

Non-alcoholic Fatty Liver Disease: Pathology, Disease Models and Therapies

Zhiyu Wu *

School of Health Sciences, McMaster University, Hamilton, Canada

* Corresponding Author Email: Wuz140@mcmaster.ca

Abstract. Non-alcoholic fatty liver disease (NAFLD) is characterized by a range of conditions induced through fat accumulation in the liver. This disease impacts population all around the world. NAFLD prevalence is rising at an alarming rate over the past years. To address the alarming increase in NAFLD prevalence, researchers are attempting to develop effective therapeutics to combat NAFLD. To develop NAFLD therapeutics, it is crucial to address current knowledge in NAFLD pathogenesis. Through summarizing current knowledge in NAFLD pathogenesis, researchers can better visualize current knowledge surrounding the disease and present knowledge gaps in the field. This review aims to deeply understand the role of three key NAFLD pathogenic factors: hepatic lipotoxicity, hepatic inflammation, and insulin resistance, and proposes potential target for NAFLD treatment. Furthermore, this review systematically summarizes current disease models and NAFLD therapies. In general, this review provides an overview of the progress of NAFLD and discusses reliable and practical models of NAFLD.

Keywords: Non-alcoholic fatty liver disease, Pathology, Disease models, Therapy.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) describes a continuum of various conditions described as an increase in fat content (>5%) in the liver. NAFLD include diseases such as fatty liver, non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. According to recent meta-analysis and systematic reviews, NAFLD prevalence has been increasing at an alarming rate over the years. Currently, NAFLD prevalence world-wide is estimated to be 32.4% (95%CI 29.9-34.9) [1]. NAFLD patients are often strongly associated with co-morbidities such as diabetes, obesity, and cardiovascular disease. Due to the heavy clinical impact, NAFLD serves as a significant burden on the economy. Economic models simulating NAFLD burden on the US economy projected \$103 billion on direct medical costs alone and \$188 billion on societal costs. Despite the high prevalence, lower quality of life, and huge economic burdens, currently, NAFLD has no approved treatment [2]. Current researchers are working to better understand NAFLD pathogenesis to improve NAFLD therapeutics. This review discusses three common NAFLD pathogenic factors: hepatic lipid accumulation, inflammation, and insulin resistance (IR) and, summarizes current disease models and therapies of NAFLD.

2. NAFLD Pathogenesis

NAFLD pathogenesis is complex and involves a multitude of factors including but not limited to environmental factors, dysregulated lipid homeostasis, and inflammation. The liver is responsible for key metabolic functions and is involved in modulating different pathways of lipid metabolism. Disorder of lipid metabolism leads to NAFLD, followed by inflammation, fibrosis, and liver injury.

2.1. Hepatic Lipid Accumulation

Hepatic lipid accumulation such as having a high concentration of lipids and lipid derivatives accumulating in hepatic cells, results in lipotoxicity. Lipids such as free fatty acids (FFA), lysophosphatidyl choline (LPC) and ceramides have been extensively researched and identified as being heavily involved in NAFLD.

2.1.1 Free Fatty Acids

Free fatty acids (FFAs) can be described as unsaturated and saturated fats. Unsaturated fats are described by having double bonds. Saturated fats are those that do not have double bonds. Palmitate (PA) is the most abundantly produced saturated FFA. PA is synthesized from de novo lipid synthesis and is triggered by a consumption of an excessive amount of carbohydrates. Another source of PA synthesis is de novo lipogenesis from glucose and fructose, which are very common components in many western diets. A large amount of glucose and fructose in hepatocytes leads to subsequent commitment to de novo lipogenesis, depleting ATP in animal and human liver. The liver can increase saturated FFA uptake as a compensatory mechanism if there is an increase in FFA concentration in the blood. Past observations show that saturated FFAs are mostly produced through lipolysis during fasting, some through de novo lipogenesis and others from hydrolysis of TG [3]. Saturated FFAs are toxic to hepatocytes and therefore an excess accumulation is unfavourable. Excess saturated FFAs in the liver triggers cellular stress. Cellular stress induces many deleterious pathways such as apoptosis among hepatocytes. Studies that examined PA found that PA is associated with disrupted insulin signalling along with peroxisome proliferator-activated receptors alpha (PPAR α) upregulation, heavily contributing to NAFLD pathogenesis. PA also activates proapoptotic p-53 upregulated modulator of apoptosis (PUMA) and tumor necrosis factor related apoptosis inducing ligand (TRAIL2) through upregulating CCAAT/ enhancer-binding homologous protein (CHOP) by generating endoplasmic reticulum (ER) stress. Additionally, FFAs induce proapoptotic signalling through death receptors. Death receptors are considered as mediators of hepatic lipotoxicity. Among patients diagnosed with NAFLD, there has been observation of increased death receptors such as Fas. Liver death receptors include TNF receptor 1 (TNFR1), TRAIL ligand receptor (TRAIL-R)1, TRAILR2, and Fas. Death receptor activation in the liver can cause receptor oligomerization which forms death-inducing signalling complex. The death inducing signalling complex then activates proapoptotic caspase 8, which then subsequently activate caspases 3, 6, and 7, leading ultimately to cell death. Death receptor TRAILR2 is also a mediator of lipotoxicity in hepatocytes. PA can activate TRAILR2 which leads to subsequent caspase dependent apoptosis. Specifically, TRAILR2 leads to the cleaving of Bid by caspase 8, leading to the formation of tBid, which translocate into the mitochondria to release cytochrome c, a well known pro-apoptotic factor, again leading to apoptosis among hepatocytes [4,5]. As seen above, saturated FFAs are shown to elicit harmful effects such as chronic ER stress, activating many proapoptotic singling molecules leading to hepatocyte apoptosis, contributing to NAFLD pathogenesis.

2.1.2 Lipid Phosphatidylcholine (LPC) & Ceramides

LPC are lipids produced from phosphatidylcholine (PC) through partial hydrolysis. It is derived from diacylglycerol (DAG). Studies have shown that FFAs such as PA can induce DAG, indirectly leading to LPC formation. LPC is formed from two major sources: extracellular LPC and intracellular LPC. LPC contributes to lipotoxicity through the induction of ER stress in hepatocytes, thereby triggering apoptotic pathways. Pathways activated by LPCs include c-jun N-terminal kinases (JNK) or glycogen synthase kinase 3 (GSK3), and transcription factor CHOP, leading to upregulation of proapoptotic proteins such as PUMA [6]. Aside from LPC, PA can also induce ceramide production. When PA is converted to palmitoyl CoA after being introduced to hepatocytes, it can lead to ceramide formation by de novo ceramide synthesis pathway. The above (PA, LPC, and ceramides) causes hepatocytes to release extracellular vehicles (EVs). EVs attract pro-inflammatory cytokines into the liver leading to inflammation. Ceramides are known to induce inflammation in the liver via various other pathways: toll like receptor (TLR) 4 and NLRP3. PA promotes TLR4 producing TNF α , interleukin-6 (IL-6) and IL1 β , inducing ceramide production and increasing inflammation. NLRP3 is a multiprotein complex involved in caspase activation after being triggered by infection or cellular damage. Ceramides activate NLRP3 inflammasomes, activating caspase 1. NLRP3 activation secretes proinflammatory cytokines IL1b and IL18, leading again to inflammation in the liver [7,8].

2.2. Inflammation

Inflammatory signalling pathways often result in insulin signalling inhibition or induce hepatocyte injury which is a risk factor to NAFLD pathogenesis. TLRs are crucial in liver inflammation. TLRs are pattern recognition receptors. Pattern recognition receptors are heavily involved in innate immune responses associated with danger associated molecular patterns (DAMPs). FFAs such as PA can activate TLRs triggering proinflammatory transcription factor nuclear factor-kb (NFkb) activation. FFAs commonly trigger TLR4 dependent proinflammatory pathways. The signalling of TLR4 leads to transcription factors such as NFkb activation. Activating these transcription factors produces inflammatory cytokines. Another pathway that can cause insulin resistance (IR) is through impairing the post receptor insulin signal. This is promoted when FFAs activate TLR2/4 and JNK proinflammatory signalling pathways. Induction of IR is a major contributor to NAFLD pathogenesis. [9]. Within hepatocytes specially, PA can autocrinally activate TLR4, subsequently activating NFkb and release proinflammatory cytokines, promoting inflammation [10]. Proinflammatory cytokines is a major contributor to IR, leading to NAFLD pathogenesis. Inflammation of the liver is detrimental but can also be beneficial to disease progression. Reviews on NAFLD inflammation found that certain inflammatory cells such as IL-17 contribute to liver injury, but other cells such as IL-22 promote liver regeneration because of liver damage. Resident Kupffer cells can play both roles depending on the disease progression. Neutrophils causes liver injury when NAFLD is in its early stages by producing pro-inflammatory cells but is essential in the liver's defense mechanism against bacterial infections which is common among NAFLD patients [11]. Understanding the role of inflammation and how pathways differ during various disease stages can help better characterize NAFLD, providing novel insights on identifying novel NAFLD therapies.

2.3. Insulin Resistance

IR is a well-known contributing factor to NAFLD pathogenesis. Epidemiological analysis found that hepatic fat accumulation is linked with hepatic and skeletal IR, and that IR is commonly observed in patients diagnosed with NAFLD [12]. IR is briefly described by reduced glucose uptake in the adipose tissue (AT) and muscle. IR in AT results in an increased release of FA through a dysregulated lipolysis, perpetuating impairment in insulin signalling thought the body. Dysregulated, or unsuppressed lipolysis, increases the amount of FFAs in the blood, forcing an adaptive response from the liver to increase FFA uptake, leading to steatosis. This simultaneously promotes de novo lipogenesis due to an increased stimulation from lipogenic enzymes by sterol regulator element binding protein -1c (SREBP-1c). IR also leads to hyperinsulinemia which increases hepatic synthesis of FA, again leading to the development of steatosis contributing to NAFLD pathogenesis [13].

3. Preclinical Models to Characterize NAFLD Pathogenesis

To effectively study the pathogenesis of NAFLD, preclinical models are employed to understand the mechanisms associated with disease development. Preclinical models are essential to researching NAFLD pathogenesis due to ethical complications involving human participants. This review summarizes common disease models employed to characterize NAFLD.

3.1. In Vivo Models

Due to the complexity presented in NAFLD, researchers must find a way to characterize NAFLD pathogenesis prior to commencing human clinical trials. Large animal models such as monkeys or pigs can present complex ethical issues and can be very costly. Therefore, through decades, researchers relied on mouse model preclinical studies to investigate the various mechanisms leading to NAFLD. Over the years, mouse models are improving in translatability to humans and are indispensable for the treatment of diseases (Table 1).

3.1.1 Genetic models

There are many animal models that undergo genetic modification to help scientists further examine the theory surrounding NAFLD. *Ob/ob* mice models induce a leptin gene mutation, which causes leptin deficiency. Leptin deficiencies lead to NAFLD due to the mice phenotype being hyperphagic, obese and diabetic. The drawback of *ob/ob* mice; however, is that it will likely not develop NAFLD with gene mutations alone but will require a second intervention such as a special diet modification to promote the development of NAFLD. The *db/db* mice possess a genetic alteration targeting the leptin receptor gene. The *db/db* mice develop characteristics like *ob/ob* mice and require a second intervention such as diet modification to induce NAFLD development [14-16]. The agouti gene mice introduce a mutation to the agouti gene. This leads to the loss of melanocortin as well as an obese phenotype. This model will develop hyperphagia (due to an impairment to the hypothalamus), obesity, IR, and liver steatosis. However, for this mice model to progress to later stages of NAFLD will require an additional intervention such as dietary challenges [17]. Melanocortin 4 receptor mice (MC4R) mice modify the MC4R gene. MC4R is critical in food and weight regulation. The MC4R mice develop obesity, hyperphagia, hyperinsulinemia, and hyperglycaemia. Studies have shown that feeding MC4R KO mice a high fat diet (HFD) exhibited hepatic steatosis and impaired lipid metabolism. Similar experiments have also demonstrated that with similar conditions, in the long term, mice developed comorbidities like what is presented by a human patient diagnosed with NAFLD [18]. Finally, mice models can target SREBP-1c and induce overexpression of the protein. Overexpression of SREBP-1c in AT prompts NAFLD pathogenesis in mice through an alteration in white AT differentiation, hyperinsulinemia, hepatic inflammation, and fibrosis [19].

3.1.2 Diet induced Models

Aside from genetic models, many mice models also attempt to replicate NAFLD using different dietary challenges. Common dietary modifications used include methionine and choline deficient (MCD) diet, high fat diet (HFD), fructose rich diet (FRD), and fast-food diet (FFD). MCD diet is one of the most common diet to investigate the pathogenesis of NAFLD. The model focuses on nutrient deficiency from eliminating methionine and choline, with a focus on providing high sucrose and low-fat content. Mice that are fed the MCD diet rapidly develop hepatic steatosis. Following that, the mice develop necrosis, inflammation, and fibrosis, along with oxidative stress (OS). OS activates TLR4 signalling pathway mentioned above, producing proinflammatory cytokines, which contributes to liver injury. Although this method is like NAFLD in humans, it is lacking some prominent risk factors like IR and obesity. Contrary to obese symptoms exhibited in human NAFLD, mice fed MCD diet is shown to lose weight. In addition, different mouse strains can also produce different degrees of NAFLD severity, rendering it hard to standardize results [20]. HFD is also a very widely used diet among mouse models. Mice that are fed an HFD develop obesity, glucose intolerance, dyslipidemia, and increased SREBP1c expression. This alteration leads to a dysregulated lipogenesis, increased production of proinflammatory cytokines and increased OS. This method is very representative of human NAFLD since the diet consists of many components like a human diet that involves energy dense macronutrients. However, it does not produce severe liver injuries and requires a longer feeding time. As such, an HFD is often complemented by a genetic mice model [21]. An FRD contains high carbohydrate content and therefore is a risk factor for NAFLD development. Studies have shown that fructose is most damaging to the liver after comparing it to different mono and disaccharides. An FRD mice exhibits IR, OS, proinflammatory cytokine production, and overexpression of SREBP1c in the liver. However, it fails to produce severe liver injuries, but it closely resembles NAFLD in humans [22]. Finally, an FFD is focused on a western diet that is fast food rich. Fast food is a risk for obesity and NAFLD. The FFD can increase fibrosis, ER stress and lipoapoptosis. All of which are also events observed in human NAFLD patients. Studies have shown that after 6 weeks, in mice fed an FFD, demonstrated hepatic steatosis along with inflammation. This diet allows faster progression to later stages of NAFLD, and weight gain is also observed in mice treated with an FFD [23].

Table 1. Current animal Models to Characterize NAFLD Pathogenesis

In Vivo Models	Model	Diabetes Mellitus	Obesity	Hyperglycemia/ Insulin Resistance	Steatosis	Limitations
Genetic models	<i>Ob/ob, db/db</i>	Y	Y	Y	Y*	May require secondary intervention to induce steatosis and further disease stages. Leptin receptor mutations are less common in humans [14-16].
	<i>Agouti</i>	Y	Y	Y	Y*	Require secondary intervention to induce late stages of NAFLD [17].
	<i>MC4R</i>	Y	Y	Y	Y*	Study relied on long-term HDF to induce non-alcoholic steatohepatitis (NASH) [18].
	<i>SREBP-1c</i>	Y	N	Y	Y*	May require additional intervention to induce later stages of NAFLD. May present differently compared to clinical human NAFLD [19].
Diet Induced Models	MCD	N	N	N	Y	Obesity not present. Reduced TG and Cholesterol levels unlike human NAFLD. Focus on nutrient deficiency, as opposed to human diet (major NAFLD risk factor) [20]
	HFD	Y	Y	Y	Y	Liver disease less severe compared to MCD. Require second intervention (often genetic mice models). Do not mimic NAFLD etiology due to forced diet [21].
	FRD	Y	Y	Y	Y	Similar to HDF, requires additional intervention to stimulate late stages of NAFLD [22].
	FFD	Y	Y	Y	Y	Proinflammatory mediators was not present. Results closer to metabolic syndrome [23]

*Steatosis can be induced by the model; however, additional intervention may be required. Y=Yes, N=No

3.2. In Vitro Models

In vitro models are purposeful in the investigation of NAFLD molecular mechanisms illustrating NAFLD pathogenesis and progression. Three in vitro models are reviewed: primary cell cultures, immortalized cell cultures, and 3D cell cultures. Primary NAFLD cell cultures consist of primary human hepatocytes, Kupffer cells, and more. These cell cultures present a better resemblance to the in vivo phenotype. However, such models present an ethical issue due to the nature of obtaining human cell cultures. As a result, cells are limited in culture time, may present heterogeneity and it is very difficult to obtain healthy control samples. Alternatives such as primary rodent cell cultures are

also used however it is less representative to human NAFLD cellular mechanisms [24-26]. Immortalized cell lines, compared to primary cell cultures, has better reproducibility, is more stable, easier to obtain, and uses consistent cell types. However, mutations induced in these cells can limit study results [27,28]. Finally, 3D cell cultures are less employed as most in vitro models are 2D monolayer cell cultures. However, unlike 2D monolayer cultures, 3D cultures can mimic the physiological processes in the liver. It is mostly used to model gene regulation in NAFLD. Most often, 3D cultures are used to assist o in vivo models as well as in vitro models [29] (Table 2).

Table 2. *In Vitro* Models to Characterize NAFLD Pathogenesis

<i>In Vitro</i> Models	Description	Pros	Cons
Primary Cell Cultures	Hepatocytes or non-parenchymal cells isolated from liver tissue	Greater resemblance to in vivo phenotype. Better model to study drug effects on metabolism [32].	Limited culture time, limited availability from human samples. Heterogeneity present in obtained samples. Difficult to obtain healthy controls [33-35].
Immortalized Cell Lines	Tumor derived	Unlimited growth, stable phenotype, standardized cell cultures, easy to reproduce [36,37].	Mutations can limit results. May not be an accurate observation compared to human NAFLD conditions [38].
3D Cultures	Maintains hepatic cell types in controlled microenvironment	Representative of organ phenotype. Mimic physiological interactions and metabolic functions. [39]	Used to assist in vitro and in vivo models. Researchers continue to prefer 2D cell cultures. [39]

4. NAFLD Therapies

There are various therapies targeting NAFLD currently undergoing clinical trials. These therapies target mechanisms described previously. Promising future treatments currently in clinical trials include apoptosis signal regulating kinase 1 (ASK1) inhibitor, C-C motif chemokine receptor 2/5 (CCR2/CCR5) antagonists, Acetyl CoA carboxylase (ACC) inhibitor and farnesoid X receptor (FXR) agonists [30]. ASK initiates the p38/JNK pathway through upregulating TNF α , thereby introducing ER stress, leading to cellular apoptosis. Clinical trials targeting ASK1 inhibition demonstrated an improvement in fibrosis over a 24-week period. In addition, there is also amelioration in liver stiffness and liver fat percentage, regressing NAFLD disease progression. One clinical trial studying the safety of ASK1 inhibitor selonsertib terminated its study due to lack of results at week 48 according to the pre-specified study protocol [31,32]. The CCR2/CCR5 antagonists are designed to target inflammation. It works to have antifibrotic properties while improving insulin sensitivity. The recruitment of inflammatory cells such as macrophages through CCR2 is a major contributor to IR. CCR2 antagonist was found to improve IR. CCR5 antagonists were observed to improve NAFLD through disrupting hepatic stellate cell expansion. One clinical trial on CCR2/CCR5 antagonist showed one-year treatment improving fibrosis without worsening steatohepatitis. Two-year treatment showed consistent results [33,34]. ACC modulates the change from malonyl CoA to acetyl CoA. This step is essential to de novo lipogenesis. Through targeting ACC, researchers were able to pharmacologically inhibit de novo lipogenesis. Simultaneously, it is able to downregulate SREBP1c, a transcriptional regulator of enzymes in de novo lipogenesis. Clinical trial (GS0976) showed that patients experienced a 45% decrease in liver fat content, a decrease in liver stiffness which is a marker for fibrosis. Another study on GS0976 found improvements to hepatic steatosis after 12-week therapy

[35,36]. Lastly, FXR is a regulator of enzymes in the de novo lipogenesis pathway. An FXR agonist, obeticholic acid (OCA) was demonstrated to improve liver disease after 72 weeks of OCA administration. In the FLINT study, a phase 2 study, OCA group showed significantly more liver histology improvements than control. Another 24-week therapy (Cilofexor) demonstrated improvements in hepatic steatosis [37-40] (Table 3).

Table 3. NAFLD Therapy in Past and Current Clinical Trials

Therapy	Clinical Trials	Results
ASK 1 Inhibitor	GS-4997+ Selonsertib (NCT02466516)	Improvements in liver stiffness, fat content, improvement in fibrosis. May reduce steatohepatitis and stage 2-3 fibrosis [31].
	Selonsertib (NCT03053050)	Terminated based on lack of efficacy from results of Week 48 analysis [32].
CCR2/CCR5 Antagonist	Cenicriviroc (NCT02217475)	One-year treatment showing improvements in fibrosis without worsening steatohepatitis. Two-year treatment corroborates findings from year 1, with greater effect in advanced fibrosis [33,34].
ACC Inhibitor	GS-0976 (NCT03449446)	12-week therapy observed 45% decrease in liver fat content, a decrease in liver stiffness [35,36]
FXR Agonist (OCA)	Obeticholic Acid (NCT01265498) Cilofexor (NCT02854605)	72-week OCA administration 45% of patients showed improvements in liver histology [37,38]. 24-week therapy resulted in improved hepatic steatosis, and liver biochemistry in patients with NASH [39,40].

5. Conclusion

NAFLD prevalence is increasing at an alarming rate around the world. Research surrounding NAFLD is imperial as there is currently no approved treatment for this condition. This condition not only present alongside multiple comorbidities such as obesity and heart disease, but also significantly decrease quality of life of these patients. Three major NAFLD pathogenesis mechanisms were discussed: hepatic lipid toxicity, hepatic inflammation, and insulin resistance. This paper also discussed promising treatments targeting specific pathways discussed within these topics. Despite promising results shown by an improvement in steatosis and reduction of NAFLD related biomarkers, more research is needed to ensure the safety of these NAFLD therapies. Several methods involving the investigation of NAFLD pathogenesis was also discussed. Many of which requires a secondary intervention to induce advanced stages of NAFLD. Additionally, all discussed NAFLD models do not accurately reflect human NAFLD (ex. comorbidities, psychology etc.) which warrants further investigation into improved, yet also ethical models of NAFLD.

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