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REVIEW

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The novel approach for non-invasive diagnostic biomarkers from an early stage of NAFLD to advanced fibrosis

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Abstract

Background Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disorders that will be started from more than or equal to 5% of fats deposited into the liver hepatocyte cells and progressively leads to steatosis, further increment in fat deposition, and signature of inflammatory markers which cause the non-alcoholic steatohepatitis (NASH) condition. Due to a lack of diagnosis and effective treatment, NASH is converted into liver cirrhosis or hepatocarcinoma, which indicates the irreversible stage of the disease and finally recommends liver transplantation for patient survival. However, nowadays, several clinical biomarkers are identified, and most of the new biomarkers are in the developmental stage, but still the diagnosis of each stage of fatty liver is unaccomplished. So, in this review article, we try to present all current mechanistic perspectives to find the non-invasive biomarkers which could be the best approach in the future to diagnose fatty liver disease in each stage.

Main text NAFLD is a growing phase disease if properly not taken care of by the patient. There are certain factors that can make fast progress in the disease stage like NAFLD to advance liver fibrosis or hepatocarcinoma. We describe to the best extent how different types of disease stages in the case of the fatty liver could be diagnosed using non-invasive biomarkers. A certain type of mechanistic pathophysiology approach is used to differentiate each stage of fatty liver disease like serum biomarkers (inflammatory cytokines), lipoproteins, micro-RNAs, gut microbiome-associated biomarkers, lipid droplet-associated perilipins, apolipoprotein E, the role of dihydroceramide, and gene expression studies.

Conclusions Recent advancements in diagnostic biomarkers research focused on non-invasive methods, but the diagnosis of different stages of fatty liver disease is still inconclusive. We tried to cover all the potential non-invasive biomarkers in our manuscript. This review helps the researchers to develop possible diagnostic biomarkers for each stage of liver disease.

Keywords Biomarkers, Dysbiosis, Fibrosis, Gut microbiome, Hepatosteatosis, Lipoproteins, Liver cirrhosis, Micro-RNA, Obesity

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Introduction

Epidemiology of NAFLD/NSAH

Non-alcoholic fatty liver disease was first recognized in 1980 as a multifaceted metabolic disorder. It is one of the most widespread illnesses affecting modern humans. According to estimates, 25.24% of the world's population now has NAFLD, with the Middle East and South America having the highest incidence rates. Over the past 20 years, NAFLD has emerged as the most prevalent chronic liver disease worldwide. In Japan, a study of 3271 individuals was conducted which suggests that NAFLD is more common in obese patients, that is, 68.5% developed NAFLD as compared to only 15.2% of nonobese patients [1]. The prevalence of NAFLD is higher in participants with T2DM and those who are obese (51.83% in diabetic participants vs. 30.76% in nondiabetic participants).

Recent studies claim that among 1812 biopsy samples of NAFLD patients between 2006 and 2019 were combined into a selected clinical dataset across nine Asian nations; 21.6% of patients with biopsy-confirmed NAFLD were not fat. Additional variables found using random forest analysis that is helpful for detecting nonobese NAFLD patients with advanced liver disease include hemoglobin, GGT, waist circumference, and cholesterol [2].

In a study conducted among US adults by the National Health and Nutrition Examination Survey from 2017 to 2018, NAFLD was independently linked to men, older age, and Hispanic race. Non-Hispanic whites between the ages of 50 and 79 had the greatest prevalence rates of NAFLD. However, compared to non-Hispanic whites, NAFLD prevalence rates were higher in Hispanics at a younger age.

Out of the 4024 individual US adults, 56.7% aged ≥ 20 years had NAFLD by CAP. With the increase in obesity among youth, it is expected to increase in the NAFLD. However, it also suggests that the prevalence of NAFLD was higher in men than in women [3].

Another clinical research analysis states the causes of chronic liver disease (CLD) in people, mainly adolescents and young adults between the ages of 15 and 29, over the past 10 years. Out of the many factors such as viral hepatitis, they found that NAFLD is the primary factor driving an increase in CLD incidence. Given the high rates of obesity and T2DM in the general population, which have made NAFLD one of the most widespread causes of liver disease in both adults and children, NAFLD can cause a considerable burden of CLD, particularly in people ages 20 to 29 [4].

In a systematic review and meta-analysis study conducted in 2019 for global NAFLD prevalence, it has been found that in a literature search of 17,244 papers, 245 of them were eligible studies involving 5,399,254 people. The combined global prevalence of NAFLD was 29.8%. The highest NAFLD prevalence was found in South America (3 studies, 5716 people), followed by North America (4 studies, 18,236 people). NAFLD grew from 21.9 to 37.3% between 1991 and 2019 according to trend analysis, with South America experiencing the largest annual change (2.7%), followed by Europe (1.1%). Despite regional heterogeneity, this suggests that NAFLD prevalence is generally rising globally [5].

Another group that studied the prevalence of NAFLD worldwide found that the estimated global prevalence of NAFLD is 32.4%. The prevalence considerably increased over time, rising from 25.5% in or before 2005 to 37.8% in or after 2016. Men were substantially more likely to have NAFLD as compared to women. According to estimates, there are 46.9 instances of NAFLD per 1000 people, 70.8 cases per 1000 people for men, and 29.6 cases per 1000 people for women. Men have much greater rates of NAFLD than women do, both in terms of incidence and prevalence [6].

According to a recent meta-analysis of NAFLD in China the following study was performed 66.21% in obese vs. 11.72% in lean. In China, the prevalence of NAFLD and the rising obesity rate are correlated (the prevalence from approximately 2% in 2000 to 7% in 2014). Additionally, T2DM and obesity raise the danger of simple steatosis developing into NASH, cirrhosis, and HCC. Notably, China has Asia's highest prevalence, incidence, and yearly mortality rate from NAFLD. According to another meta-analysis of 93 research from 24 countries or regions, lean NAFLD affects 5.1% of the general population, while nonobese NAFLD affects 12.1% of persons. It is important to remember that NAFLD can occur in slim adults with normal waist circumference (12.9%) [7]. Another study in the US population suggested that high-quality diet (HQD), more physical activity, and college education were linked to lower NAFLD risk. Those with HQD who were physically active showed the lowest probability of developing NAFLD [8].

When compared to the demographics of the USA, where African-Americans (AA) make up 12% of the population, it was seen that they were underestimated in the study of 1026 people with NAFLD, making up just about 3% of the sample population. In reality, NAFLD may be present in 10 to 20% of nonobese Americans.

In Asian people, where the majority of "lean" NAFLD investigations have been done, "lean" NAFLD is most frequently observed. NAFLD may affect between 7 and 18% of Asia's nonobese population (including China, Korea, and Japan) [1].

The burden of NAFLD is anticipated to expand further in the years to come, driven by continued rises in the incidence of obesity and T2DM as well as an aging population [9]. With increase in NAFLD, it is also becoming

the root cause of hepatocellular carcinoma and cirrhosis [10] (Fig. 1).

Non-alcoholic fatty liver disease

When fat continuously accumulates in over 5% of hepatocytes, then it is known to be non-alcoholic fatty liver disease (NAFLD) [11]. The emergence of NAFLD occurs due to disposition of fats. As per data gathered from epidemiological studies globally, related to obesity and type 2 diabetes cases, it leads to proportionality increment in NAFLD or NASH patients [12]. Modern lifestyle and diet patterns are responsible for the epidemic growth. It is proposed that in the recent times, more than 25% of the world population is suffering from NAFLD initial stage, whereas previously only 25% were suffering [5, 13–15]. The lack of specific non-invasive biomarkers to detect the exact stage of fatty liver disease is a major concern as this leads to the increasing severity of NAFLD and fibrosis. This increasing severity leaves no option other than liver transplantation to save the patient [16].

There are numerous diagnostic procedures and biomarkers on the market that can be used to identify the different stages of fatty liver disease. However, as of now, neither a distinct illness profile at later stages nor an early-stage diagnosis has been accurately identified. Understanding each step of the NAFLD is crucial [17].

NAFLD stages

Beginning of the fatty liver disease is marked with the continuous disposition of fat into the hepatocytes cell, and if there is coverage of more than 5% area in the cells, then there is emergence of early stage of non-alcoholic fatty liver disease (NAFLD) [18]. In the absence of remedies, the fat disposition progresses to the next stage of the disease where high amount of fats are accumulated. The accumulation of fats leads to formation of balloons, and this stage is referred to as liver steatosis [19].

When there is continuous growth pattern observed in steatosis, then the liver cells start entering into the red zone, and inflammatory damage occurs to the hepatocytes. This damage alters the normal physiology of the cells that leads to the marred state of the normal hepatic cellular system known as non-alcoholic steatohepatitis condition. In this stage, various types of inflammatory cytokines are activated and also activate the hepatic stellate cells which is further leading to increased expression of IL-6, IL-beta, C-reactive protein (CRP), and TNF alpha and activation of Toll-like receptor-4 (TLR4) [20, 21]. Then massive destruction of the hepatic cells changed into the liver fibrosis [22].

The liver fibrosis stage begins with an increasing load of extracellular matrix (ECM) proteins which indicate the massive damage in hepatocytes, and the persistent rise of

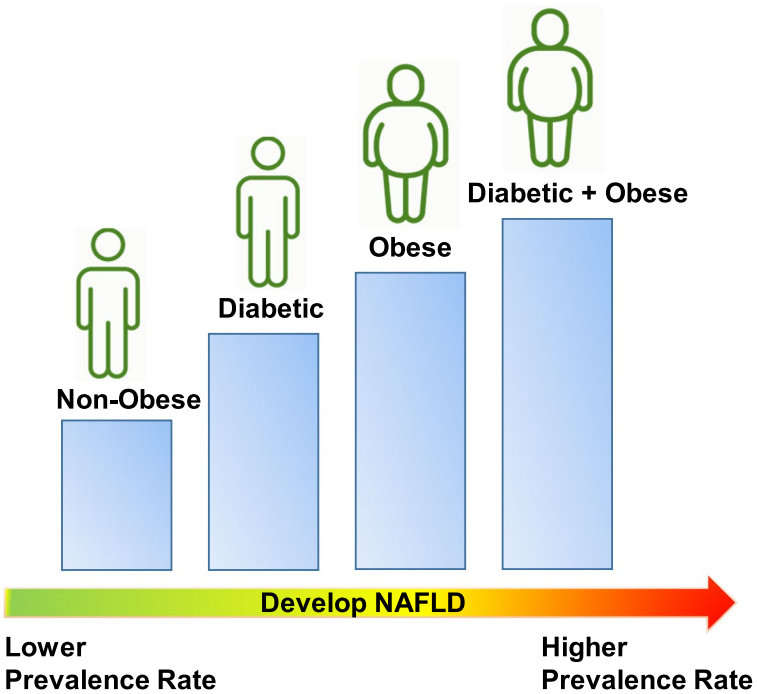


Fig. 1 Showing the conclusive current trend followed by the patient of nonalcoholic fatty liver disease based on their epidemiological data globally. The nonobese patient shows a very lower prevalence rate to develop the NAFLD when we compare with patients with already type 2 diabetes mellitus and obesity patient

liver injury leads to the advanced stage of liver fibrosis. Furthermore, it has been proven that the advanced fibrosis stage consists of 6 times more ECM when compared with the normal. Along with extra deposition of ECM, some associated genes are also expressed such as collagen I, III, and IV, fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans [23–28] (Table 1).

Non-invasive serum biomarkers

Current trend in research of metabolic disorders is trying to reveal the cellular signaling altered in diseased state to find the possible diagnostic biomarkers for non-alcoholic fatty liver disease. Liver biopsy is the gold standard even after long research in metabolic domain to detect the exact stage of fatty liver disease. However, we tried to present all possible serum biomarkers which is specific to each stage of the liver disease as per latest data present on diagnostic biomarkers. There are certain type of the genes and proteins expressed in different pattern such as inflammatory cytokines like (IL-6, TNF-α, NF-kappa β, TLR-4, and IL-1β), fatty acid transporters like proteins (FATP1, FATP2, FATP5), lipid droplet-associated proteins or perilipins (PLIN1, PLIN2, PLIN3, PLIN4, and PLIN5), and extracellular matrix proteins in different stages of the disease. Figure 2 shows the expression of certain serum biomarkers from the early stage of NAFLD to NASH or liver fibrosis in an elaborative pattern.

Multiomics approach on oxidation process in hepatocytes

Recently, the multiomics approaches are targeted digging out the molecular pathway of fatty liver disease, which will be helpful to discover novel biomarkers. The oxidation process is one of the main processes which occur in hepatocyte cells during the disease severity. There are certain fatty acid-derived products that play a keen role in non-alcoholic steatohepatitis conditions. Based on the lipid omics data, it has been proved that the oxidation product of arachidonic acid, 11 hydroxyeicosatetraenoic acid (11-HETE), and linoleic acid oxidation products are significantly increased in NASH patients [34, 35].

Scope of micro-RNAs as biomarkers

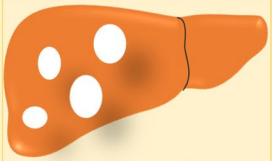
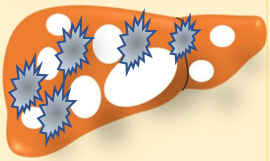
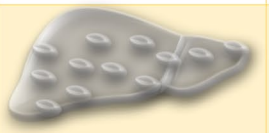
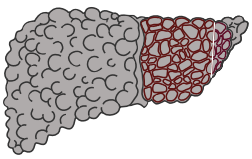
Micro-RNAs have special functions to control the post-transcriptional gene silencing and maintain the normal cellular and metabolic homeostasis by regulating the gene expression. After comparing data collected from the serum studies of NAFLD patients and the studies of analysis of the expression of micro-RNA of NAFLD patient with the healthy population, there is a huge difference noticed in both the results. One of recently published data shows the Micro-RNA 29 have the dominant role in non-alcoholic fatty liver disease [36]. However, there are three subtypes of Micro-RNA 29 which are Micro-RNA 29a, Micro-RNA 29b, and Micro-RNA 29c. Among them, Micro-RNA 29a is highly upregulated in NAFLD patients, but Micro-RNA 29b and Micro-RNA 29c do not show any promising effect in clinical specimen of NAFLD patient when compared with the healthy control. Another Micro-RNA such as Micro-RNA 16, Micro-RNA 21, Micro-RNA 122, Micro-RNA 34a, and Micro-RNA 375 show their upregulation in non-alcoholic fatty liver disease patient. An animal study using the apolipoprotein E-deficient mice which is used as a NAFLD model shows the increased expression of Micro-RNA 34a-p and Micro-RNA 375-3p [37]. However, if we look into the later stage of the disease, the expression of Micro-RNA is not the same when we compare with NAFLD patient data. The severity of disease such as early stage of hepatocarcinogenesis shows downregulation of this gene. Apart from the clinical data of NASH patients, it also revealed some new Micro-RNA, which are overexpressed in serum sample like Micro-RNA 21, Micro-RNA 23a, Micro-RNA 26a, Micro-RNA 155, Micro-RNA 200c, Micro-RNA 222, Micro-RNA 224, Micro-RNA 374a, and Micro-RNA 423. These types of research advancement in area of micro-RNA could come up with breakthrough in discovery of new non-invasive serum biomarkers for NAFLD/NASH [38].

Another study shows that micro-RNA that are specific for the fatty liver disease, which is elaborated more below as mentioned in Fig. 3. So, these disease stage-specific miRNAs could be beneficial to discover the non-invasive serum biomarkers for the non-alcoholic fatty liver disease.

Gut microbiome-associated biomarkers

Gut microbiome science related to human physiology is continuously making the remarkable footprints to solve the role of trillions of bacteria present in the gut which play an impactful role in different types of disorders in our body system [51]. Among them, it has been proved that there is a cross talk that happens between the liver and gut with the help of portal vein which connects the entire gut system to the liver [52]. We are aware that the consumption of Western diet or high-fat diet leads to alteration in entire bacterial population in gut microbiota, and this state is well known as dysbiosis [53, 54]. The study of this specific type of bacterial shift could bring the breakthrough to diagnose the non-alcoholic fatty liver disease. There are certain bacterial phyla that are upregulated or downregulated in each stage of the NAFLD. Most of the bacterial phyla follow the same pattern in all stages of the disease, such as *Bacteroides* which shows their increased expression in early NAFLD and later stage of the disease. Contrastingly, some of the

Table 1 Showing the progressive stage of non-alcoholic fatty liver disease which is started from the initial stage of 5% of the fat deposition in hepatocytes and progressively follows the sequence of the disease like this non-alcoholic fatty liver disease (NAFLD) → steatosis (without inflammation) → non-alcoholic steatohepatitis (NASH) with inflammation → F0 stage without fibrosis → F1 stage portal fibrosis without septa → F2 stage periportal fibrosis → F3 stage bridging fibrosis → liver cirrhosis [29–33]

S.No.	Liver Fibrosis Stages	Features	Histopathology	Pathogenesis
1.	Non-Alcoholic Fatty Liver Disease (NAFLD)	Hepatocyte fat deposition more than or equal to 5%		Alteration in Lipids profile Increase in LDL Decrease in HDL Increase in TGs Increase ALT/AST
2.	Steatosis or Hepatocellular Ballooning (Without Inflammation)	Highest amounts of fats get deposited and show macrovesicular characteristics.		Continuously increase in Lipid profile leads to: Fats deposition Ballooning in hepatocyte Fat related pathways activation
3.	Non-Alcoholic Steatohepatitis (NASH)	Hepatocellular ballooning with inflammatory cells Mega mitochondria observed		Inflammatory pathway activation ER stress, Mitochondrial dysfunction, Oxidative stress, Proinflammatory cytokines activation. e.g., IL-6, IL-1β, TNF-α etc.
3.1.	F-0	No Fibrosis		Extracellular Matrix Deposition but the fibrosis not observed in beginning of this stage.
3.2.	F-1	Portal Fibrosis Without septa Sometimes fibrosis covered zone 3 of parenchyma		Initial stage of fibrosis started, the various ECM genes expression increased like MMP9, TIMP, Hyaluronic
3.3.	F-2	Periportal Fibrosis/ or portal fibrosis with rare septa		Fibrosis cover also periportal region with progressively increased expression of metalloproteinase
3.4.	F-3	Bridging Fibrosis		Highly ECM associated genes activation Variceal bleed Ascites Hepatic encephalopathy Hepatocellular carcinoma
3.5.	F-4	Liver Cirrhosis		Scar tissue formation Excessive: Variceal bleed Ascites Hepatic encephalopathy Hepatocellular carcinoma

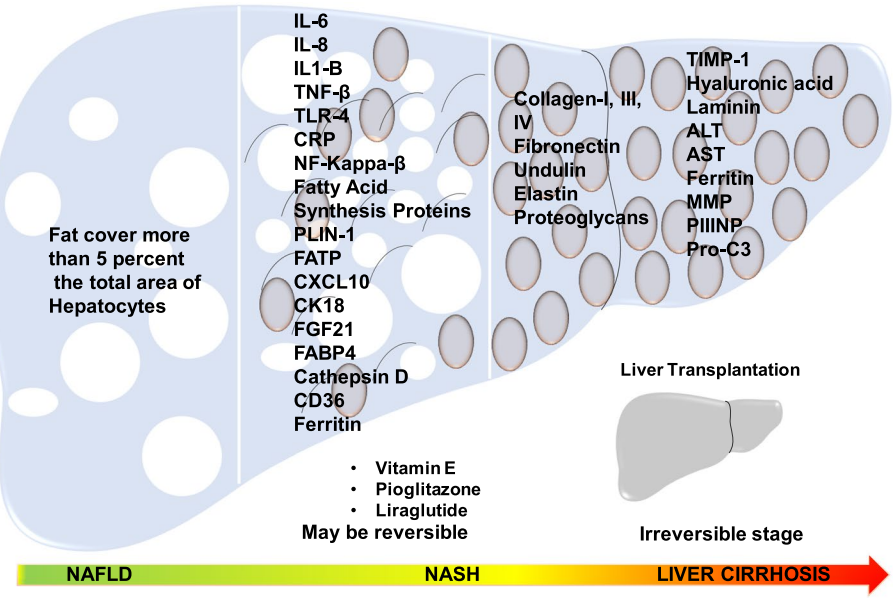


Fig. 2 Showing that the increased expression of certain genes and proteins related to inflammatory, fats, and extracellular matrix (ECM) pathways in early stage of NAFLD to liver-fibrosis stage. The early stage of the disease started from the small amount of fat deposition in liver parenchyma, but progressively, mode of liver disease converted into non-alcoholic steatohepatitis with inflammatory signatures shows increased expression of interlekin-6 (IL-6), interleukin-8 (IL-8), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), Toll-like receptor-4 (TLR-4), C-reactive protein (CRP), and nuclear factor-kappa beta (NF-kappa β). Activation of fat-related pathways like fatty acid-binding protein-4 (FABP4) and cluster of differentiation 36 (CD36). At the stage of NASH, the lipid droplet-associated proteins perilipin-1 (PLIN1) is highly expressed. Some other NASH-related proteins expression like cytokeratin 18 (CK18), cathepsin D, and ferritin. At this stage, some studies indicate that the disease can be reverse using the combine therapy of vitamin E, pioglitazone, and liraglutide. But after crossing the NASH stage, the liver entered into the emergency zone; the stage is known as liver fibrosis or cirrhosis condition where only liver transplantation is the single option to save the life of the patient. The fibrosis stage begins with increased expression of extracellular matrix proteins like hyaluronic acid, laminin, tissue inhibitor of metalloproteinase (TIMP-1), ferritin, alanine transaminase (ALT), aspartate transaminase (AST), procollagen peptide type III (PIIINP), matrix metalloproteinase (MMP), and procollagen C3 (Pro C3)

bacterial phyla are very specific to each stage, such as *Allisonella* which is particularly detected in NASH, and increased expression of Verrucomicrobia is seen in early stage of the NAFLD, but downregulation is seen in the later stage of the disease (liver fibrosis) [55]. Recently, studies show that the bacterial phyla show an abundance in hepatocyte fibrosis, such as Tenericutes and Actinobacteria, and a decrease in the abundance of Bacteroidetes [56] (Fig. 4).

Interestingly, some bacterial phyla are specifically detected abundantly in early stage of NAFLD, whereas those bacterial phyla not observed significantly change in later stage of the diseases such as Fusobacteria, Verrucomicrobia, and Lentisphaerae which could lead to discovery of the scope of diagnostic markers for the NAFLD [57].

Lipid droplets perilipin family protein

Lipid droplet-associated protein like perilipin 1–5 (PLIN1, PLIN2, PLIN3, PLIN4, and PLIN5) and another linked pathways involved in synthesis and fate of lipid droplets could be another approach to discover the biomarkers for the non-alcoholic fatty liver disease [58, 59]. Most of the in vivo study already proved that the expression of PLIN1 is highly upregulated in NAFLD. The macrovesicular steatosis is specific to PLIN1 expression, and PLIN1 stabilized the large lipid droplets and dominantly present in white adipose tissue [60, 61]. PLIN2 is abundantly found in the liver organ, and most of the studies previously claimed its direct involvement in fatty liver disease progression due to their important role in lipid accumulation in hepatocytes. These studies somewhere show their participation in inflammatory pathways activation, but the whole mechanism is not clearly defined until now [62–64]. Furthermore, PLIN2 knockout studies show improvement in NASH condition. PLIN3 sometime called as mannose-6-phosphate-binding proteins shows their importance in lipid droplets biogenesis [65]. However, inhibition of PLIN3 presents their role in reduction of inflammation proportionally reducing the action of prostaglandin synthase and improves the hepatosteatosis condition by suppressing the inflammation [66]. Upregulation of PLIN4 reveals the micro-steatosis state in hepatocytes, and PLIN4 expression in nascent lipid droplets

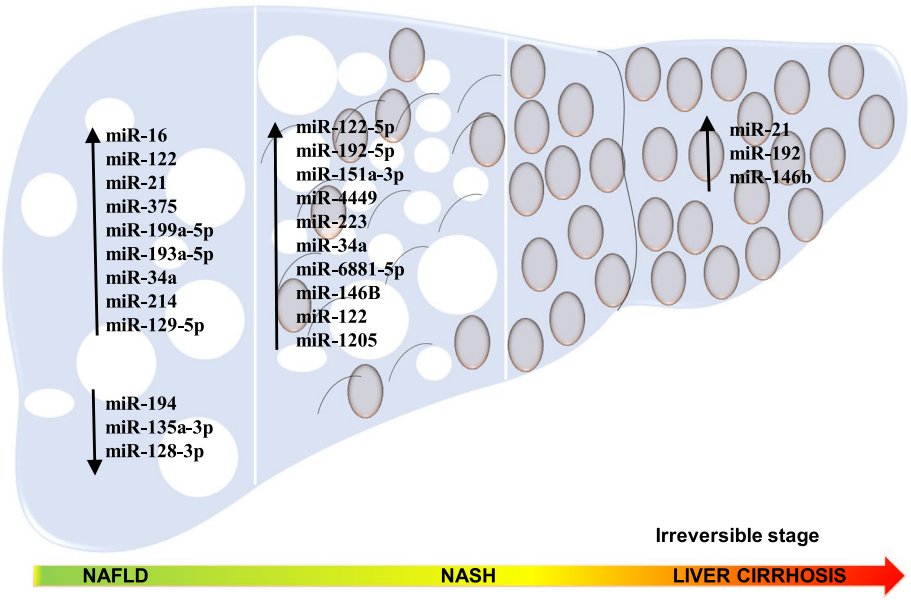


Fig. 3 Showing that micro-RNA alteration was observed in each stage of the fatty liver disease. Some early-stage-specific micro-RNA like miR-16, miR-1991-5p, miR-193a-5p, and miR-129-5p were highly expressed, and miR-194, miR-135a-3p, and miR-128-p were downregulated [39–43]. Comparatively, in case of NASH, the miR-122-5p, miR-192-5p, miR-4449, miR223, miR-34a, miR-6881-85p, miR-146b, miR-122, and miR-1205 found abundantly expression [44–49]. Later stage of the disease like fibrosis/cirrhosis stage also shows some specific upregulation of miRNAs like miR-21, miR-192, and miR-146b [43, 50]

indicated that it is integrated into nascent lipid droplets together with freshly generated triacylglycerols, so it may be possible the inhibition of PLIN4 shows their preventive action in NAFLD and also could be helpful to design the further study as an biomarkers for micro-steatosis condition [59]. Unlike other perilipins, the PLIN5 expression is observed at the stage of hepatocellular carcinoma (HCC) and also execute the function to regulate the lipid homeostasis by inhibiting the lipolysis [67]. Surprisingly, murine model of HCC also presents the evidence to play a keen role in tumor progression, but most of the cellular signaling is still unclear [68]. In future, this could be another best consideration to find the novel biomarkers for the later stage of liver diseases such as advance fibrosis or HCC. In conclusion, although the lipid droplets perilipin family proteins (PLIN 1–5) are understudied proteins, understanding their mechanisms may pave the way for the development of effective treatments and biomarkers for NAFLD/NASH.

Lipoproteins

The liver is the major organ for the metabolic process which is also involved in fat metabolism, and it highly participates in the transport of hydrophobic lipoprotein with the help of apolipoproteins involvement in their transport mechanism. Lipoproteins are broadly categorized into three classes in the human body: chylomicron, very low-density lipoprotein, and high-density lipoprotein. NAFLD and dyslipidemia, a collection of abnormalities in plasma lipoproteins including triglyceride-rich very low-density lipoproteins, are interlinked with each other, but their mechanism of action is still unknown. Apolipoproteins are a group of predominantly liver-derived proteins found in serum lipoproteins; they not only play an essential intracellular role in hepatic lipoprotein but also play an essential extracellular role in lipid transfer between key organs through circulation. Various clinical studies prove that hepatic lipoproteins are the major player to regulate the fat deposition in hepatocytes.

Apolipoprotein E

Adipose tissue that stores too much energy contributes to the development and spread of obesity, which has a number of harmful side effects including metabolic syndrome and cardiovascular illnesses. There are several lipoproteins and their components that are directly or indirectly linked with the lipolysis process in adipocytes, but some of them play a very keen role to regulate the fat metabolic process such as apolipoprotein E. Apolipoprotein E is the core component of lipoproteins which is dominantly expressed in adipose tissues and sometimes called arginine-rich apolipoprotein due to high content of arginine amino acids. Previously recorded data proved

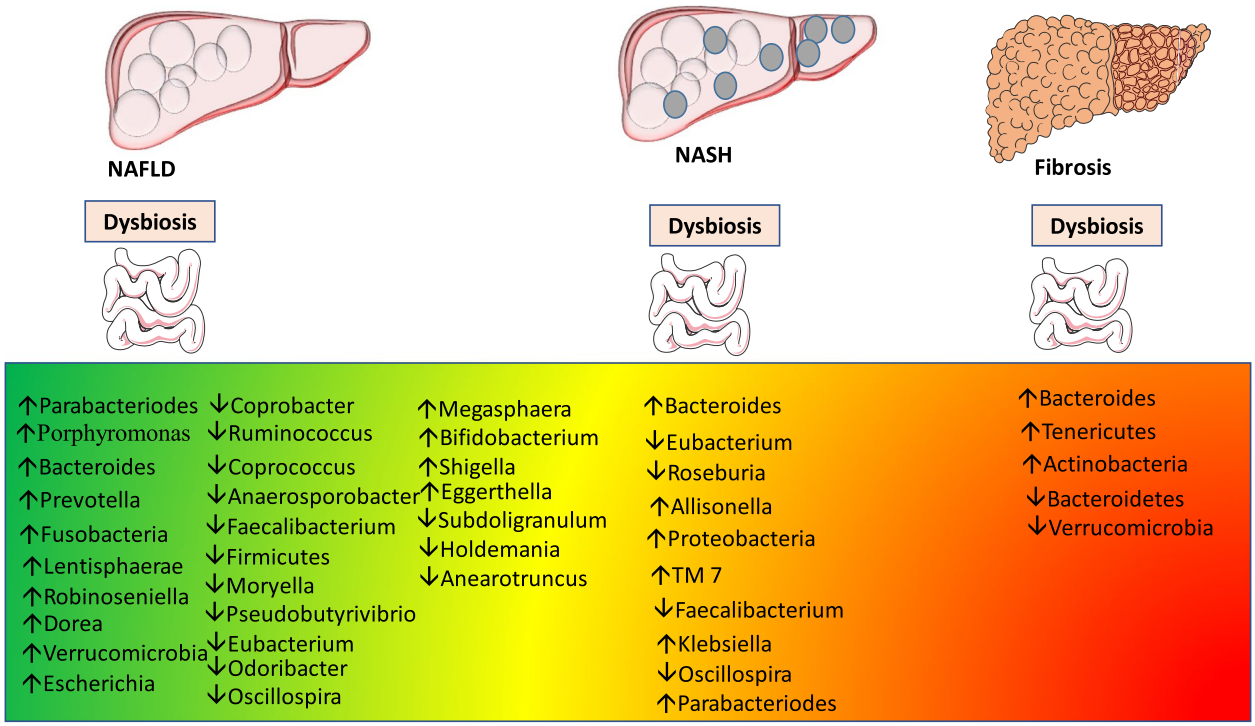


Fig. 4 Showing that the dysbiosis condition after the patient diagnosed with non-alcoholic fatty liver disease, which results imbalance in gut bacterial population. Initial phase of NAFLD shows upregulation of certain bacterial phyla which are *Parabacteroides*, *Porphyromonas*, *Bacteroides*, *Prevotella*, Fusobacteria, Lentisphaerae, *Robinsoniella*, *Dorea*, Verrucomicrobia, *Escherichia*, *Megasphaera*, *Bifidobacterium*, *Shigella*, and *Eggerthella* and decreasing population of *Coprobacter*, *Ruminococcus*, *Coprococcus*, *Anaerosporobacter*, *Faecalibacterium*, *Moryella*, *Pseudobutyrivibrio*, *Eubacterium*, *Odoribacter*, *Oscillospira*, *Subdoligranulum*, *Holdemania*, and *Anaerotruncus*. When we focus on the NASH stage, several new bacterium phyla detected on progressive stage of the disease and show the increased population of *Bacteroides*, *Allisonella*, Proteobacteria, TM7, *Klebsiella*, and *Parabacteroides*. Oppositely, there are certain bacterial phyla population which was decreasing which includes *Oscillospira*, *Roseburia*, *Eubacterium*, and *Faecalibacterium*. Liver fibrosis stage shows decrease bacterial phyla of Bacteroidetes and Verrucomicrobia, whereas the increased population of *Bacteroides*, Actinobacteria, and Tenericutes reported in end stage of the fatty liver (liver fibrosis)

that apolipoprotein E contributes to lipoprotein internalization and degradation via the process of endocytosis which compels that there could be a better role of apolipoprotein in metabolic disorders, especially in fat accumulation in hepatocytes [69]. Recently, in data gathered from the animal study, the apo E-deficient mouse with methionine choline-deficient (MCD) diet or Western diet (WD) shows severe steatosis and high content of VLDL in a hepatic system with the increased expression profile of proinflammatory cytokines such as IL-1β, TNF-α, and MCP1 [70].

Role of dihydroceramide

Recently, a study claims that the dihydroceramide in triglycerides-enriched VLDL shows involvement in the signature of non-alcoholic fatty liver disease which was earlier considered for the type 2 diabetes-associated markers, and these TGs-enriched VLDL particles participate in the progression of atherogenic dyslipidemia [71, 72]. Contrarily, increased expression of dihydroceramide in diabetic patients also hampers the normal homeostasis of metabolic pathways of fat which results in the excessive amount of fat deposition in the hepatic system and ultimately leads to non-alcoholic fatty liver disease. The whole mechanism of dihydroceramide is still unclear, but the all-associated genes and proteins of dihydroceramide could be a better approach to finding the novel biomarker of non-alcoholic fatty liver disease associated with type 2 diabetes.

Gene expression studies

The genes involved in tissue remodeling processes and play their role in cell–matrix interaction show highly expression in NASH (F1–F4). Contrastingly, the genes involved in encoding of lipid protein metabolism seem to be downregulated. The differentiation between the early stage of NAFLD and NASH is the major question to find the novel biomarkers of the progressive stage of the liver disease. Conclusively, in early stage of NAFLD, most of the inflammatory pathways are activated and genes expressed abundantly, comparing with the NASH or the

Table 2 Showing some less studied biomarkers to differentiate between the early stage of NAFLD and NASH [73, 74]

Early stage of NAFLD and steatosis	NASH (F1–F4) stages
	↑Collagen, type 1, alpha 1 (COL1A1)
↓Complement component 4 binding protein beta	↑Collagen, type 1, alpha 2 (COL1A2)
↓Fibrinogen gamma/gamma chain (FGG)	↑Connective tissue growth factor
↓Lipid droplet-associated genes expression	↑Cytokeratin 18
↓Fibrinogen beta/gamma chain (FBG)	↑Chemokine C–C motif ligand 21 (CCL21)
↓Serpin family SERPING1	↑Chemokine C–C motif ligand 5 (CCL5)
↓SERPINC1	↑Chemokine C-X-C motif ligand 8 (CXCL8)
Lipoprotein A	↑Chemokine C-X-C motif ligand 9 (CXCL9)
↓Paraoxygenase family protein	↑Galectin 3
↓SERPINA1	↑A disintegrin and metallopeptidase 10 (ADAM 10)
	↑A disintegrin and metallopeptidase 17 (ADAM 17)
↑Low-density lipoproteins (LDL)	Oxidized low-density lipoproteins (Ox LDL)
	↓Paraoxonase 3 (PON3)
↑NF-kappa beta activation	↓Peroxisome proliferator-activated receptor alpha (PPAR-α)
↑Proinflammatory cytokines like Toll-like receptor-4 (TLR-4)	↓Cluster of differentiation 14 (CD 14)
	↓Lipoprotein A

later stage of the disease the certain new genes and proteins activated which are shown in Table 2 below.

Conclusion

Non-alcoholic fatty liver disease is a highly complicated mechanism that is linked to a wide range of metabolic disorders. As a disease progresses, it develops a variety of signs and cellular changes that disrupt the network of genes and proteins results to make complicated diagnostic criteria of each stage of the non-alcoholic fatty liver disease. We are unable to conclude the gold standard non-invasive diagnostic methods for the non-alcoholic fatty liver disease to their most severity form of liver cirrhosis till date. This review will open a window of new speculative notions in the field of non-alcoholic fatty liver disease to find the novel biomarkers by concentrating on the numerous genes and proteins that have previously demonstrated their potential involvement in disease state. Newly studies on gut microbiome show a lot of bacterial population and are altered in different stages, some of the bacterial population expression level is same in all stages of NAFLD, but some could be more specific, for example, the study on 16 NASH patient shows higher abundancy of *Allisonella* present in fecal [55]. These types of research need further study evidence on higher population for data reproducibility and accuracy which can be impactful to design a diagnostic kit using the stool sample. Another leading research studies on micro-RNAs in field of metabolic diseases, such as fatty liver disease, have shown highly encouraging results that can be used to find serum biomarkers for NAFLD/NASH. Apart from this, some of the miRNAs are detected at the later stage of the disease specially fibrosis, like miR-21, miR-192, and miR-146b. These could be good biomarkers to detect the fibrosis stage. Furthermore, that increased population of Tenericutes and Actinobacteria could be best findings for the diagnosis of liver fibrosis. Previously recorded data defined the role of dihydroceramides in type 2 diabetes mellitus; however, it participates in metabolic pathways through high content of TGs in VLDL particles and helps in disease progression, and it may be possible there could be a strong chance that patient with type 2 diabetes mellitus may develop fatty liver disease at later stage. Some less focused serum gene expression alteration in NAFLD including serpin family, lipid droplet-associated proteins (PLIN1-5), and paraoxygenase family proteins also need further study to prove the exact regulatory pathway hampered by those genes and the way in which better diagnostic biomarkers methods could be adopted that could replace the invasive techniques in diagnostic area.

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Author statement
We the undersigned declare that this manuscript is original, has not been published before, and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors, and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.
We understand that the corresponding author is the sole contact for the editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions, and final approval of proofs.

Aman Sharma
Corresponding author and on behalf of other authors.

Authors' contributions
All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by AS, PD, and TP. The first draft of the manuscript was written by AS, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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