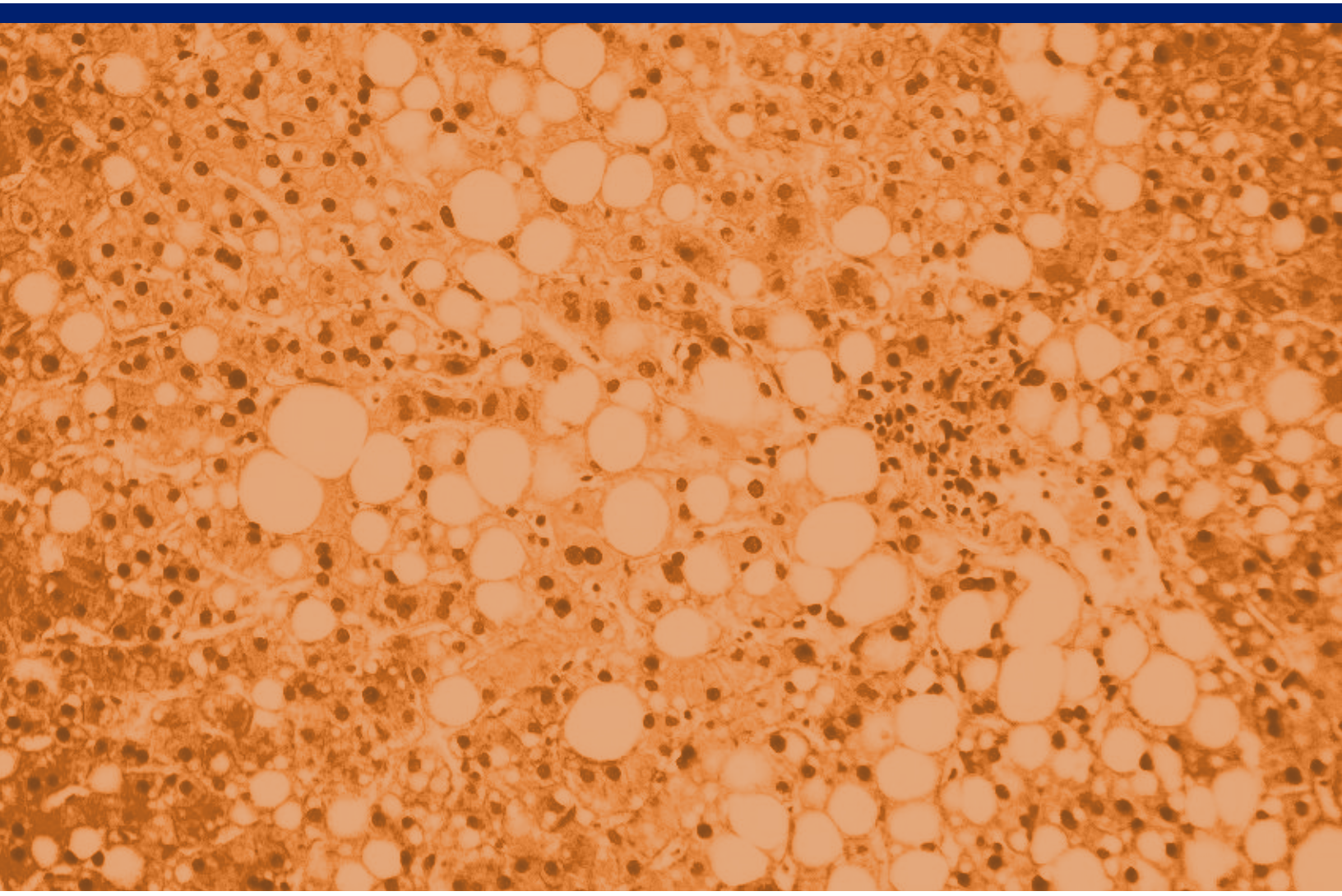
WHITE PAPER

NONALCOHOLIC FATTY LIVER DISEASE:
overview of biology and drug development landscape

Eurofins Central Laboratory



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DISEASE PREVALENCE AND ECONOMIC BURDEN

Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent chronic liver diseases, with a global incidence of around 25 % in the adult population – highest in South America and the Middle East, followed by Asia, the USA, and Europe[1,2] – and it is a major cause of mortality and morbidity worldwide. Its pathology can range from steatosis – abnormal fat retention – with or without inflammation, to the more aggressive form of nonalcoholic steatohepatitis (NASH), characterized by ballooning of hepatocytes, inflammation, and fibrosis, that can lead to cirrhosis or even hepatocellular carcinoma (HCC).

NAFLD is increasingly becoming a leading cause of end-stage liver disease and liver transplantation, in parallel with the rise in the general population of obesity, type 2 diabetes, and other metabolic disorders; prevalence is around 63 % in type 2 diabetes patients, and as high as 80 % in those who are obese.[3,4] Although individuals with a healthy body mass index (BMI) can also develop non-obese NALFD, this is usually associated with a pre-existing metabolic disease or a genetic predisposition.[5,6] The condition more commonly affects adults in middle age, however, the huge increase in child obesity in the western world has seen its prevalence in children rise to a recent all-time high of 7.6 %. Diagnosis at an early age also carries with it an added risk of developing other liver-related pathologies and comorbidities in adulthood.[7] A significant percentage of patients diagnosed with NAFLD will go on to develop cirrhosis and HCC over the course of the disease, which has a substantial impact on its economic burden, especially as the current rise in incidence is set to continue over the coming decade (**Figure 1**).

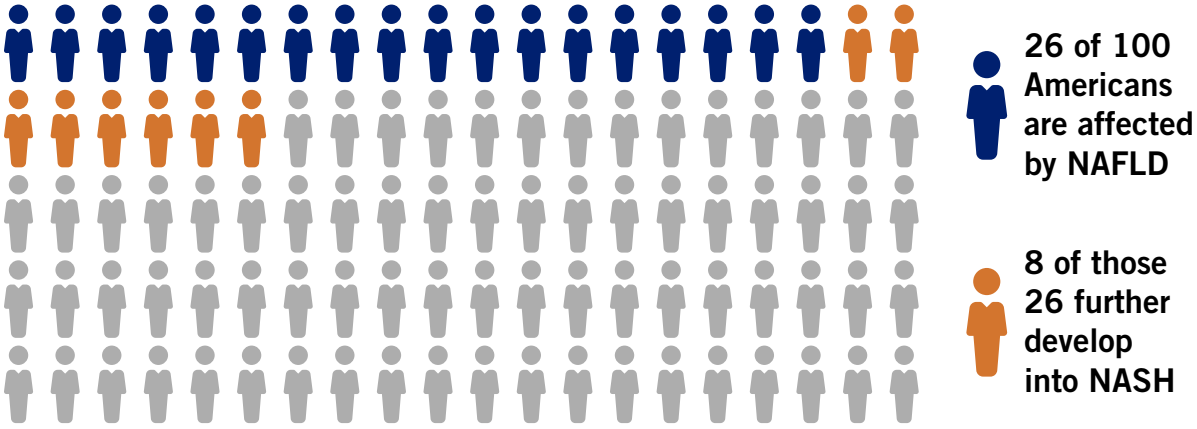


Figure 1: NAFLD affects up to 26% of all Americans, while up to 30% of those with NAFLD will further develop into NASH.[8]

In the USA, higher annual costs of care and resources for NAFLD patients have been affected hugely by the need for more outpatient appointments, imaging tests and liver biopsies.[9] In the UK, the cost of NASH alone ranged from between £2.3 and £4.2 billion in 2018, and the total associated wellbeing costs were estimated to reach up to £10.5 billion.[10] The impact of these diseases is such that retrospective clinical trials – like the global assessment of the impact of NASH (GAIN) in 2017 – were purposefully designed to address the socio-economic burden of NASH in adult patients, in both the USA and Europe. The NASH-associated cost per patient in the GAIN study was estimated at €25,521 and €73,255 for Europe and the USA, respectively. Costs were also found to correlate with the fibrosis stage reached by each patient, driven by the associated increase in hospitalization rates and comorbidities.[11]

Despite the health implications and economic burden of NAFLD/NASH, a targeted drug approved by the Food and Drug Administration (FDA) is yet to emerge. Current management of disease progression relies solely on diet and lifestyle modifications, which are subject to varying levels of compliance and so unreliable as a long-term treatment option.

NAFLD TO NASH: MECHANISMS OF DISEASE PROGRESSION

NAFLD is medically defined as the presence of over 5 % hepatic steatosis that is not caused by excessive alcohol consumption but, in reality, it encompasses a whole disease spectrum that can have different presentations and clinical outcomes. While most patients have stable liver disease for decades, a small proportion will go on to develop advanced fibrosis, putting them at greater risk of progressing to end-stage liver disease and HCC.[12]

The initial development of NAFLD is usually sparked by the deposition of fat in the liver, a process that can be directly associated with metabolic conditions such as obesity, type 2 diabetes, dyslipidemia, and hypertension. So far, three causes of excessive lipid accumulation have been identified: increased visceral adipose tissue lipolysis; hepatic de novo lipogenesis; and a diet rich in high-calorie fat.[13] Several factors are now known to correlate with the initiation of NAFLD and its progression to NASH (**Figure 2**):

Fat accumulation

One of the drivers of NAFLD is an unhealthy lifestyle, with low physical activity and a high-calorie diet causing an increase in adipose and fat accumulation that, combined with general low-grade inflammation, promotes insulin resistance, triglyceride (TG) breakdown and the formation of fatty acids (FFA). These events result in a general imbalance in glucose homeostasis and lipid metabolism; more FFAs need to be processed by the liver, and hepatic de novo lipogenesis increases, leading to general liver toxicity.[14]

Lipotoxicity and mitochondrial dysfunction

Lipid overload contributes to lipotoxicity and inflammation, and increases oxidative stress and the endoplasmic reticulum (ER) stress response, both leading to the release of reactive oxygen species (ROS). The rise in available FFA in NAFLD, that are typically broken down in the mitochondria, leads to further dysregulated ROS production by this organelle with a damaging effect on the hepatocytes.[14]

Dysbiosis and inflammation

Dysbiosis – changes in the gut microbiota – have been shown to influence the development of NAFLD, and its progression to NASH and HCC. Microbiome modifications associated with a dysregulated lipid metabolism, cholesterol production, glucose homeostasis, and changes in the intestinal barrier that allow the passage of bacteria or bacterial products into the portal circulation, have all been shown to increase liver inflammation. Obesity is also an important factor that can alter intestinal permeability and reduce the expression of antimicrobial peptides in the gut.[15,16] An altered immune response and abnormal activation of the inflammasome, as well as immune cell infiltration in the liver, also contribute to the typical hepatic inflammation seen in NASH.[12]

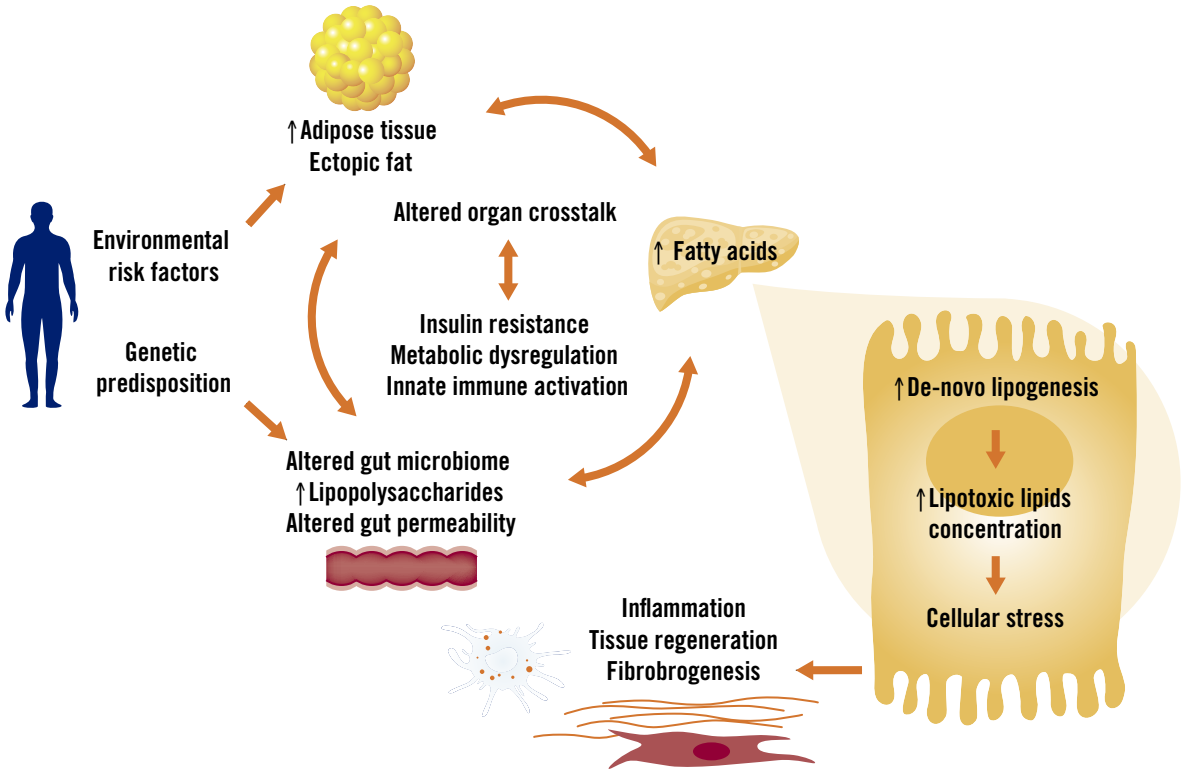
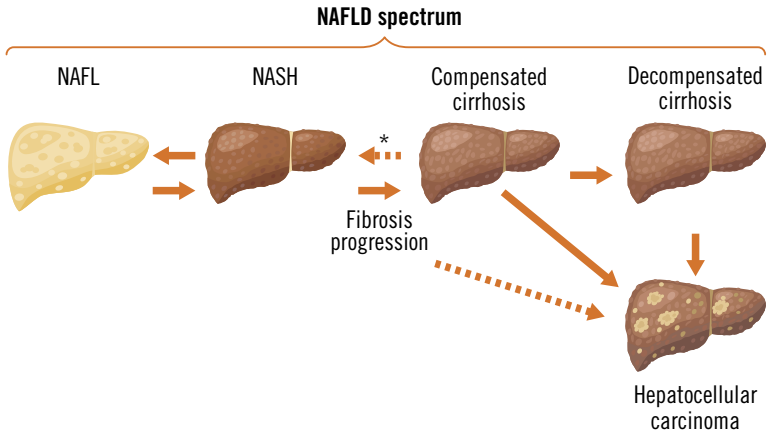


Figure 2: Pathways that affect the pathogenesis of NALFD. The genetic and environmental crosstalk between liver, adipose tissue and the gut can trigger inflammation and insulin resistance, resulting in fatty acid deposition and de novo lipogenesis. The formation of lipotoxic lipids then contributes to cellular stress, increased inflammation, and fibrosis.[17]

The evolution from the initial steatosis of NAFLD to NASH can also be affected by an orchestration of complex signals from the surrounding adipose tissue and the gut, with chronic inflammation and an abnormal microbiome being linked to obesity.18 Increased death of hepatocytes, inflammation, and fibrosis can all be caused by adipose tissue secreted factors or leakage of intestinal pathogens. The final events in the progression to NASH then see an activation of hepatic stellate cells (HSC) that, in turn, synthesize and deposit extracellular matrix (ECM), two of the main features of fibrosis.

RISK FACTORS AND DISEASE OUTCOMES

NAFLD diagnosis typically takes the course of a rule-out exercise for other liver conditions and alcoholic-related liver disease, and there are usually signs of hypertension, splenomegaly (enlarged spleen) and thrombocytopenia (low platelet levels). Clinicians rely on a combination of imaging – an abdominal ultrasound (US) scan, computerized tomography (CT) or magnetic resonance imaging (MRI) – and histology. The progression to NASH takes time – each disease stage lasting on average 7.7 years[19] – and can be dependent on each patient's risk factors (**Figure 3**).



Factors associated with NAFLD and NASH progression

Comorbid illness	Genetic factor	Environmental factors
• Type 2 diabetes	• PNPLA3	• Fructose
• Insulin resistance	• TM6SF2	• Cholesterol
• Dyslipidaemia	• GCKR	• Alcohol
• Obesity	• MBOAT7	• Exercise
• Hypertension	• HSD17B13	• Coffee
• Hypopituitarism		

Figure 3: NAFLD disease spectrum and risk factors associated with progression from NAFLD to NASH. Protective factors are highlighted in green.[17]

The leading cause of death in patients with NAFLD tends to be cardiovascular disease (5-10 %), followed by extrahepatic malignancy – colorectal or breast cancer – most likely due to the shared risk factors between these diseases, although the exact biological mechanism is still largely undetermined.[20] It is thought that type 2 diabetes, very low-density lipids, glucose overproduction, inflammatory factors, c-reactive protein, coagulation factors, and insulin resistance are all factors present in NAFLD/NASH that contribute to an increased risk of a negative outcome from a cardiovascular event. Liver-specific mortality has been reported at 0.77 and 11.77 per 1,000 person-years for NAFLD and NASH, respectively[21], and overall mortality tends to be three times greater in patients in later stages of fibrosis compared to those with no liver disease.[22] Finally, NAFLD-associated HCC carries a higher mortality risk and is predicted to become one of the major contributors to HCC in the US in the future. Despite these associated comorbidities and high mortality rates, a survey has found that NASH patients are the least likely to be screened for the development of HCC and the most likely to die while awaiting a liver transplant.[6, 23] It is also important to note that in a recent patient-reported outcomes survey, researchers revealed that NAFLD sufferers tend to have a reduced health-related quality of life compared to the general population, with higher rates of depression, emotional fatigue, and other systemic symptoms significantly reported.[24]

It is essential to find a better way of understanding the diversity of this disease – as well as the factors that influence its progression – in order to develop effective patient management programs that avoid worse outcomes and reduce the high economic burden. As it stands, it is still relatively common for individuals to remain undiagnosed for decades, even after cirrhosis is fully established, by which time treatment routes are significantly limited. Orthotopic liver transplant is still the only therapeutic option open for some patients with end-stage liver disease, despite it having limited success rates and associated risks.[25]

DIAGNOSIS AND DISEASE MANAGEMENT

Primary care is the first point of contact for all metabolic issues, and family physicians have an important role to play in the prevention, diagnosis, risk stratification, and management of NAFLD. Prevention advice for NAFLD/NASH generally includes a balanced diet in combination with physical activity. Once a patient is diagnosed, a risk stratification strategy is initiated by evaluating the degree of fibrosis or the presence of cirrhosis. In addition, because of the heterogeneity of this disease, management of NASH needs an all-inclusive approach that oversees cardiovascular risk, steatosis, general inflammation, and fibrosis. Since obesity is a central driver of the disease, a weight loss target of 5-7 % is typically recommended, but factors such as financial constraints and other comorbidities can make this difficult for some patients on a long-term basis. However, with no current FDA or European Medicines Agency (EMA) approved therapy for NASH, a lifestyle change is the default treatment for most patients, regardless of its limited success.

NAFLD is typically diagnosed using imaging techniques such as abdominal US or CT, which help to characterize the degree of hepatic steatosis in the liver. However, these methods have very low diagnostic specificity and are typically not used in isolation. MRI is more accurate and can measure low levels of fat deposition, but its high cost means it is currently only used in research and clinical trials, and rarely for diagnosis.[26,27] There are also a number of biomarker panels available that physicians can use to evaluate and assess disease staging. These include the fatty liver index – BMI, waist circumference, TG and gamma-glutamyl transferase (GGT); the hepatic steatosis index – BMI, diabetes, and liver enzyme panels; the SteatoTest – biochemical panel, age, gender, BMI; and several NAFL screening scores – stratification approaches combining a series of measurements.26 Once an NAFLD diagnosis is made, it is critical that a patient's fibrosis staging and scoring is performed – and that any other liver conditions are ruled out – for an appropriate management therapy to be put into place.

The gold standard methodology for the diagnosis of liver fibrosis is a biopsy. This test is invasive, it is known to be prone to high variability and operator-dependent, it has significant associated risks, and cannot be repeatedly performed throughout the course of the disease. For these reasons, other methods for fibrosis scoring of NAFLD patients that are more manageable and less invasive are now under rapid development. These include the NAFLD fibrosis score, the Fibrosis-4 (FIB-4) index for liver fibrosis, and the aspartate aminotransferase-to-platelet ratio index, all of which measure a variety of clinical and demographic factors, as well as routine laboratory parameters.[26] These scoring systems have been shown to have a high predictive value for advanced fibrosis in primary care settings.

Aside from these, blood biomarkers are also used to evaluate the degree of fibrosis in NAFLD patients such as the Enhanced Liver Fibrosis (ELF) score system that measures hyaluronic acid, tissue inhibitor of metalloproteinase 1 (TIMP1), and amino-terminal propeptide of type III collagen (PIIINP).[28] Despite being in use globally, the ELF scoring system is not yet FDA approved for fibrosis risk assessment in NAFLD patients. Imaging technologies too can be used to measure liver stiffness, such as ultrasound-based elastography and MRI, with transient elastography being the first choice for point-of-care testing.[26] More recently, a study has developed a novel scoring system – the FibroScan-aspartate aminotransferase (FAST) score – to identify patients with NASH and advanced fibrosis. This has shown some promising results in identifying high risk patients and, when approved by the appropriate regulatory authorities, it may help to select patients for specific therapeutics without the need for invasive liver biopsies.[29]

Inexpensive fibrosis scoring systems are typically the ones selected to stratify risk in NAFLD patients who can be easily managed in primary care. Patients with moderate to high risk will then go through a more comprehensive second-line fibrosis staging testing (**Figure 4**) before referrals are made to specialized clinics.

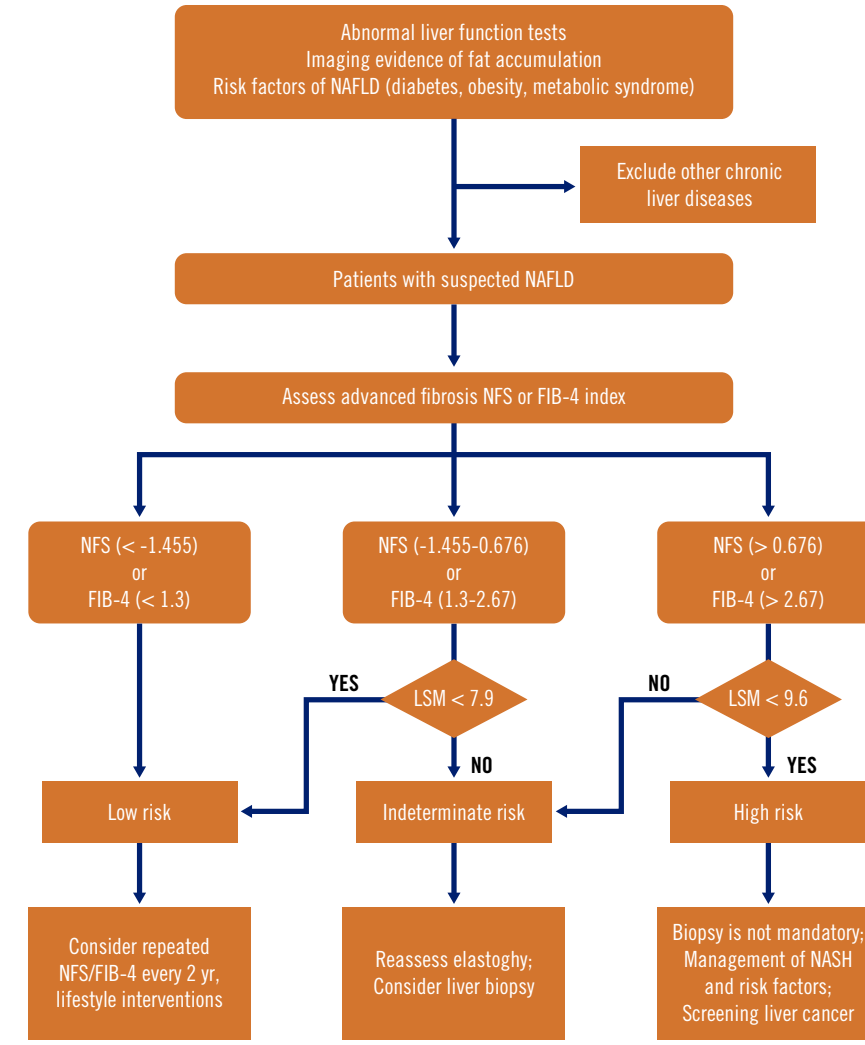


Figure 4: Diagnostic pathways for NAFLD/NASH using noninvasive scoring systems.[27] LSM – liver stiffness measurement.

Despite the availability of noninvasive biomarkers, there are currently no clear recommendations for a population-wide screen for NAFLD/NASH, most likely due to the lack of approved therapeutics. An exception is the American Diabetes Association, which has issued guidelines for monitoring liver enzymes on a regular basis in high-risk patients with type 2 diabetes in order to evaluate them for the early development of NAFLD.[30]

THE THERAPEUTIC LANDSCAPE OF NAFLD/NASH

While our knowledge of NAFLD and liver fibrosis mechanisms continues to increase, there are still many unknown variables surrounding diagnosis, progression, staging, and disease management. So far, the most effective therapy for NASH is weight loss, which has been shown to decrease hepatic steatosis and inflammation, and result in the resolution of early fibrosis, in around 50 % of patients. Patients who increase their weekly physical activity have also been shown to have lower liver enzymes, and this is associated with a reduction in all-cause mortality.[31]

However, for some, lifestyle changes are either not adequate or unsuccessful. There are a number of clinical trials currently underway for some new therapies and these are expected to emerge in the next three to five years. In addition, there are several off-label drugs that are available for other indications, which have been studied in phase 2b trials for NAFLD. Of these, ursodeoxycholic acid (UDCA), omega-3 fatty acids, and metformin have not shown much histological benefit but, in contrast, both vitamin E and pioglitazone have had some success, and are now endorsed by current guidelines as possible treatments for some NASH patients (**Figure 5, Table 1**).

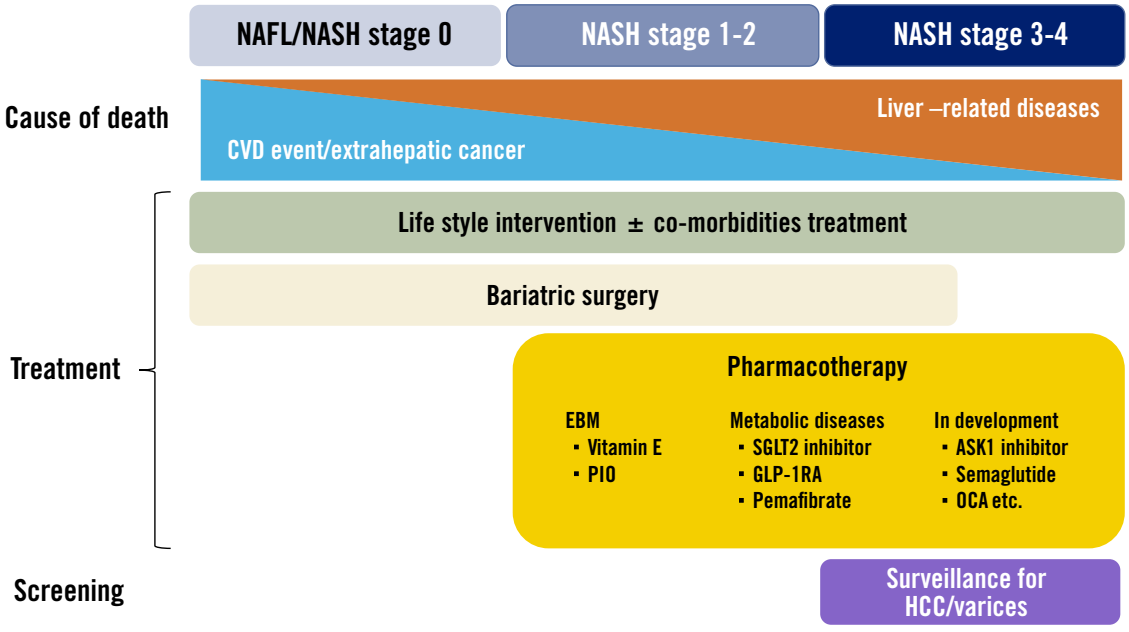


Figure 5: Fibrosis stage-based treatment selection for NAFLD/NASH.[32]

EVIDENCE OF SUCCESS IN OFF-LABEL THERAPIES IN NASH

Vitamin E

With antioxidant and free radical scavenging properties, vitamin E has been shown to improve steatosis and inflammation when taken for up to 96 weeks compared to placebo. It did not, however, have an impact on the reduction of fibrosis, and some adverse side effects were reported, including a link between high doses and an increase in cardiovascular events.[33]

Pioglitazone

Pioglitazone was shown to improve NASH activity and some studies point to a potential prevention of development of type 2 diabetes in these patients. A factor that limits its widespread use however is that weight gain is a known side effect, as well as the risk of bone loss related to negative effects on peroxisome proliferator-activated receptor (PPAR)-γ activation.

The reported side effects mean that it is unlikely that either vitamin E or pioglitazone will continue to be studied as potential therapies for NASH in phase 3 trials, though they can be prescribed at a clinician's discretion.[17]

UDCA, omega-3, and metformin

Although UDCA was shown to have an effect in improving alanine aminotransferase (ALT) levels in some patients, it had no impact on the progression of NASH. Omega-3 fatty acids reduced oxidative stress, inflammation and lipotoxicity in some patients, and they are currently used to manage hypertriglyceridemia but not as a treatment for NASH. Metformin, a commonly-used weak insulin sensitizer, was shown to diminish the progression to type 2 diabetes but had no significant effect on NASH.[33]

Glucagon-like peptide (GLP)-1 receptor agonists and sodium glucose co-transporter-2 (SGLT2) inhibitors

GLP-1 receptor agonists and SGLT2 inhibitors are currently under development in phase 2 and phase 3 trials to assess their efficacy in NASH resolution or improvement in fibrosis in patients with type 2 diabetes.[17]

	Effects on the liver	Quality of evidence	Other benefits	Key adverse events	Contraindications and cautions
Pioglitazone	Improves hepatic steatosis and necroinflammation, and can improve fibrosis	Several small* to moderate† phase 2 randomised controlled trials[116]	Improves insulin sensitivity and diabetic control	Weight gain, fluid retention, bone loss, and might increase bladder cancer	Contraindicated in patients with NYHA class III or IV heart failure; maximum dose 15 mg if used in combination with gemfibrozil or other strong CYP2C8 inhibitors
Vitamin E	Improves hepatic steatosis and necroinflammation; might prevent liver decompensation and mortality in patients with advanced liver fibrosis	Several small* to moderate† randomised controlled trials; data on clinical outcomes based on a retrospective cohort study with propensity score matching[116,117]	Neutral metabolic effects	A meta-analysis suggests a small increase in overall mortality at high doses; might increase risk of bleeding, prostate cancer, heart failure, and haemorrhagic stroke	Caution in patients with high cardiovascular risk and those at high risk of bleeding
GLP-1 agonists‡	Improves hepatic steatosis and necroinflammation	Several small* to moderate† randomised controlled trials[118]	Improves diabetic control, reduces major adverse cardiovascular events and weight	Nausea, vomiting, dyspepsia, diarrhoea, and constipation	Discontinue GLP-1 agonists immediately in case of acute pancreatitis; might cause acute kidney injury rarely; semaglutide might increase diabetic retinopathy complications
SGLT2 inhibitors§	Improves hepatic steatosis, necroinflammation, and liver enzymes	Several small* randomised controlled trials with non-invasive tests; two small* uncontrolled paired liver biopsy studies[119]	Improves diabetic control; modest weight reduction; might have renoprotective benefits; canagliflozin and empagliflozin reduce major adverse cardiovascular events	Genitourinary infection, acute kidney injury, and euglycaemic diabetic ketoacidosis; might increase the risk of fractures and limb amputations	Contraindicated if estimated glomerular filtration rate is less than 45 mL/min per 1·73 m²

NYHA=New York Heart Association. *Small was defined as less than 50 participants in the active group. †Moderate was defined as 50–100 participants in the active group.

‡For example, liraglutide and semaglutide. §For example, canagliflozin, dapagliflozin, and empagliflozin.

Table 1: Potential off-label therapies for NASH.[17]

Several other drugs are currently in the pipeline as potential therapies for NASH, with some trials showing promising results. Most of them are targeting pathways involved in disease progression, from lipid metabolism to inflammation and fibrosis (**Table 2**).

Metabolic Targets	Trial	Trial Name	Phase	Mode of Diagnosis	Determination of Resposnse to Treatment
FXR agonists					
Obeticholic acid – non-cirrhotic NASH	NCT02548351	REGENERATE	Phase 3	Biopsy	Histologic improvement
Obeticholic acid – compensated cirrhosis	NCT03439254	REVERSE	Phase 3	Biopsy	Histologic improvement
EDP-305	NCT03421431		Phase 2	Biopsy, MRI, PDFF, ALT levels	ALT
Tropifexor, in combination with Cenicriviroc (CCR 2/5 antagonist)	NCT03517540	TANDEM	Phase 3	Biopsy	Histologic improvement
Cilofexor, in combination with Firsicistat and Selonserib (acetyl CoA and ASKI inhibitor)	NCT03449446	ATLAS	Phase 2	Biopsy	Histologic improvement
Thyroid beta receptors (THR-β) selective agonists					
MGL-3196/Resmetriom	NCT03900429	MAESTRO-NASH	Phase 3	Biopsy	Histologic improvement
VK 2809	NCT02927184		Phase 2	MRI-PDFF	LDL-C
FGF-21					
BMS-986036, PEG-FGF21/Pegbelfermin - compensated cirrhosis	NCT03486912	FALCON 2	Phase 2	Biopsy	Histologic improvement
BMS-986036, PEG-FGF21/Pegbelfermin - stage 3 fibrosis	NCT03486899	FALCON 1	Phase 2	Biopsy	Histologic improvement
BIO89-100	NCT04048135		Phase 2	MRI-PDFF, Biopsy, centrals obesity + DM, ALT elevation, and or +fibrosis (>7KPa)	MRI-PDFF, triglycerides, LDL, ALT
Efruxifermin	NCT03976401		Phase 2	Biopsy	MRI-PDFF
FGFR-19					
NGM282, aldafermin	NCT02443116		Phase 2	Biopsy	MRI
FGFR-1/β-Klotho					
BFKB8488A	NCT04171765		Phase 2	Biopsy	Histologic improvement
PPAR agnostics					
Saroglitazar – PPAR α/γ	NCT03061721	EVIDENCES IV	Phase 2	Imaging or Biopsy	ALT level
Lanifibranor – PPAR α/γ	NCT03008070	NATIVE	Phase 2	Biopsy	Histologic improvement
Seladelpar – PPAR δ	NCT03551522		Phase 2	Biopsy	MRI-PDFF
Aldosterone receptor antagonist					
MT-3995	NCT02923154		Phase 2	Not specified	ALT levels
Lipid modulator/SCD-I inhibitor					
HTD-1801	NCT03656744		Phase 2	MRI	MRI
Aramchol	NCT01094158/NCT04104321	Aramchol1003/ ARMOR	Phase 2/ Phase 3	Biopsy/Biopsy	Magnetic resonance spectroscopy/Biopsy
AMPK (adenosine monophosphate-activated protein kinase)					
PXL770	NCT03763877		Phase 2	MRI-PDFF	MRI-PDFF
FASN					
TVB-2640	NCT03938246		Phase 2	Biopsy	MRI-PDFF
IBAT (ileal bile acid transporter) inhibitor					
Elobixibat	NCT04006145		Phase 2	Biopsy	Serum LDL-C
MPC (mitochondrial pyruvate carrier) modulator					
MSDC-0602K	NCT02784444		Phase 2	Biopsy	Histologic improvement
Glucose homeostasis					
GLP-I R (glucagon-like peptide-I receptor), Semaglutide	NCT02970942		Phase 2	Biopsy	Histologic improvement
NST-4016, Icosabutate	NCT04052516	ICONA	Phase 2	Biopsy	Histologic improvement
Anti-inflammatory/anti-fibrotic targets	**Trial**	**Trial Name**	**Phase**	**Mode of Diagnosis**	**Determination of Resposnse to Treatment**
BI 1467335, AOC3 inhibitor	NCT03166735		Phase 2	Biopsy, imaging, fibroscan	Levels of amine oxidase copper-containing 3
Hepastem	NCT03963921	PANASH	Phase 2	Biopsy	
GRI0621, NK cell R antagonist	NCT02949375		Phase 2	Diagnosis of liver disease	ALT
Galectin-3 inhibitor	NCT02704403	RESOLVE-IT	Phase 3	Biopsy	Histologic improvement and long term outcomes
Cenicriviroc – a CCR 2/5 antagonist	NCT03028740	AURORA	Phase 3	Biopsy	Histologic improvement

Table 2: Summary of therapeutic targets and novel emerging therapies in NASH.[34]

For a trial to succeed and attain drug approval by the FDA, it needs to show clinically meaningful benefit. The longevity of NASH progression means it can take decades for a liver-related death to occur and therefore trials in NASH need to use other surrogate endpoints, such as histology, to evaluate meaningful benefit. Typically, endpoints for NASH will include resolution without worsening of fibrosis, or improvement of fibrosis by one stage or more without the worsening of NASH. The EMA, however, still requires both endpoints to be met for drug approval. Current ongoing trials for NAFLD and NASH include several drugs that target various different pathways that have been shown to be mechanistically relevant for disease establishment and progression.

Drugs targeting insulin, glucose and lipid metabolism

Drugs that target the farsenoid X receptor (FXR) are currently under development in both phase 2 and 3 trials, including obeticholic acid (OCA). Although OCA is already approved for the treatment of primary biliary cholangitis, it has failed to achieve NASH resolution, despite showing improvement in some histological features. This observation, combined with noted side effects potentially related to cardiovascular events, has delayed its approval for the treatment of liver diseases.35 Other FXR agonists are currently under evaluation, both alone or in combination with other drugs. Thyroid hormone receptor agonist is also being tested as it is thought to improve NASH by increasing liver fat metabolism. Fibroblast growth factor (FGF)-21 and FGF-19 are under development to test their ability to improve glucose homeostasis and insulin sensitivity as well as fibrosis. Several PPAR agonists, involved in ketogenesis, lipid uptake and cholesterol catabolism, are now under phase 2/3 development with mixed results being reported.[34] In addition, statins are also under consideration and have shown some improvement in liver enzymes in a group of patients with a particular genetic predisposition. Finally, Firsocostat, an acetyl-coA carboxylase inhibitor, is showing promising data, with a reduction of liver fat by 29 % in a proportion of trial participants.[36,37]

Drugs modulating inflammation, apoptosis and fibrosis

Antagonists of the chemokine receptor (CCR)2 and CCR5 are currently being evaluated in a phase 3 trial after achieving one of its endpoints in phase 2. The mode of action of these drugs is based on their ability to lower macrophage infiltration in the liver, with a corresponding decrease in hepatic inflammation.[38] A vascular adhesion protein (VAP)-1 inhibitor is being studied in phase 2 to evaluate its ability to reduce the levels of specific biomarkers including amine oxidase copper-containing 3 (AOC3), ALT, aspartate aminotransferase (AST), alkaline phosphatase and GGT.[39] In addition, toll-like receptor

(TLR)-4 antagonists and a natural killer (NK) cell antagonist are also under development as potential therapeutics, combining the use of specific biomarkers to evaluate response to treatment.39 Finally, drugs that target cell apoptosis, and aim to reduce liver cell death – such as caspase inhibitors and apoptosis signal-regulating kinase 1 (ASK1) inhibitor – are currently under phase 2 and phase 3, respectively, to evaluate their potential in decreasing liver fibrosis, and linking histology with the NAFLD activity score (NAS) system.[39]

Drugs targeting the gut microbiome

Recent studies have found an association between microbiome composition and advance stages of fibrosis in NAFLD, and have identified several bacterial species that can vary according to disease stage. For this reason, some probiotics, prebiotics and antibiotics have been investigated preclinically and in human studies to understand their influence in disease progression. The use of antibiotics has had mixed results in studies and will need careful consideration in order to prevent potential antibiotic resistance. Fecal transplantation has been a promising treatment for microbial dysbiosis in NAFLD but has not been able to show any significant effects on insulin resistance or steatosis. Lastly, short chain fatty acids were found to influence NASH progression, by increasing GLP-1 and other insulin sensitizing peptides and reducing gut permeability in preclinical studies, however, results have been disappointing and inconsistent in patients so far.[40]

The realization that NASH is a growing cause of advanced liver disease has encouraged the development of new therapies. However, one of the main challenges in this field is still the continued reliance on liver biopsies for diagnosis, staging and study endpoint evaluation. As further studies – such as those mentioned above – help to clarify the pathways involved in disease progression, the number of targets to explore will also rapidly increase. A reliable biomarker or panel that could accurately help diagnose and stage NAFLD across the whole disease spectrum is yet to become available, but some that would help identify high-risk individuals are currently under evaluation.

BIOMARKERS

Any biomarkers for use in a clinical trial must adhere to the qualification program developed by the FDA center for drug evaluation research (CDER) to establish standards for validating their analytical measurements and clinical value.

Noninvasive tests currently available for the diagnosis of NAFLD and fibrosis

Evaluating the fibrotic stage of the liver is the most important determinant factor for liver-related disease progression, and for the development of other comorbidities like type 2 diabetes and cardiovascular disease. Some methods evaluate the degree of fibrosis by measuring liver stiffness in combination with generalized clinical and blood markers, for example, the AST-to-platelet ratio index (APRI) was initially devised for hepatitis C infection, but has been suggested as a predictor of fibrosis in NASH. The NAFLD fibrosis score (NFS) is now a demonstrated prognostic tool for fibrosis and the FIB-4 scoring system includes routinely available laboratory tests that measure AST, ALT and platelets (**Table 3**). In addition, the ELF scoring platform is also widely used, including an additional panel consisting of PIIIP, HA and TIMP1.

Test	Description	Accuracy	Advantages	Disadvantages	Guideline recommendation
Ultrasound	Hyperechoic texture or a bright liver	AUROC 0.93, Sn 85%, Sp 94% for diagnosis of steatosis[33]	Cheap; No radiation; Available; Easy to perform	Low sensitivity in individuals with steatosis < 20% or BMI > 40 kg/m^2; Observer-dependency; Influenced by fibrosis or iron overload	The first-line diagnostic test for diagnosing moderate and severe steatosis[32]
Computed tomography	Measurement of liver steatosis with attenuation values of liver and spleen	AUROC 0.99, Sn 100%, Sp 82% for diagnosis of steatosis > 30%[29]	Visualize the whole liver; Higher applicability; Quantify moderate-severe steatosis	Low sensitivity for light-moderate steatosis; Radiation exposure	NA
CAP	Measurement of liver steatosis with ultrasound attenuation by Fibroscan	AUROC 0.82, Sn 69%, Sp 82% for diagnosis of any steatosis[44]	Immediate assessment; Can be used in ambulatory clinic setting; Measure LSM simultaneously	Operator-dependency; Limited sensitivity; High failure rates in obesity patient; Low accuracy for quantifying steatosis; Uncertain cut-off values	The role of CAP for steatosis assessment is inclusive, more future studies are needed to define the role of CAP[32]
Magnetic resonance based techniques	Quantitative measurement of steatosis over the entire liver by adding parameter to MRI scanners	MRI-PDFF: AUROC 0.99, Sn 96%, Sp 100% for diagnosis of any steatosis[49] MRS: Sn 80%, Sp 80% for diagnosing steatosis ≥ 5%[58]	Not affected by obesity; Quantify assess steatosis over the entire liver; Lower sampling variability	Expensive; Time consuming; Device- and operator-dependency; Not suitable for patients with implantable devices	It is excellent to quantify steatosis, but the high price limits its application

AUROC: Area under the receiver operating characteristic curve; Sn: Sensitivity; Sp: Specificity; BMI: Body mass index; CAP: Controlled attenuation parameter; NA: Not applicable; MRI: Magnetic resonance imaging; MRI-PDFF: Magnetic resonance imaging-derived proton density fat fraction; MRS: Magnetic resonance spectroscopy; LSM: Liver stiffness measurement.

Table 3: Biomarker panels for the diagnosis of NAFLD-related fibrosis.[27]

Finally, FibroMeters, a family of blood tests that have been specifically designed for each liver disease, have a FibroMeterNAFLD specific for NAFLD and a FibroMeterV2G for hepatitis C, both of which have shown accuracy as noninvasive blood tests for the diagnosis and fibrosis staging in NAFLD. These panels measure analytes, such as urea, platelets, prothrombin time, hyaluronic acid (HA) and alpha 2 macroglobulin (A2M), in addition to AST, ALT, glucose and ferritin.[41] Other biomarkers that have been proposed for the staging of NAFLD-related fibrosis are PRO-C3 and NIS4. The first measures type III collagen neo-epitopes and it has been shown to have some accuracy in assessing disease stage and activity as a single diagnostic marker, performing similarly to the FIB-4 panel. NIS-4 comprises a panel of NASH-associated biomarkers such as miR-34a-5p, YKL-40, A2M, and glycated hemoglobin (HbA1c) and, although the role of the first two factors in disease are not yet determined, they are known to be elevated when patients develop liver fibrosis.

A study investigating and comparing these different options has found that the NFS and FIB-4 scoring systems faired better in determining the degree of fibrosis than others such as the APRI and BARD (**Table 3**).[42] However, there is still no clear noninvasive test that is both sensitive and specific, and that can be used for risk assessment without the need for biopsy.

The future of biomarkers for NAFLD-related fibrosis

Finding new biomarkers to detect fibrosis development in NAFLD patients is a huge focus for current research. Potential markers under consideration include Mac 2-binding protein glycan isomer (M2BPGi) – a factor secreted by stellate cells during fibrosis progression – autotaxin (ATX) – an enzyme that transforms lysophosphatidylcholine to lysophosphatidate and is involved in smooth muscle contraction – platelet aggregation and wound healing mechanisms, and thrombospondin 2 (TSP2) – involved in collagen/fibrin formation.[43]

In addition, a number of studies are looking into genetic factors that associate and predispose patients to NAFLD. Genome-wide association studies (GWAS) have shown correlations between some genetic variants and the development and severity of NALFD, with the most extensively characterized being PNLA3 1148. Carriers of this variant have double the risk of developing NAFLD and triple the risk of disease progression to NASH and HCC.[44] It is still early days for routine genotyping of NAFLD patients, and it is not yet recommended as a methodology for the diagnosis or prognosis assessments in NALD/NASH. However, with further research and availability of advanced technologies, there may be a future for genetic testing in these diseases.

Despite the availability of many potential biomarker and other noninvasive testing for fibrosis staging, a recent study surveying thousands of primary care physicians and specialized consultants found that knowledge of available noninvasive testing for NAFLD and NASH and referrals based on disease suspicion to specialized services were extremely low.[45] This shows there is an urgent need to raise awareness of NAFLD/NASH across all specialties and of available biomarkers for diagnosis and fibrotic assessment, to improve patient identification and address current difficulties related to NASH management and clinical trial enrollment.

LIMITATIONS OF CLINICAL TRIALS AND FUTURE CONSIDERATIONS

Before a drug is approved, it typically goes through preclinical trials, phase 1 safety studies and phase 2 dose escalation trials. Phase 2 trials aim to find the correct dose for a particular drug, and its efficacy. A phase 3, much larger, trial is then planned to compare the efficacy and safety of the new drug when compared to other existing therapies. Each stage has specific endpoints, or targets, that need meeting before proceeding to the next phase and being approved by independent regulators. Once approved, new therapies are considered to be available in the clinic as part of a phase 4 trial. This aims to test the drug in the real world, a process called 'post-marketing surveillance', in order to understand its efficacy and register any rare side effects. A successful phase 3 endpoint for NASH would require showing disease resolution, defined as the disappearance of ballooning and significant reduction of inflammation, with or without lessening of fibrosis.[46] As previously stated, the longevity of liver disease means this is impractical, and clinical trials are now incorporating other surrogate endpoints alongside liver stiffness, typically including some of the biomarker panels mentioned in (**Table 4**).

Outcomes	Hard endpoints	Surrogate markers
Clinical	All-cause mortality Liver-related mortality Hepatic decompensation Progression to cirrhosis	Child-Pugh score, MELD score HVPG TE, MRE Liquid biomarkers
Metabolic	Reduction of hepatic fat Improvement of insulin resistance Change of lipid profile Change of BMI	MRI-PDFF, multiparametric MRI CAP in TE HbA1c, fasting glucose, HOMA-IR
Inflammatory	Change of necro-inflammation Change of hepatocyte ballooning	Multiparametric MRI Liver enzymes
Fibrosis	Change of fibrosis stage	TE, MRE Liquid biomarkers

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; MELD, model for end-stage liver disease; HVPG, hepatic venous pressure gradient; TE, transient elastography; MRE, magnetic resonance elastography; MRI-PDFF, magnetic resonance imaging-estimated proton density fat fraction; CAP, controlled attenuation parameter; BMI, body mass index; HbA1c, glycosylated hemoglobin; HOMA-IR, homeostasis model assessment for insulin resistance.

Table 4: Endpoints in NAFLD and NASH clinical trials.[47]

Most trials in NASH still require liver biopsies to define participants and establish efficacy, and so patient recruitment is understandably limited. There are still challenges to overcome in defining appropriate clinical endpoints due to the lack of specific symptoms in NASH. This, added to other potentially confusing factors – for example, from alcohol intake, diet and physical activity – high placebo effects, and slow disease progression, makes trials in NASH challenging. Some studies have already started to try to incorporate noninvasive markers; the PROMETEO study aims to assess the repurposing of the oral antifibrotic Pirfenidone, a drug already approved by the FDA; and the CENTAUR study is focusing on cenicriviroc, a dual CCR2 and CCR5 antagonist.[48,49]

There is undoubtedly still a long way to go; more data is required to couple histology and biomarker panels, and to define specific criteria of response, including the magnitude of change, thresholds for assessment of risk, and to specify what changes are meaningful in response to different treatment. In addition, it is necessary to assess the cost-effectiveness of these methods.

NAFLD/NASH: THE FUTURE

A huge amount of progress has been made in the past decades on the understanding of the underlying disease biology of NAFLD, but there are still many challenges to overcome. There is a clear need for the implementation of strategies to identify and manage at-risk patients in primary care, tackle public awareness of these diseases, and to address risk factors, especially under the current obesity epidemic. One of the major barriers to the development of effective therapeutic drugs has been the continued use of liver biopsies for diagnostic purposes and the lack of reliable noninvasive biomarkers for the entire disease spectrum. However, a large number of phase 3 trials are underway, and there is the potential for novel and more effective therapies, along with better and less invasive tools for diagnosis and prognosis. A successful therapeutic for the treatment NAFLD/NASH will undoubtedly become available in the near future.

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