stimulating an important hepatic signaling system, the AMPK-SIRT1 axis and consequently by reducing lipogenesis, oxidative stress and
inflammation in NASH liver. Furthermore, by inhibiting the progression of NASH to HCC, LC might be useful in the management of NASH and may constitute a powerful approach for the novel NASH-HCC model.
CHAPTER FOUR

Comparison between diabetes and NASH
1. Introduction

NAFLD is a global health problem, affecting 6-45% of the general population, rising up to 70% with type 2 DM and 90% in morbidly obese patients [224]. NASH is part of the spectrum of NAFLD that leads to progressive liver disease, which can progress to cirrhosis and its associated complications, including hepatic failure and HCC. In NASH patients, advanced fibrosis is the major predictor of morbidity and liver-related mortality. High incidence of perisinusoidal hepatic fibrosis is also reported in experimental type 1 DM, while in humans perisinusoidal fibrosis often parallels with diabetic microangiopathy [33]. Recent studies suggest that NAFLD may be more common in type 1 DM and may serve as an independent risk marker [27], although the attention given to NAFLD in type 2 DM [225]. It is also clearly established that diabetes acts as an independent risk factor for HCC. Since NASH is considered as a hepatic issue of metabolic syndrome and highly associated with diabetes [226]. NASH and DM frequently coexist as they share the pathogenic abnormalities within the liver, including hepatocyte apoptosis, ER stress, lipotoxic mediators, oxidative stress and inflammations are key contributors to hepatocellular injury. However, there is no direct evidence that sequentially depicts the liver pathogenesis from type 1 diabetes to NASH. To investigate the causal involvement of diabetes in NASH, I considered a novel progressive NASH model in mice, which established under...
diabetic condition [227]. In this study, I have shown the pathogenic correlation between the experimental type 1 diabetic rat liver and the NASH mouse liver.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were of analytical grade and purchased from Sigma (Tokyo, Japan).

2.2. Animals and Experimental design

All animals were treated in accordance with the guidelines for animal experimentation of our institute [91]. Male Sprague-Dawley rats (weight 250-300 g) were obtained from Charles River Japan Inc. (Kanagawa, Japan). C57BL/6J mice were bred in my laboratory. Animals were housed in a temperature of 23 ± 2 °C and humidity of 55 ± 15%, and were submitted to a 12 hour light/dark cycle, and allowed free access to standard laboratory chow and tap water. Rats were allowed to fast for 4 hours and then induced diabetes by a single intraperitoneal (i.p.) injection of freshly prepared solution of STZ (Sigma-Aldrich, Inc. Saint Louis, MO, USA) at a dose of 55 mg/Kg, diluted in citrate buffer 20 mM (pH 4.5). Forty eight hours later, blood glucose was measured by tail-vein sampling using Medi-safe chips (Terumo Inc., Tokyo, Japan). Diabetes was defined as a morning blood glucose reading of ≥ 300 mg/dL. Twelve rats were randomly divided into two
groups (n = 6/group): nondiabetic normal control group (Normal), and diabetic rats (DM). NASH was induced in male mice by a single subcutaneous injection of 200 µg STZ (Sigma, MO, USA) at 2 days after birth. They were being started feeding with HFD32 (CELA Japan) ad libitum at the age of 4 weeks, and continued up to 14 weeks age of mice. Mice were randomly selected into two groups (n=6/group): the normal group (Normal) were normal mice subjected to the normal diet; and the NASH group (NASH) were STZ injected mice subjected to the HFD32. All rats and mice were sacrificed at 11 and 14 weeks, respectively after the induction of STZ for analysis of liver tissues.

2.3. Biochemical analysis

Fasting blood glucose was measured using G-checker (Sanko Junyaku, Tokyo, Japan). Serum ALT, AST, ALP, TG and TC were measured by FUJI DRI-CHEM 7000 (Fujifilm, Tokyo, Japan) as previously described [150].

2.4. Histological examination

Liver sections (4 µm) of different groups of animals were immediately fixed in 10% formaldehyde solution, embedded in paraffin, cut into several transversal sections and mounted on glass slides. Then the liver tissue sections were deparaffinized and stained with hematoxylin and eosin (H&E). Morphological analysis was done by computerized image analysis system on ten microscopic fields per section examined in a 40-fold magnification (AmScope, USA), with the observer blind to the study group [150].
2.5. Western blot analysis

The frozen liver tissues were weighed and homogenized in an ice-cold buffer (50 mM Tris·HCl, pH 7.4, 200 mM NaCl, 20 mM NaF, 1 mM Na₃VO₄, 1 mM 2-mercaptoethanol, 0.01 mg/mL leupeptin, 0.01 mg/mL aprotinin).

Homogenates were then centrifuged (3000 × g, 10 min, 4°C) and the supernatants were collected and stored at -80°C. The total protein concentration in samples was measured by the BCA method [150]. Samples with equal amounts of protein were separated in polyacrylamide gel, transferred and identified on nitrocellulose membrane with specific antibodies followed by HRP-labelled secondary antibodies. The antibodies applied: SREBP1c, PPARα, p-PERK, PERK, p-IRE1α, IRE1α, CHOP, p47phox, CYP2E1, IL-1β, TLR4, VEGF-B, glypican-3, and β tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-AMPKα, AMPKα, cl.caspase-12, cl.caspase-3 and GAPDH (Cell Signaling Technology Inc., Beverly, MA, USA), prothrombin (Abcam, Cambridge, UK). The levels of AMPKα (for p-AMPKα), PERK (for p-PERK), IRE1α (for p-IRE1α), and GAPDH or β-tubulin were estimated in every sample to check for equal loading of samples.

2.6. Statistical analysis

Data are shown as means ± SEM and were analyzed using unpaired t test. A value of \( p < 0.05 \) was considered statistically significant. GraphPad Prism 5 software (San Diego, CA, USA) was used.
3. **Results**

3.1. **Biochemical parameters in experimental animals**

There was no huge difference in energy consumption and body weight between the NASH mice and its respective normal mice. In case of DM rats the energy consumption was significantly increased and body weight was significantly decreased when compared to the normal rats (Table 1). The ratio of liver weight to body weight (g/Kg) was significantly increased both in DM and NASH groups when compared to their respective normal group (Table 1). Fasting blood glucose level, serum TG, TC, ALT, ALP were also markedly elevated in both groups DM and NASH group than their respective normal group (Table 1). And serum AST level was trended to increase in DM group but significantly increased in NASH group comparing to their normal group (Table 1).
Table 1: Changes of biochemical parameters in DM rats and NASH mice.

<table>
<thead>
<tr>
<th></th>
<th>SD Rats</th>
<th>C57BL/6J mice</th>
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<tbody>
<tr>
<td></td>
<td>Normal group (n=6)</td>
<td>Normal group (n=6)</td>
</tr>
<tr>
<td></td>
<td>DM group (n=6)</td>
<td>NASH group (n=6)</td>
</tr>
<tr>
<td>Food intake /day (g)</td>
<td>22.71 ± 0.53</td>
<td>36.36 ± 0.89***</td>
</tr>
<tr>
<td></td>
<td>4.0 ± 0.6</td>
<td>3.27 ± 0.055</td>
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<tr>
<td>Energy consumption/day (Kcal)</td>
<td>91.75 ± 2.14</td>
<td>146.89 ± 3.6***</td>
</tr>
<tr>
<td></td>
<td>16.16 ± 2.5</td>
<td>16.59 ± 0.28</td>
</tr>
<tr>
<td>Body weight (BW) (g)</td>
<td>539.5 ± 38.48</td>
<td>316.33 ± 15.7***</td>
</tr>
<tr>
<td></td>
<td>24.8 ± 2.7</td>
<td>20.7 ± 5.9</td>
</tr>
<tr>
<td>Liver weight (LW)/BW (g/Kg)</td>
<td>28.57 ± 1.47</td>
<td>43.23 ± 1.35***</td>
</tr>
<tr>
<td></td>
<td>40.1 ± 0.21</td>
<td>89.1 ± 1.5***</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>116.25 ± 22.1</td>
<td>761.8 ± 50.8***</td>
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<tr>
<td></td>
<td>101.3 ± 23.4</td>
<td>488.0 ± 19.9***</td>
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<tr>
<td>Serum TG (mg/dL)</td>
<td>94.75 ± 5.72</td>
<td>431.25 ± 118.92**</td>
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<tr>
<td></td>
<td>17.5 ± 1.3</td>
<td>46.3 ± 12.1**</td>
</tr>
<tr>
<td>Serum TC (mg/dL)</td>
<td>58.50 ± 3.8</td>
<td>105.5 ± 10.9***</td>
</tr>
<tr>
<td></td>
<td>81.8 ± 3.8</td>
<td>253.8 ± 18.9***</td>
</tr>
<tr>
<td>Serum ALT (IU/L)</td>
<td>39.75 ± 2.36</td>
<td>124 ± 28.82*</td>
</tr>
<tr>
<td></td>
<td>38.0 ± 9.0</td>
<td>304.3 ± 218.7*</td>
</tr>
<tr>
<td>Serum AST (IU/L)</td>
<td>141.33 ± 16.74</td>
<td>187.8 ± 99.28</td>
</tr>
<tr>
<td></td>
<td>72.4 ± 5.4</td>
<td>270.5 ± 84.4**</td>
</tr>
<tr>
<td>Serum ALP (IU/L)</td>
<td>180 ± 0.0</td>
<td>554.25 ± 351</td>
</tr>
<tr>
<td></td>
<td>322.8 ± 49.3</td>
<td>653.8 ± 141.9***</td>
</tr>
</tbody>
</table>

Normal, age matched normal rat or mice; DM, untreated type 1 diabetic rats; NASH, untreated NASH-HCC mice; TG: triglyceride; TC: total cholesterol, ALT: alanine aminotransferase, ALP: alkaline phosphatase, AST: aspartate aminotransferase, NAFLD: non-alcoholic fatty liver disease. Values are expressed as means ± SEM. Statistical analysis was carried out using unpaired two t-test where, *p <0.05, **p<0.01, ***p <0.001 vs respective normal group.

3.2. Histopathological findings

Normal histology was found in the normal rats and mice liver (Figure 1).
H&E staining showed extreme steatosis, enlarged hepatocytes, scattered lobular inflammatory cells in NASH mice. Microvesicular vacuolization, focal necrosis and inflammation in the portal area were also found in the liver of DM rat but in lesser extent than the NASH mice (Figure 1).

**Figure 1. Histopathological changes.** Histological staining with H&E in liver (A) shows light microscopic photograph of normal rat and DM rat; (B) normal mice and NASH mice.

### 3.3. AMPKα expression in the liver of experimental animals

The phosphorylation of AMPKα in the diabetic liver was trended to reduce while in NASH liver it was significantly reduced (Figure 2A-B).
3.4. Hepatic lipogenesis in experimental animals

The hepatic protein expression of lipogenic controllers including SREBP1c was significantly increased both in DM and NASH group than their respective normal group (Figure 3A & B). But expression of PPARα was
significantly reduced in DM liver while trended to reduce in NASH liver (Figure 3C &D).

**Figure 3: Hepatic lipogenesis in DM and NASH liver.** Western blots show specific band for hepatic (A) SREBP1c (for β-Tubulin) for SD rats and (B) SREBP1 in mice; (C) PPARα for SD rats and (D) PPARα for mice and the representative histograms show the band densities with relative to that GAPDH. Each bar represents mean ± SE. Normal, age matched normal rat or mouse; DM, STZ injected type 1 diabetic rats; NASH, STZ injected mice subjected to HFD. Statistical analysis was carried out using unpaired t test where, *p<0.05, **p<0.01 vs respective normal group.
3.5. Expression of UPR signaling proteins

Diabetic rats and NASH mice both have displayed a significant upregulation in the hepatocytes expression levels of p-PERK (p<0.01) compared with their respective normal liver (Figure 4A-B). The phosphorylation IRE1α (p<0.05) was also significantly increased in DM liver (p<0.001) and NASH liver (p<0.05) when compared to their respective normal group (Figure 4C-D).

**Figure 4:** Hepatic UPR signaling protein expression in DM and NASH liver. Western blots show specific band for hepatic (A), p-PERK (PERK) for SD rats;
(B) p·PERK (PERK) for mice; (C) p·IRE1α (for IRE1α) for SD rats and (D) p·IRE1α (for IRE1α) for mice and the representative histograms show the band densities. Each bar represents mean ± SE. Normal, age matched normal rat or mice; DM, STZ injected type 1 diabetic rats; NASH, STZ injected mice subjected to HFD. Statistical analysis was carried out using unpaired t test where, *p<0.05, **p<0.01, ***p<0.001 vs respective normal group.

3.6. Expression of apoptotic markers in experimental animals

Hepatic protein expression of cleaved caspase-12, cleaved caspase-3 and CHOP were significantly increased in the liver of diabetic rats (non-significant, p<0.01, p<0.001) and the NASH mice (p<0.01, p<0.01, p<0.05) when compared to their representative normal groups (Figure 5A-F).
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**Figure 5: Hepatic apoptotic protein expression in DM and NASH liver.**

Western blots show specific band for hepatic (A-B), cl.caspase-12 for SD rats and mice; (C-D) cl.caspase-3 for SD rats and mice; and (E-F) CHOP in SD rats and mice (for Lamin A) and the representative histograms show the band densities with relative to that β-Tubulin or GAPDH. Each bar represents mean ± SE. Normal, age matched normal rat or mice; DM, STZ injected type 1 diabetic rats; NASH, STZ injected mice subjected to HFD. Statistical analysis was carried out using unpaired t test where, \*p<0.05, \**p<0.01, \***p<0.001 vs respective normal group.

3.7. **Oxidative stress in experimental animals**

The hepatic expression of NAD(P)H oxidative subunits p47phox and CYP2E1 were significantly increased both in DM (p<0.05) and NASH (p<0.05, p<0.01) group when compare to their respective normal group (Figure 6A-D).
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Figure 6: Hepatic oxidative stress in DM and NASH liver. Western blots show specific band for hepatic (A·B) p47phox for SD rats and mice; (C·D) CYP2E1 for SD rats and mice and the representative histograms show the band densities with relative to that GAPDH. Each bar represents mean ± SE. Normal, age matched normal rat or mice; DM, STZ injected type 1 diabetic rats; NASH, STZ injected mice subjected to HFD. Statistical analysis was carried out using unpaired t test where, *p<0.05, **p<0.01 vs respective normal group.

3.8. Hepatic inflammation in experimental animals

Inflammation is involved in metabolism related diseases including, diabetes and NASH. Here, I found the protein expression of pro-inflammatory
cytokine IL-1β, and inflammatory cascade TLR4 were significantly increased both in DM and NASH liver when compared with their respective normal liver (Figure 7A-D).

**Figure 7: Hepatic inflammation in DM and NASH liver.** Western blots show specific band for hepatic (A-B), IL-1β for SD rats and mice and (C-D), TLR4 for SD rats and mice; and the representative histograms show the band densities with relative to that β-Tubulin or GAPDH. Each bar represents mean ± SE. Normal, age matched normal rat or mice; DM, STZ injected type 1 diabetic rats; NASH, STZ injected mice subjected to HFD.
Statistical analysis was carried out using unpaired t test where, $^* p<0.05$, $^{**} p<0.01$, $^{***} p<0.001$ vs respective normal group.

3.9. Hepatic protein expression of VEGF-B, glypican-3 and prothrombin in experimental animals

I investigated the hepatic protein expression level of VEGF-B, glypican-3, and prothrombin in all groups, I found that these expression were significantly increased both in DM and NASH groups than their respective group (Figure 8A-B, D-F), except the glypican-3, was trended to increase in DM group (Figure 8C).
**Figure 8:** Hepatic VEGF-B, glypican-3, and prothrombin expression in DM and NASH liver. Western blots show specific band for hepatic (A-B), VEGF-B for SD rats and mice; (C-D), glypican-3 for SD rats and mice, and (E-F), prothrombin for SD rats and mice and the representative histograms show the band densities with relative to that GAPDH. Each bar represents mean ± SE. Normal, age matched normal rat or mice; DM, STZ injected type 1 diabetic rats; NASH, STZ injected mice subjected to HFD. Statistical analysis was carried out using unpaired t test where, \* \(p<0.05\), \** \(p<0.01\) vs respective normal group.

4. Discussion

In the present study, I have demonstrated that the correlative effect of DM and NASH on liver. Since diabetes and NASH both act as the critical risk factor for HCC; the third common cause of cancer related death. Although, NASH is a cause of serious health issue, there is no effective treatment as well as diagnostic marker at present [228]. To
investigate the pathogenesis and finding the treatment, suitable animal model is the most important.

In this study, I found the similar pathogenic abnormalities in the liver of both DM and NASH animals. In NASH, first chemical intervention induced mild inflammation, a key pathogenesis of diabetes and metabolic disorders [229]. As β-cell replication and neogenesis are actively found early after birth [230], low-dose of STZ at this time causing of both islets islet injury and regenerative responses, leading to diabetic conditions. Continuation of dietary intervention enhances fat deposition in the liver with increased lipogenesis and fatty acid oxidation, leading to the hepatocellular injury.

Accordingly, in H&E staining, I found the severe fat deposition in NASH liver when compared to the DM liver and both livers showed the inflammation and cellular hypertrophy (Figure 1). Fat accumulation is the prerequisite for the development of NASH. AMPK is well established for the lipid metabolism and found the decreased levels of p-AMPKa both in DM and NASH liver (Figure 2A-B). The extent of lipogenesis marker expression including, SREBP1c and serum lipid profile were significantly elevated in both DM and NASH group compared to their respective normal liver (Figure 3A-B, Table 1). While the expression fatty acid regulatory gene significantly reduced in DM liver and slightly in NASH liver (Figure 3C-D). The serum ALT and AST level indicator of liver damage markedly elevated in NASH group but in DM
group AST level was trended to increase but non-significantly while ALT was increased significantly when compared to its respective normal group (Table 1). Transition of steatosis to steatohepatitis is the crucial event in NASH pathophysiology, although it is still unclear. Several studies suggested that lipotoxicity play important role in this action, which associated with ER stress or prolonged UPR stress, oxidative stress and inflammation [53]. In this study, it is found that the significant elevation of phosphorylated PERK and IRE1α in both DM and NASH liver (Figure 4). Prolonged UPR stress leads to hepatocyte apoptosis, and generation of apoptotic bodies has been recognized as a potent inflammatory and fibrogenic stimulus [231], direct link between apoptosis and NASH has been demonstrated by a number of experimental studies. Several pathways lead to lipotoxic apoptosis, here significant elevation of hepatic cleaved caspase 12, cleaved caspase 3 and ER stress induced apoptotic protein CHOP were found in both groups DM and NASH (Figure 5), while in diabetic rats the elevation of cleaved caspase 12 did not reach a significant level (Figure 5A). Oxidative stress has been considered one of the of the responsible pathway for the progression of steatosis to steatohepatitis [232]. Oxidative subunit p47phox and oxidative marker CYP2E1 were significantly increased both in DM and NASH liver (Figure 6). CYP2E1 promotes oxidative stress and inflammation, resulting in hepatocyte injury and progression to NASH [40]. Inflammation represents a crucial aspect in NASH pathogenesis. Excess of toxic lipids causes cellular
stress which triggers the hepatocyte apoptosis, the prevailing mechanism of cell death in NASH, associated with the degree of liver inflammation [41]. The protein expression pro-inflammatory cytokine IL-1β and inflammatory marker protein TLR4 were significantly elevated in the liver of DM rat and NASH when compared with their respective normal group (Figure 7).

Oxidative stress and inflammation pathways lead to the switch of a normal cell to a tumor cell, its survival and proliferation [175]. Since diabetes and NASH both are risk factor for the HCC, here I checked the protein expression of VEGF-B, glypican-3 and prothrombin, while VEGF may help to regulate hepatic tumorigenesis [176], glypican-3 [177] and prothrombin [178] are elevated in most HCC liver. The expression of VEGF-B and prothrombin were significantly elevated in both groups, DM and NASH with their respective normal group (Figure 8A-B, E-F) while, the gypican-3 was significantly elevated in NASH group but in DM it was very slightly increased than its respective normal group (Figure 8C-D). From the other studies and my data, it is confirmed that glypican-3 is a potential liver cancer therapeutic target as it is over-expressed in HCC but not expressed or expressed at a low levels in normal and DM liver tissue.

In conclusion, my data indicate that diabetes is closely linked with the NASH and its pathophysiology. Thus, type 1 diabetic liver and the STZ-induced NASH liver are showing the similar pathogenic abnormalities, this novel
NASH model is comprised under diabetic background. This an open a new resource to understand the mechanism and novel therapeutic strategies against NASH.
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SUMMARY
SUMMARY

From my study and others it has been speculated that NASH will overtake HCV/HBV as the most common associated risk factor for HCC in the next decade due to the prevalence of diabetes and NAFLD. Because an increasing number of patients are being diagnosed with NASH, a more comprehensive understanding of this disease mechanism is critical to developing new clinical strategies for early detection, treatment, and even prevention.

Hyperglycemia, hyperlipidemia, and inflammation are the main metabolic abnormalities in diabetes, which are able to stimulate generation of ROS. All of these metabolic abnormalities along with ER stress are closely associated with the building of steatosis in liver and lipotoxicity in steatohepatitis. Oxidative stress and pro-inflammatory cytokines progress the NASH to HCC in some cases. Inflammatory cascade NF-κB-HMGB1 signaling are attractively involved in the pathogenesis of NASH and its progression to HCC. Where, AMPK is well established for the metabolic activity and inhibition of the phosphorylation AMPK also associated with the carcinogenesis.

Curcumin regulates blood glucose and antioxidant. Curcumin treatment protects the liver against ER stress and apoptosis in experimental type 1 diabetes. It ameliorates liver damage and reduce the progression of NASH to HCC through modulating HMGB1-NF-κB translocation in experimental NASH-HCC under diabetic condition. Where, Le Carbone improves the metabolic abnormalities and through activating AMPK and SIRT1 in the NASH-HCC liver. Le Carbone attenuates oxidative stress and inflammation along with preventing the lipogenesis in the experimental NASH-HCC liver.

In conclusion, curcumin and Le Carbone might be useful in the management of NASH may constitute a powerful approach in treatment and prevention.
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