

Current NASH Pathogenesis and Diagnostic Techniques and Treatments

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Abstract. Nonalcoholic fatty liver disease (NAFLD) is defined by excessive triglyceride deposition in liver, excluding alcoholic or drug factors. NAFLD has grown into a public health concern since it is a leading cause of chronic liver disease in wealthy areas due to obesity and its associated metabolic syndrome. NAFLD has two typical types: non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NASH significantly impacts the life quality and life expectancy of chronic liver disease. Moreover, NASH is fatal since it involves the onset of multiple chronic disease and may cause cirrhosis and liver failure. Potentially more worrying, NASH does not show any obvious symptoms in early stages, therefore, a more effective and precise NASH diagnostic technique is necessary. This review introduces the current research status of NASH and summarizes the obstacles and potential solution in this research.

Keywords: Non-alcoholic steatohepatitis, Pathogenesis, Protein inhibition, Models.

1. Introduction

NAFLD is an abnormal accumulation of triglyceride in the liver that has no other known cause, such as alcohol usage. NAFLD drastically decreases the life qualities of patients, it could not only potentially trigger the onset of type 2 diabetes and atherosclerosis but also has a chance to develop into life-threatening stage. NAFLD is a general term that includes NAFL and NASH. Typically, NAFL poses far fewer risks than NASH and is unlikely to develop into NASH [1]. However, if NAFL continues to progress to NASH, the occurrence rate of serious disorders such as cirrhosis, liver cancer, and liver failure will be significantly increased. As the more lethal form of NAFLD, NASH involves cell damaging, inflammation, hepatocyte ballooning and 5-15% of patients could eventually develop to cirrhosis and hepatocellular cancer which are determined as end-stage liver diseases [1]. NASH is now becoming one of the major causes of chronic liver disease and health problem in the Western world [1]. Patients with NASH typically have no symptoms at all. Therefore, NASH has usually already progressed to end-stage when patients become aware of their liver issue. Even though liver biopsy is considered as the most precise method to diagnose NASH, there lies a chance that the location being sampled does not represent the entire liver. Technology of surrogate biomarkers may provide a reliable and non-invasive diagnose result. It is particularly significant since the incidence of cirrhosis and end-stage liver disease is expected to increase unless public health measures are implemented, given the aging population and extended duration of exposure to NASH. This emphasizes the significance of developing approaches to accelerate the invention of NASH treatment and prevention strategies. Insufficient research of validated preclinical disease models that accurately reflect the biology and consequences of human disease is the major barrier to the development of NASH therapies. In order to better understand disease etiology, identify possible therapeutic targets, and test the potential of a single medication or combination of therapies prior to human studies, the need for effective and safe therapy encourages the development of NASH both *in vitro* and *in vivo*.

2. Pathogenesis of NASH

Further researches are still required to comprehensively understand the pathogenesis of NASH. Typically, long term excess lipid accumulation cause steatosis and therefore damage liver cell and cause inflammatory lesion. Two major hypothesis -- “two-hit” and “multiple hits” are commonly

recognized [1]. Both of hypothesis acknowledge the progression from steatosis to cell damage. “Multiple hits” hypothesis suggests in detail that NASH is resulted from several conditions acting simultaneously. Genetic predisposition, abnormal lipid metabolism and protein secretion, oxidative stress, lipotoxicity, endoplasmic reticulum (ER) stress are all responsible for NASH development [1]. Generally, consumption of dietary sugar would increase the fatty acids (FA) level in human body. Lipotoxic lipids are produced as the consequence of increased fatty acids. Lipotoxic lipids are responsible for ER stress, oxidant stress (OS) and inflammasome activation which further triggers apoptosis. During apoptosis, cytokine and chemokine release. The released protein, majority are interleukins (IL), transforming growth factor (TGF) and tumor necrosis factor (TNF). By combining with the VLDL exported to extracellular region as a consequence of increasing level of FA, hepatic stellate cells are activated as shown in Figure 1. Due to the involve of macrophages, fibrosis occurs. Liver cell fibrosis will make cells more fragile. The damaged cell accumulation would cause irreversible liver lesion such as cirrhosis.

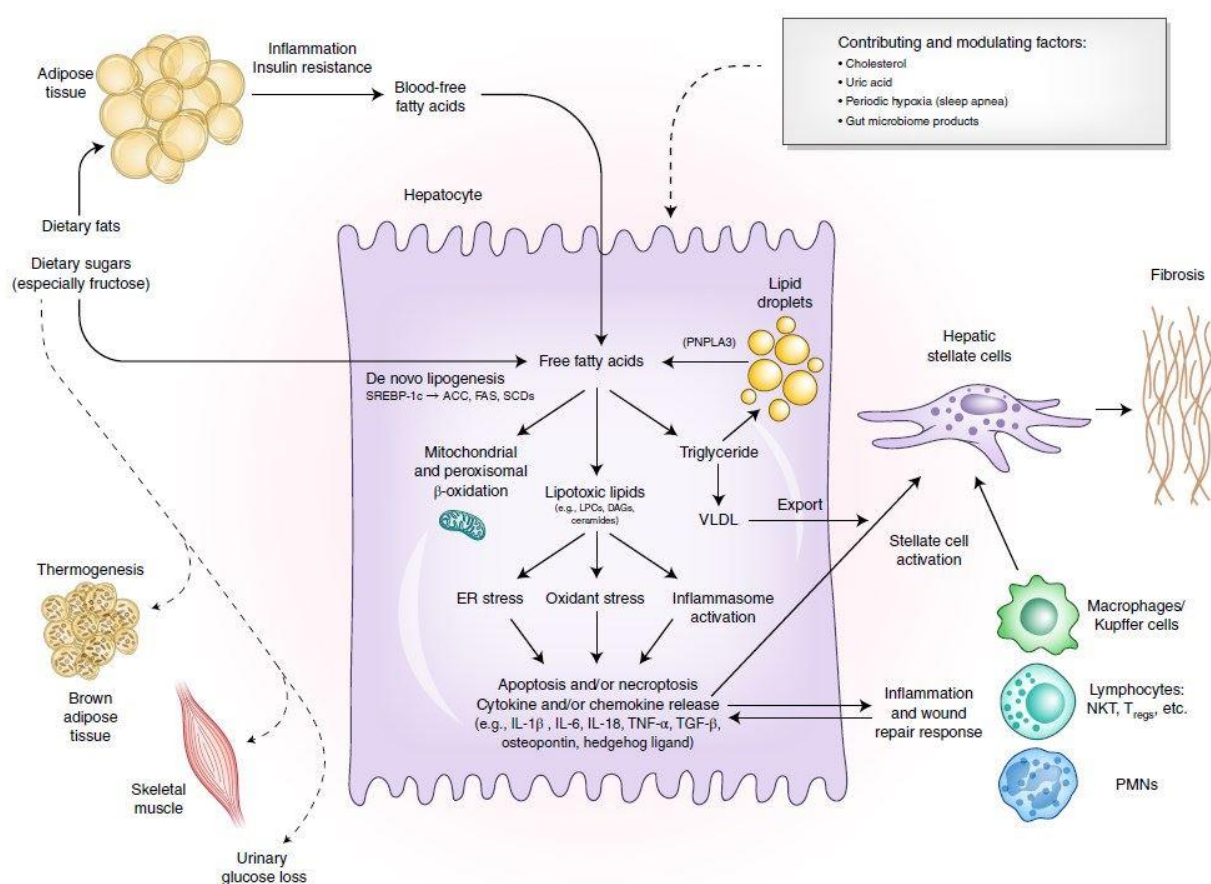


Figure 1. Hypothesized Pathogenesis of NASH [1]

NASH is characterized by lipid buildup, cell damage, inflammation and variable degrees of fibrosis. As mentioned in former, NASH is an invisible killer. It seldomly cause noticeable symptoms to its patients. Therefore, patient’s condition can only be determined by detecting the change of indicators. The appearance of steatosis is a critical indicator of NASH diagnose. After discover the appearance of steatosis, several steps need to be taken in order to determine whether NASH developed. The diagnoses steps are showed in Figure 2.

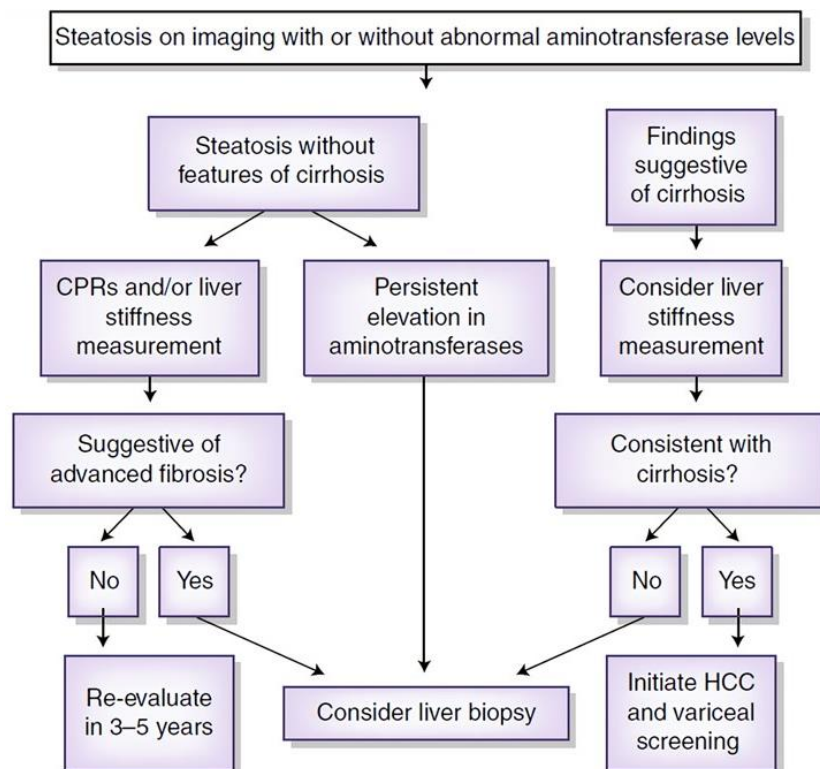


Figure 2. The Work Flow of Pre-clinical Diagnose of NASH [1]

3. Biomarker Tracing as A Diagnostic Technique

The most commonly used diagnostic method is liver biopsy which measures the concentration of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [1]. The presence of low concentration of ALT and AST indicates the end-stage of NASH. That is because when NASH progresses into the end-stage, serum transaminase level drops and necrotic inflammation conditions occurred. Yet there is a potential that the site being sampled does not accurately reflect the entire organ. Biomarkers could be utilized as a more comprehensive and precise diagnostic tool, predicting a patient's disease development and pinpoint the patients who might respond to a specific treatment. Currently available biomarkers in field show no superiorities than monitoring the ALT and AST levels in diagnosing low sensitivity NASH patients [1]. Particularly for identifying NASH from simple steatosis and estimating the potential of disease development, the optimal biomarkers should be simple to evaluate, repeatable, economical, and reliable. According to the hypothesized pathogenesis, apoptotic, oxidative stress, inflammation and extracellular vesicles (EV) biomarkers have been suggested that they might serve as ideal diagnostic indicators for NASH.

3.1. Apoptosis Biomarker Tracing

The degree of apoptosis is relevant to the severity of steatohepatitis and fibrosis, which are why NASH is lethal. Increasing liver cell apoptosis trend indicates NASH progression [2,3]. Apoptosis can happen in both extrinsic and intrinsic pathway. In extrinsic pathway, activation of a apoptosis ligand-induced receptor is involved forming a apoptosis complex. The intrinsic pathway is triggered by cellular stress. Both pathways activate the intracellular proteases, caspases 3 (CP3), which cleaves cells into small apoptotic bodies. Then the fragments are degraded by phagocytosis. The process of phagocytosis promotes inflammation and release cytokines. The released cytokines activate the stellate cells which result in fibrosis. During the development of NASH, the CP3 level would gradually increase. Cytokeratin-18 (CK-18) function as "executioners" to cleave CP3s [4]. Thus, the concentration of CK-18 has positive correlation to the concentration of CP3. Steatosis and normal liver can be differentiated from steatohepatitis by monitoring the CK-18 level. Since only NASH

involves inflammation so that NASH patient would tend to have higher level of CK-18 present in their serum. Presumably, the lipid peroxidation is potentially involved and being a part of the features in NASH pathogenesis [4].

3.2. Oxidative Stress Biomarker Tracing

One of the most essential biomarkers of liver oxidative stress is metallothionein (MT). It is observed that the necrotic inflammatory response in NASH is accompanied with a decline in MT concentration [5]. Hence, the drop in the level of MT could indicate the occurrence of lipid peroxidation, which may further suggest NASH progression. The onset of NASH is correlated with decreased adiponectin and increased levels of IL-6 and tumor necrosis factor (TNF). Adiponectin is considered to be protective [5]. In NASH patient, the level of adiponectin is lower than that of the control. Therefore, the level of adiponectin could not only be able to predict the inflammation and fibrosis, but distinguish NASH from steatosis. EVs can be recognized as reliable biomarkers for liver damage during the development of NASH based on the biogenesis and composition, which play a critical pathophysiological role in liver damaging [6,7]. The EVs derived from NASH have abundant miRNA-122 and miRNA-192 inside. These miRNAs are released to EVs from the stimulated or damaged liver cells as responses. Therefore, EVs could be utilized to monitor the liver damage degree as a non-invasive measure. Each of these biomarkers may help with diagnosis or even treatment methods in future.

Considering the fact that NASH causes multiple inflammatory lesions such as balloon-like degeneration and steatosis, researches have been conducted to find out the signaling protein that might be responsible for the onset. It has been observed that the level of TNF- α , IL-6 and IL-8 are leveled up as inflammation gradually develops [8]. Yet, further researches still required to test the feasibility of these signaling protein.

4. NASH-Related Models

To comprehend the pathogenesis of NASH deeper, identify potential therapeutic targets and evaluate the potential of individual drugs or combinations prior to human studies, the need for effective and secure treatments for human diseases incentive the development of *in vitro* and animal models of NASH.

4.1. In vitro Models

The mature models for study include primary cell cultures, immortalized cell lines, liver slices, and perfused liver [9]. Other cell lines, such sinusoidal endothelial cells and Kupffer cells, have also been created to aid in the understanding of the molecular processes behind liver illnesses. Extracellular matrix (ECM) production by hepatic stellate cells (HSC), the primary cause of liver fibrosis, might aid in the discovery of novel treatments that may change the course of all chronic liver illnesses [9]. In order to convert the basic cell into clinical applications, a model which mimics the tissue's 3-dimensional structure and multicellular complexity is required to be built. Co-culture and microfluidics are two methods for *in vitro*-engineered liver models that are commonly used. One key problem of the traditional processes for *in vitro* model culture has the incorrect physiological ways of delivering and removing metabolites. Therefore, the importance of researching cell matrix biochemistry and mechanism is highlighted. The complex interactions between HSCs and hepatocytes and other non-parenchymal cells have been gradually revealed by the development of well-defined 3D multicellular organoid cultures *in vitro* models that mimic the *in vivo* ECM structures [9].

4.2. In vivo Models

Multiple rat models have been established. Following the logic of controlling variable, more than 10 models are studied. Mice are fed by different foods to develop different symptoms and

conditions. The synthesis and secretion are especially focused, since it would vary the steatosis and inflammation conditions of mice livers. Also, the transgenic mice are studied. The gene edited mice show different resistances to steatosis or fibrosis. SREBP transgenic mice model suggest a potential therapeutic method of curing NASH (Table 1).

Table 1. Established Rat Models for NAFLD research [3]

Types	Influence to NASH Progression
Dietary Regulation	
Methionine and choline deficiency (MCD) dietary model	Choline and methionine deprivation will inhibit secretion of VLDL and cause hepatosteatosis, oxidative stress, liver cell death but only cause minor inflammation and fibrosis. This model is suitable for study advanced NASH
Choline-deficient, high-fat dietary model	Similar to MCD model, can exhibit insulin resistance and also develop fibrosis
High-steroid (cholesterol) dietary model	Mice fed with high-cholesterol diet has reduced VLDL synthesis and beta-oxidation of fatty acids. Apoptosis and hepatic oxidative stress increased.
High dietary sugar model	Mice fed with high sucrose and fructose develop typical hepatic steatosis
Trans-fatty acid dietary model	Trans-fatty acid intake can cause fat accumulation and inflammatory lesion. Mimics human intake of trans-FA
Gene Edited Mice	
ob/ob	Leptin-deficient mice exhibit insulin resistance and steatosis. Resistant to fibrosis
db/db	Leptin receptor-deficient mice exhibit glucose intolerance and hepatosteatosis
foz/foz	Mice show insulin resistance, increased cholesterol levels and steatosis
SREBP-1c transgenic	Severe insulin resistance, spontaneous steatohepatitis and oxidative stress
MUP-uPA transgenic	Severe ER stress, hepatosteatosis with TNF driven inflammation on a high-fat diet fed mice
Phosphatase and tension homolog deleted	
Mat 1A deleted	Methionine adenosyl transferase deficient mice could develop NASH
LDL receptor knockout	Steatosis worse in mice fed with high cholesterol food
Combined	Mimic the NASH progression in human body

5. Treatments Against NASH

NASH involves multiple symptoms simultaneously. The most noticeable one is the fibrosis which significantly increases the risk of liver cancer and cirrhosis. There are no current accountable treatment options to reverse the late-stage fibrosis in NASH patient. However, correcting or eliminating the underlying fibrosis factors to reverse cirrhosis has a great potential to become a reliable strategy. The most of therapeutic methods will be discussed are inhibitory strategies.

5.1. Protein Inhibition Treatment That Reduces Fibrosis

Heat shock protein 47 (HSP47) is a chaperone protein that function as a mediator in fiber collagens folding. Thus, inhibition of HSP 47 allows collagen to misfold and accumulate in cell which will trigger apoptosis instead of secreting. Hence, the accumulation of collagen in fibrotic tissues decreased. Besides, small molecule compound AK778 shows inhibitory ability to the interaction of HSP 47 and collagen. The product Col003 can competitively inhibit the interaction and cause the inhibition of collagen secretion [1]. The competitive binding between Col003 and HSP 47 may provide a foundation for more effective fibrosis medicine research. The other strategy to reverse fibrosis is inhibiting caspases (CP). During NASH progressing, CP degrades cells and the cell fragments cause inflammation. CP activation in NASH requires CP activities. Based on this feature, an inhibitor that can inhibit a wide variety of CPs can be designed. With the introduction of CP inhibitor, apoptosis is repressed and inflammation symptom is reduced. “VX-166” is a CP inhibitor

that has been implemented in rat model and exhibited the ability to slow the development of fibrosis [10]. The experiment has proved that fibrosis can be repressed by CP inhibitor.

5.2. Protein Inhibition Treatment That Reduces Lipotoxicity

Other than fibrosis, lipid accumulation is another common symptom in NASH. Recent data suggests that the development from steatosis to NASH may not determine by the amount of triglyceride in the liver but by the accumulation of lipotoxic intermediates occurred during synthesis of triglyceride and lipid degradation [11]. The major regulator for intracellular cholesterol homeostasis is sterol regulatory element-binding protein 2 (SREBP2) [12]. The inhibitor of SREBP2 could interrupt sterol metabolism. The NASH's lipotoxicity is consequently reduced. Another enzyme inhibition theory was also purposed. Triacylglycerols (TG) are essential energy storage in human tissues. Imbalanced energy intake and consumption are susceptible to cause TG build up, resulting in NASH. DAG O-Acyltransferase (DGAT) has enzymic activity that catalyze the formation of TGs [13]. This transferase exists in two isoforms: DGAT1 and DGAT2. DGAT1 is expressed at lower levels in the liver and adipose tissue than it is in skeletal muscle. In a randomized clinical study that included individuals with NAFLD, the small molecule DGAT1 inhibitor was found to ameliorate hepatic steatosis. DGAT2 controls lipid metabolism and is mostly expressed in the liver and adipose tissue [13]. DGAT inhibitors could prevent the excess formation of TGs. Recent clinical experiment has proved that vitamin B3 is an effective DGAT inhibitor [13]. c-Jun-N-terminal kinase (JNK) mediates transcriptional process involved in inflammation and cell apoptosis [14,15]. JNK and its relevant signaling pathway are critical element in NAFLD onset. JNK induce the NASH progress through damaging liver cell, causing inflammatory lesion and promoting fibrosis. In animal model, it has been proved that JNK augment could accelerate steatosis. Moreover, JNK cause inflammatory lesion through activating the activator protein to promote the transcription of inflammatory cytokines. Thus, JNK deletion could slow steatosis progress and improve circulating triglyceride level. In further, preventing liver cell death. Comparing to the CP inhibitors, this strategy cut off the hepatocyte injury signaling pathway two steps before.

5.3. Protein Inhibition Treatment That Reduces Inflammation

NASH is characterized by hepatocyte damage, death, and inflammation. If inflammation response can be detained, it would help to reduce the symptoms of patients. Multiple inhibitors that can block the signaling pathway of inflammation development have been studied and tested in animal model. One of the major inhibitors is ASK-1 inhibitor. The A protein named GS-4997 is able to the sterically block the assemble of activated ASK-1 complex, which in further promote the ubiquitylation of ASK-1 [16]. Without ASK-1, stellate cells will not be activated. On the other side, protein that could promote cell proliferation, theoretically, would reduce inflammation as well as apoptosis inhibitors. However, none of feasible proteins have been addressed.

6. Conclusions

The researches and treatments of NASH have provided many feasible strategies for researchers and doctors to cure NASH. Biomarkers tracing as a newly purposed diagnostic technique would reveal a more accurate liver disease status to patients and doctors. According to the researches and observation of NASH, four typical symptoms have been determined-- lipid accumulation, cell damage, inflammation and fibrosis. Each symptom has been developed with specific treatments. Among all treatments, protein inhibition is the most commonly implied method. That is, the key enzymes or coenzymes in each signaling pathway could be inhibited. Thus, the biochemical reaction that promote NASH development will be consequently slowed or terminated. HSP-47, DGAT and ASK-1 are all promising treating targets that can significantly reduce NASH symptoms. However, due to the complexity of NASH's onset conditions, potential inhibitors all required further assessments to prove to their effectiveness. The current researches are mostly still stay in hypothesis.

Few of them have found the suitable protein to work with. For biomarker diagnostic techniques, introduced biomarkers all required further researches and tests to solidify their accuracy and feasibility in order to be clinically implemented. Treatment with great varieties have been addressed, yet it may consume a long period of time to determine the most effective and economical treatment. However, it is obvious that the research of NASH has stepped into a new stage. More promising clinical solution will occur in recent years.

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