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TITOLO TESI

DIAGNOSTIC PERFORMANCE OF SEVERAL INDIRECT AND DIRECT BIOMARKERS OF FIBROSIS AND FIBROSCAN-BASED SCORES FOR THE DETECTION OF LIVER DISEASE SEVERITY IN PATIENTS WITH METABOLIC DYSFUNCTION-ASSOCIATED STEATOTIC LIVER DISEASE (MASLD)

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SUMMARY

CHAPTER 1

ABSTRACT

Background: The term Metabolic-associated steatotic liver disease (MASLD) refers to a wide range of phenotypic manifestations of liver disease associated with hepatic steatosis. These manifestations can vary from simple and indolent hepatic steatosis (MASL), to forms of liver damage characterized by necro-inflammatory activity, with or without the presence of liver fibrosis (Metabolic-associated steatohepatitis, MASH), and may progress to liver cirrhosis and hepatocellular carcinoma. MASLD is the most common liver disease in the general population, with a global prevalence of 37.8% [1]. The primary causes of mortality in these patients are cardiovascular diseases, followed by extrahepatic malignancies, and then by liver-related complications such as cirrhosis and liver cancer [2]. MASLD confers a high cardiovascular risk profile, not only due to the shared cardiovascular risk factors such as arterial hypertension, dyslipidemia, insulin resistance, and abdominal adiposity, but also through specific pro-atherogenic mechanisms [2]. The stage of liver fibrosis in MASLD patients has been identified as the strongest predictor of morbidity and mortality in this population [3]. Given its prognostic implications, the assessment of liver fibrosis has become the central focus for risk stratification in MASLD patients, as well as a target for therapeutic interventions and patient recruitment in experimental trials. Recent studies have found a prevalence of MASH with advanced fibrosis in 0.3%-1% of the general population; however, this prevalence is significantly higher in high-risk groups such as patients with obesity, type 2 diabetes mellitus, or endocrinopathies [4,5]. For this reason, recent guidelines recommend screening patients with evidence of steatosis and at least one metabolic syndrome factor to determine the presence of advanced liver fibrosis [6]. However, it remains challenging to distinguish which patients with MASLD have advanced fibrosis. Liver biopsy is still considered the gold standard for diagnosing both the severity of the disease and the staging of liver fibrosis, but it is associated with potentially serious complications and is costly, making it impractical for screening in the general population. To address this issue, in recent decades, non-invasive tests have been developed with the aim of predicting which patients are more likely to have advanced liver fibrosis. Some of these non-invasive tests are based on biochemical and anthropometric/anamnestic data, such as the AST/ALT ratio, the BARD score, the Fibrosis-4, the NAFLD fibrosis score, the HEPAMET fibrosis score, and the FORNS index. Others use elastographic ultrasound data, such as liver stiffness, AGILE 3+, and AGILE 4. Additionally, there are commercially available kits that assess certain molecules involved in the fibrogenesis process, such as the aminoterminal propeptide of procollagen 3 (PIIIP), hyaluronic acid, laminin, coliglycin, tissue inhibitor of metalloproteinases 1, and collagen IV. Some of these tests can be used in combination to predict advanced fibrosis, while others are still used in research settings.

Methods: In this context, a single-center prospective study was conducted with the aim of determining the prevalence of severe MASH (defined as MASH with at least F3 fibrosis and NAS ≥ 4) and evaluating the effectiveness of non-invasive tests for advanced fibrosis in patients at high risk for severe MASH. Between October 2021 and May 2024, 84 patients were enrolled at the Internal Medicine Unit with a metabolic focus at the Baggiovara Civil Hospital. These patients were referred by endocrinologists, obesity specialists, and diabetologists due to clinical, biochemical, or radiological suspicion of severe MASH. Anamnestic, anthropometric, biochemical, and elastographic ultrasound data were collected for these patients, and they underwent liver biopsy.

Results: MASH was found in 61 (72.6%) patients, at least F2 liver fibrosis in 44 (52.4%) patients, at least F3 fibrosis in 34 (40.5%) patients, and severe MASH in 20 (23.8%) patients. Individual metabolic syndrome risk factors were highly prevalent, as 77 (91.7%) patients were overweight/obese, arterial hypertension was present in 65 (77.4%) patients, the median HOMA was 4.08 [1-61], and overall, 59 (70.2%) patients had a confirmed metabolic syndrome. Among simple indirect biomarkers (GOT, GPT, platelets, albumin, APRI, and AST/ALT ratio), only GOT and APRI were able to significantly discriminate patients with severe MASH (AUROC 0.73 and 0.70, respectively, with p-value < 0.05), while among complex indirect scores (FIB-4, NFS, BARD, FORNS, and HEPAMET), only FIB-4 was able to significantly distinguish patients with severe MASH (AUROC 0.66, p-value 0.03). All direct biomarkers of fibrosis (collagen IV, colyglycin, hyaluronic acid, laminin, and PIIIP), except for laminin, were able to discriminate patients with severe MASH; however, the better diagnostic performance was demonstrated by PIIIP (AUROC 0.75, CI 95% 0.63-0.87, p-value < 0.01). Liver stiffness, as well as surrogate scores (AGILE 3+ and AGILE 4), demonstrated the ability to discriminate patients with severe MASH, but with modest diagnostic performance (AUROC ranging from 0.68 to 0.69, p-value < 0.05).

Conclusions: The conducted study demonstrated that the diagnostic performance of the scores proposed in the literature is inferior when applied to the detection of severe MASH, particularly in the context of a high prevalence of metabolic syndrome-related diseases. Additionally, the diagnostic performance of a diagnostic algorithm created by sequentially utilizing liver stiffness and PIIIP, with cut-off values determined through Youden's test, was evaluated. This algorithm showed improved diagnostic accuracy (81%), with a need for biopsy occurring in 21% of patients, yielding better results compared to the diagnostic algorithms proposed by current guidelines, which recommend the use of FIB-4 or NFS in conjunction with liver stiffness.

CHAPTER 2

INTRODUCTION

Epidemiology

The prevalence and incidence of metabolic-associated steatotic liver disease (MASLD) have progressively increased over the past 20 years. This trend is underpinned by the parallel rise in the prevalence of its risk factors in the general population, such as arterial hypertension, obesity, diabetes, insulin resistance, and dyslipidemia, as well as the concurrent reduction in the proportion of chronic liver diseases due to therapeutic advancements in the treatment of viral hepatitis. In fact, while nearly two decades ago the global prevalence of NAFLD was around 25%, by 2016, the prevalence had risen to 37.6% [7]. In Italy, the prevalence of NAFLD is estimated at 25.4% [8] and is projected to reach 29.5% by 2030 [9]. The incidence of MASLD is approximately 47 cases per 1,000 people per year in the general population [9]. In certain subgroups of populations, the prevalence of MASLD is significantly higher. In fact, among populations with type 2 diabetes mellitus, obesity, or metabolic syndrome, the prevalence of MASLD can reach as high as 70-90%, not to mention that MASLD can also be found in 10% of lean patients [7], who, when compared to their counterparts without MASLD, exhibit an increased risk for metabolic syndrome [10,11]. Several studies on MASLD patients with a BMI of less than 25 kg/m² have shown that this category of patients has an increased risk of all-cause mortality compared to the general population, and that liver disease tends to be more severe in lean MASLD patients compared to those with a BMI greater than 25 kg/m² [12,13]. Patients with severe obesity subsequently undergoing bariatric surgery have a MASLD prevalence that can reach 95% [14], while a cohort study conducted on 4 million people has shown that overweight or obese individuals have up to 7 times greater risk of MASLD [15]. Regarding global distribution, Latin America, along with the Middle Eastern and North African regions (44.4% and 39.9%), has the highest prevalence of MASLD, while North America has a prevalence of 32.7% and Western Europe 24.6% [16]. MASLD affects women to a lesser extent than men (25.6% vs. 32.9%) [1], while the age group with the highest prevalence is middle-aged men in their sixth decade, reaching a prevalence of 29.3% [17]. The peak prevalence in females tends to occur later, around the seventh decade, showing an increasing trend following the end of the fertile age, with a maximum prevalence of approximately 25% around the age of 60 [17,18].

Metabolic-associated steatohepatitis (MASH) has a more challenging epidemiology to estimate because, by definition, it requires liver biopsy for diagnosis. In the general population, the prevalence is estimated to be between 3% and 6% [7], with an increase in prevalence in at-risk subpopulations such as diabetics, the obese, or those with metabolic syndrome. Specifically, in patients with type 2 diabetes mellitus (T2DM), MASH may affect up to 37% of patients, with a prevalence of advanced fibrosis of 17% [19]. Liver fibrosis in MASLD has

become critically important in recent decades, as it has been shown to be the strongest predictor of mortality from both cardiovascular causes and liver-related causes [20].

In detail, patients with MASLD and advanced liver fibrosis have a cardiovascular mortality rate that is 1.5 times higher than that of the general population and a liver-related mortality rate that is 5 times higher [20]. Factors associated with liver fibrosis include low HDL levels, diabetes mellitus, and visceral obesity [21]. The difference between MASLD and MASH is also clearly expressed in terms of mortality. While the all-cause mortality and liver-related mortality rates in patients with MASLD are 17.15 and 0.77 events per 1,000 persons per year, respectively [22,23], in patients with MASH, these values are 25.56 and 11.77 events per 1,000 persons per year [24]. Hepatocellular carcinoma (HCC) is significantly more frequent in patients with MASH [25].

Etiopathogenesis

The presence of steatosis in more than 5% of hepatocytes, as observed in histological examination, or a proton density fat fraction greater than 5.6% defines MASLD. However, MASLD encompasses a broad spectrum of disease, with MASL and MASH at its extremes [6]. These two conditions have distinctly different prognoses [6].

MASH differs from MASL in that histological examination reveals lobular inflammation and liver damage, such as hepatocyte apoptosis and ballooning degeneration [26]. The progression of liver damage, which manifests as a worsening of the fibrotic component of the liver to the complete alteration of the original histological structure, as seen in cirrhosis, is still under investigation from a pathophysiological standpoint. It is believed to arise from various pathogenic stimuli and involve multiple cellular actors [27,28].

The liver contains various cell populations. Although the most represented cell type is the hepatocyte, which generally constitutes 80% of the overall population, the liver also includes sinusoidal endothelial cells, tissue macrophages (Kupffer cells), Ito's stellate cells, and Natural Killer (NK) cells [29,30]. In recent years, studies aiming to explain the genesis of fibrosis have identified Ito's stellate cells and tissue macrophages as key players, focusing on the balance these cells regulate—specifically, the maintenance of either a pro-inflammatory or anti-inflammatory state [31–34]. Kupffer cells are divided into two subtypes: the M1 subtype has a pro-inflammatory activity, while the M2 subtype has anti-inflammatory functions [35]. Normally, these macrophages perform the removal of bacteria and bacterial products arriving in the liver from the splanchnic circulation. However, when appropriately stimulated, through the secretion of cytokines and chemokines such as Interleukin (IL)-18, IL-12, IL-6, tumor necrosis factor-alpha (TNF-α), and IL-1β, they can mediate pro-inflammatory stimuli and direct an inflammatory response [36]. Ito's stellate cells, on the other hand, are generally quiescent but can be activated by inflammatory stimuli from molecules released by dying

hepatocytes. Once activated, they transform into fibroblast-like cells and contribute to collagen deposition [37]. In the context of MASH, the initiation of this cascade may be driven by lipotoxicity [38].

The Western diet, rich in fatty acids and calories, combined with a sedentary lifestyle, significantly contributes to the development of the disease. In fact, non-esterified fatty acids (NEFAs) reaching the liver originate from three different sources: endogenous, produced through lipolysis (60%), exogenous (14%), or de novo synthesis (26%) [29,39]. Another key factor in the pathogenesis of MASLD is insulin resistance. Insulin resistance occurs when, due to chronically elevated plasma glucose levels, the body is forced to increase insulin secretion to reduce blood sugar. Continuous insulin stimulation triggers adaptive mechanisms in insulin-sensitive cells, leading to a reduced number of receptors. Consequently, the previous insulin stimulus becomes ineffective in controlling plasma glucose concentrations, requiring further increases in insulin secretion. However, the effects of insulin extend beyond blood glucose regulation. At the level of adipocytes, insulin promotes lipolysis of fatty acids, leading to the release of NEFAs into the plasma, where they bind to albumin. NEFAs are taken up by hepatocytes through both active transport via fatty acid transport proteins and caveolins, as well as by passive diffusion [40].

Among fatty acid transport proteins, CD36 may play a significant role, as obese mice that were knockout for the gene encoding CD36 showed normal fatty acid transport within hepatocytes. Additionally, MASLD patients demonstrate an overexpression of messenger DNA for CD36 [41,42]. At the hepatocyte level, insulin stimulation leads to an increase in de novo lipogenesis, particularly using fructose as a substrate [43]. Within hepatic mitochondria, NEFA can be esterified into triglycerides or converted into phospholipids or ceramides. The fate of triglycerides can be twofold: either they are exported into the bloodstream via very low-density lipoproteins (VLDL) or internalized into the cytoplasm in the form of lipid droplets as a NEFA reserve. These two fates are decided at the level of the endoplasmic reticulum, influenced by various proteins such as microsomal triglyceride transfer protein (MTTP), transmembrane 6 superfamily 2 (TM6SF2), and the cargo receptor surfeit 4 (SURF4) [29,44]. The mutation of the genes encoding these proteins leads to forms of progressive liver damage such as hypobetalipoproteinemia and abetalipoproteinemia [45]. Regarding the storage of lipid molecules in the form of droplets, the presence of proteins associated with lipid droplets is required. Certain genetic mutations, the most well-known being that affecting the gene for patatin-like phospholipase domain-containing protein 3 (PNPLA3), result in resistance to the degradation of lipid droplets and are associated with progressive forms of hepatic steatosis [46].

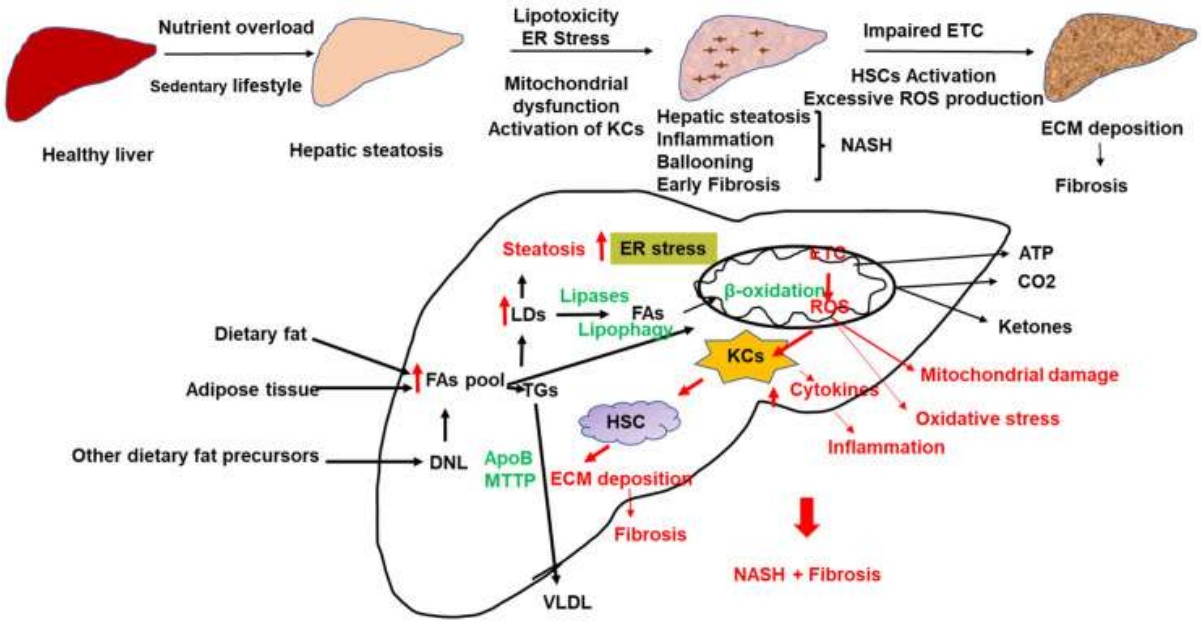


Figure 1. The pathogenic pathway of MASLD: Fatty acids in the liver are esterified into triglycerides and assembled into VLDL to be secreted into circulation, β-oxidized in the mitochondria, or stored in lipid droplets (LDs). With chronic lipid overload and insulin resistance, the hepatocyte becomes unable to dispose of the surplus fatty acids, leading to oxidative stress in the endoplasmic reticulum, which results in the release of damage-associated molecules and subsequent activation of the immune system. From Nassir et al. [29]

The composition of fatty acids within intrahepatic lipid droplets is predominantly made up of saturated fatty acids, including 16-carbon palmitate and 18-carbon stearate, which are implicated in lipotoxic effects and thus in disease progression, along with other lipid species such as diacylglycerol, lysophosphatidylcholine (LPC), and free cholesterol [47,48]. These molecules have damaging potential on hepatocytes and other liver cells, with the intensity of this damage being modulated by factors such as cytokines and gut dysbiosis [49]. Palmitate, for example, has a pro-apoptotic potential through positive feedback on factors like JNK, BIM, and p53 up-regulated of apoptosis, as well as through the degradation of normally anti-apoptotic proteins like Bcl-XL and Mcl-1 [39,50]. Palmitate can also upregulate the TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2), which may lead to activation of the extrinsic apoptotic pathway [51]. Furthermore, palmitate, with its long and saturated acyl chains, can induce oxidative stress in intracellular organelles [52]. The resulting cell death is another fundamental element in the pathogenesis of MASH; it has been demonstrated that knockout mice for the TRAIL receptor, despite being fed a high-fat diet and being obese, exhibited a reduced inflammatory response [53]. The role of palmitate as a co-protagonist in liver damage due to lipotoxicity is summarized in Figure 2.

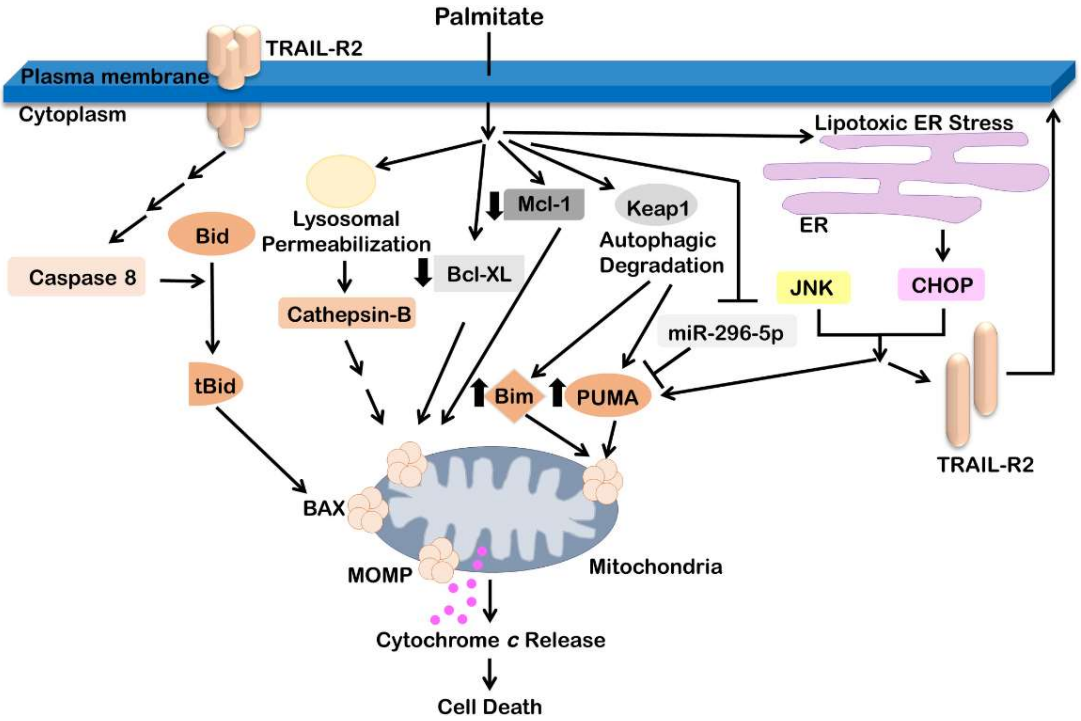


Figure 2. *Palmitate-Induced Lipotoxicity. Palmitate activates apoptotic receptors of both the extrinsic and intrinsic pathways, contributing to cellular dysfunction. The compromised integrity of the lysosomal membrane facilitates the release of the protease cathepsin B, while endoplasmic reticulum (ER) stress increases the expression of the pro-apoptotic transcription factor CHOP. This stress also promotes the activation of JNK kinase, which, in synergy with CHOP, induces the expression of the death receptor TRAIL-R2 and pro-apoptotic proteins from the Bcl-2 family, such as PUMA and Bim. The upregulation of these proteins is amplified by palmitate-induced autophagy, which degrades Keap-1. Concurrently, palmitate reduces the levels of anti-apoptotic proteins such as Mcl-1 and Bcl-XL. The oligomerization of TRAIL-R2 activates caspase 8, which converts Bid into tBid, leading to the activation of Bax on the mitochondrial membrane. The activation of Bax increases the permeability of the outer mitochondrial membrane, causing the release of cytochrome c, the activation of effector caspases, and culminating in cell death. BAX = B-cell lymphoma 2-like protein 11, Keap1 = Kelch-like ECH-associated protein 1, Mcl-1 = induced myeloid leukemia cell differentiation protein, MOMP = major outer membrane protein, tBid = truncated p15 BID. From Parthasarathy et al.* [39].

As previously mentioned, however, apoptosis is not the only mechanism implicated in the pathophysiology of MASH [31,32,54]. In fact, in a synergistic manner, apoptosis and inflammation mutually sustain the perpetuation of hepatocellular damage induced by lipotoxicity [39]. In MASH, as well as in other fibrosing liver diseases, the inflammasome plays an important role. The inflammasome contains intracellular pattern recognition receptors that induce the maturation of pro-inflammatory cytokines. One of these, the Nod-like receptor 3 (NLRP3), shows increased expression in patients with MASH [55]. Furthermore, experimental studies with NLRP3 inhibition have demonstrated a reduction in inflammation and fibrosis [39]. Similarly, the reduction of the expression of Toll-like receptors (TLRs) 4 and 9 has shown a decrease in liver steatosis and inflammation [56,57]. The activation of the inflammasome can also occur due to damage-associated molecular patterns (DAMPs) released, for example, from cellular apoptosis, pathogen-associated molecular patterns (PAMPs) increased in cases of increased intestinal permeability and intestinal dysbiosis, but also by lipopolysaccharides, mitochondrial DNA, cholesterol, and palmitate [58,59].

Role of the Immune System in MASLD/MASH

Fibrosis is a para-physiological process aimed at isolating a pathogenic *noxa* in such a way that it cannot lead to the progression of damage to other healthy areas. In the early stages of the pathophysiological process of MASLD/MASH, the role of the inflammatory response is to attempt to eliminate the pathogenic stimulus or at least limit the intensity of the damage. However, with the persistence of the *noxa*, there is a progression of damage that contributes to the development and severity of the disease. A pivotal role in the immune response in MASLD/MASH is played by Kupffer cells. These cells are tissue macrophages that contain elements of the inflammasome, including NLRP3, the activation of which leads to the secretion of IL-1β [60]. The Kupffer cell, when activated in this manner, becomes the conductor of the immune response and stimulates the deposition of fibrotic tissue [60]. Kupffer cells are capable of releasing substances that promote the chemotaxis of bone marrow monocytes; an important effector of this process appears to be CCL2 and its receptor, the inhibition of which in experimental models has shown an improvement in MASH [61].

Polymorphonuclear cells also participate in the immune response mechanism, especially in MASH. These cells can release extracellular traps composed of amino acids and antimicrobials (NETs) capable of confining pathogens. The concentrations of NET markers have been found to be increased in patients with MASH, while the inhibition of myeloperoxidase and neutrophil elastase—enzymes contained within neutrophils and released under appropriate stimulation—leads to a lower degree of inflammation and hepatic fibrosis in murine experimental models of MASH [61–63]. However, neutrophils do not only operate in the genesis and maintenance of liver damage; they also play a fundamental role in the removal of dead cells and promote the formation of new vessels [39].

Other effectors of the immune response are T helper (Th) lymphocytes. These cells can be divided into three subclasses with different roles: Th1 lymphocytes perform a pro-inflammatory role through the release of cytokines such as interferon gamma (INFγ), Th2 lymphocytes exert an anti-inflammatory role via the release of IL-4, IL-5, and IL-13, while Th17 lymphocytes can perform both actions depending on their environment. In MASH, a pro-inflammatory environment seems to prevail, stimulated by a predominance of Th1, which consequently stimulates inflammatory activity in Th17, leading to the release of IL-17 by the latter. IL-17 is an interleukin responsible for the fibrogenic action of hepatic tissue macrophages and hepatic stellate cells [64]. The role of CD8+ T lymphocytes in MASH has also been demonstrated by Ghazarian M. et al., showing that their suppression leads to reduced secretion of INFγ and TNFα, resulting in decreased inflammation, steatosis, insulin resistance, and activation of hepatic stellate cells [65]. The potential role of B lymphocytes in the pathophysiology of the disease remains unknown; however, B lymphocytes secreting TNFα and IL-6, with potential activators of T lymphocytes, have been observed in murine models of MASLD [66].

Cross-talk between adipose tissue and liver, and between intestine and liver

Adipose tissue has taken on an increasingly important role in understanding the pathophysiology of various diseases. Historically viewed as merely a means of storing energy in the form of lipids, it has been discovered that adipose tissue also produces several mediators capable of interacting with inflammation and metabolism. Adipose tissue plays a significant role in the development of MASH. In fact, it has been noted that a reduced ability to expand leads to increased lipolysis, resulting in the release of NEFA, which are subsequently converted by the liver into triglycerides and glucose [67]. However, the function of adipose tissue is not limited to its inability to expand. Molecules such as lectin and adiponectin secreted by adipose tissue itself can regulate body fat composition, insulin sensitivity, inflammation, and the amount of food consumed [68]. However, the important action seems to be performed by tissue macrophages, which, by releasing molecules such as TNF-α, IL-1β, IL-6, and CCL2, can induce insulin resistance and consequently alter lipid metabolism, initially locally and then systemically [69]. The homeostasis of lipids in adipose tissue is regulated by the action of three lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase, and monoglyceride lipase [29]. Insulin resistance can modify the lipolytic activity of adipose tissue, resulting in the release of a greater quantity of fatty acids into the bloodstream.

There is also a dialogue between the intestine and the liver. The intestine plays a role, not only in the absorption of nutrients and water, but also as a filter against bacteria and PAMPs. An altered permeability at this level results in an increased load of these products at the hepatic level, leading to the activation of macrophages and consequently triggering an inflammatory stimulus [54–57]. Moreover, bile acids secreted by the liver into the intestine and reabsorbed via enterohepatic recirculation, by acting on the Farnesoid X nuclear receptor, influence lipid and carbohydrate metabolism [66]. Lastly, it is worth noting that glucagon-

like peptide 1 (GLP-1), which regulates absorption, metabolism, and the amount of food ingested, is released by the intestine.

Genetic mutations responsible for MASLD/MASH

As previously reported, certain mutations in genes involved in lipid metabolism can lead to genetically determined forms of steatosis and liver damage. The most well-known is that of PNPLA3; however, other mutations have been reported in the literature. The variant of the gene for the regulatory protein of glucokinase (GCKR, P446L) results in an altered response of glucokinase to fructose-6-phosphate. The variant of Transmembrane 6 superfamily member 2 (TM6SF2) does not allow the dissociation of lipids from VLDL, consequently leading to their intracellular accumulation and an increase in histological damage [70–72]. Other genetic variants capable of causing hepatic fibrosis but not MASLD/MASH include the variant of ectozyme nucleotide pyrophosphate phosphodiesterase (ENPP1) and the variant of insulin receptor substrate-1 (IRS-1) 972Arg, genes normally involved in insulin receptor activity. The gene for membrane-bound O-acyltransferase domain-containing 7 (MBOAT7) codes for a protein that enables the remodeling of phospholipid fatty acid chains in healthy individuals, but in individuals with the rs641738T variant, it is associated with MASH and advanced fibrosis [73,74]. Variants of hydroxy-steroid 17-beta dehydrogenase 13 (HSD17B13) have shown an increase in intrahepatic fatty acids [75], while polymorphisms of superoxide dismutase 2 (SOD2), an enzyme important in preventing damage from reactive oxygen species, are responsible for MASH, steatosis, and hepatic fibrosis, similar to the polymorphisms of uncoupling protein 2 (UCP2) [76]. Finally, genetic polymorphisms involving other hepatic cells apart from hepatocytes can also lead to hepatic fibrosis. An example is the rs3480 A>G variant of fibronectin III, which is important in the formation of fibronectin by activated Ito cells and, if present, correlates with a higher stage of hepatic fibrosis [77].

Natural History of the Disease

Although it was traditionally believed that patients with hepatic steatosis without inflammation or signs of liver damage do not progress to MASH or advanced fibrosis, this progression can occur in 25% of cases [78]. A histological diagnosis of MASH correlates with fibrosis progression in 35% of cases and with stable fibrosis in 40%, maintaining the stage of fibrosis similar to that observed at diagnosis [79]. Some studies have shown that patients with MASH experience fibrosis progression by one stage within a median time of 7 years, while for patients with MASLD, this timeframe appears to be 14 years [80]. One of the contributing factors to disease progression is inflammatory activity, particularly as seen in histological examinations [79,81]. Indeed, an improvement in disease severity occurs when there is histological resolution of inflammatory activity and hepatocellular damage [82,83]. Some pharmacological trials have demonstrated that the progression from fibrosis stage F3 to F4 occurs in 22% of patients within a median time of 29 months. However, a regression

from cirrhotic stages to F3 has been observed in 10% of patients, and from F3 to more moderate stages of fibrosis in one-fifth of patients [84]. The terminal stage of the disease is liver cirrhosis, which affects 15-30% of patients with MASLD/MASH [79]. Compared to other chronic liver diseases (e.g., viral, autoimmune, or toxic liver diseases), cirrhosis occurs later in patients with MASH [79]. Factors such as diabetes mellitus and visceral obesity are strongly associated with disease progression. Histological improvements are seen with a 5% reduction in body weight, and complete resolution of steatohepatitis is possible with a 10% reduction in body weight [85]. Fibrosis remains the strongest predictor of mortality in MASLD patients, both for liver-related events and for all-cause mortality [86].

MASLD is also associated with other comorbidities; indeed, patients with MASLD have a 1.5-fold increased risk of chronic kidney disease (stage ≥ 3) compared to the general population, a 2-fold increased risk for liver cancer, and a 2.2-fold increased risk of diabetes mellitus [86,87]. Simon et al. demonstrated a 2-fold higher risk for malignancies in MASLD patients compared to the general population, with a predominance of extrahepatic malignancies [88]. Other studies evaluating the association between extrahepatic malignancies and MASLD have shown a link between colorectal cancer and MASLD [89]. However, the most significant association is between MASLD and cardiovascular diseases. Between 5% and 10% of patients with MASLD die from cardiovascular diseases, and MASLD patients have twice the risk of developing cardiovascular disease compared to the general population [90,91]. This strong relationship between cardiovascular diseases and MASLD is due to the fact that both conditions share many risk factors. Nevertheless, in this case, the most robust predictor of cardiovascular risk in MASLD patients is liver fibrosis [92].

Diagnosis

A clarification is necessary to better understand the terminology. Until September 2024, the European guidelines regarding the liver disease discussed thus far referred to it as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) [93]. The diagnosis of NAFLD/NASH is primarily one of exclusion, as it requires ruling out other liver diseases, despite its potential to worsen the prognosis of coexisting liver conditions. Currently, the diagnosis of NASH remains histological, with liver biopsy being the cornerstone of diagnosis, as it allows for staging and differentiating between uncomplicated NAFLD and more advanced forms. Steatosis affecting at least 5% of hepatocytes without evidence of liver injury defines NAFLD in its initial stage, where the risk of progression is minimal. However, if hepatic injury and lobular inflammation are present alongside this level of steatosis, with or without fibrosis, disease progression becomes possible [94]. When NAFLD/NASH is suspected, various liver pathologies must be excluded as secondary causes of liver disease. These include viral hepatopathies (hepatitis B and C), autoimmune liver diseases (primary biliary cholangitis, primary sclerosing cholangitis, autoimmune hepatitis), cholestatic liver diseases (progressive familial intrahepatic cholestasis), storage disorders (hemochromatosis, Wilson's

disease), endocrinopathies (hypopituitarism, hypothyroidism), iatrogenic causes, alcoholic liver disease (with alcohol consumption <30 g/day in men and <20 g/day in women), parenteral nutrition, celiac disease, α-1 antitrypsin deficiency, and malnutrition. In NAFLD, it is possible to observe altered values of markers typically associated with other liver diseases. For instance, mild elevations in ferritin levels and the presence of smooth muscle or antinuclear antibodies (ASMA and ANA, respectively) can be detected, with the latter found in up to 21% of patients [94,95]. However, antibody positivity in NAFLD/NASH patients is not associated with more severe disease [95]. In the context of a patient with NAFLD/NASH, it is essential to evaluate commonly associated comorbidities, such as central obesity, diabetes mellitus or insulin resistance, dyslipidemia, polycystic ovary syndrome, hypertension, hypothyroidism, and obstructive sleep apnea syndrome. These conditions should be appropriately managed to reduce the cardiovascular risk in NASH patients.

In September 2024, the European societies for the study of liver diseases (EASL), diabetes (EASD), and obesity (EASO) provided a more inclusive definition of NAFLD, now referred to as metabolic dysfunction-associated steatotic liver disease (MASLD) [6]. As previously mentioned, NAFLD/NASH was defined as a condition primarily characterized by the exclusion of other liver diseases. In contrast, MASLD is defined as a hepatic steatosis associated with one or more cardiometabolic risk factors, in the absence of harmful alcohol consumption [6]. The phenotypic spectrum of MASLD essentially mirrors that of the previous definition, ranging from isolated hepatic steatosis (Metabolic dysfunction-associated steatotic liver, MASL) to metabolic dysfunction-associated steatohepatitis (MASH), progressing to fibrosis, cirrhosis, and MASH-induced hepatocellular carcinoma [6].

This concept stems from two decades of evidence that MASLD is the hepatic manifestation of a systemic metabolic disorder [96]. Moreover, when reevaluating cohorts of patients with NAFLD using the inclusion criteria for MASLD, 99.8% of the patients fall under this definition [6]. For the diagnosis of MASLD, in addition to evidence of hepatic steatosis, one of the following criteria is required:

- Overweight or obesity: BMI ≥ 25 kg/m² or waist circumference ≥ 94 cm in men and 80 cm in women.
- Dysglycemia or T2DM: Prediabetes (if hemoglobin A1c is between 39 and 47 mmol/mol, or if fasting blood glucose is between 100-125 mg/dL, or blood glucose at 2 hours from the oral glucose tolerance test is between 140-199 mg/dL) or T2DM (hemoglobin A1c ≥ 48 mmol/mol, or fasting blood glucose ≥ 126 mg/dL, or blood glucose at 2 hours from the oral glucose tolerance test is greater than 200 mg/dL, or the patient is already on antidiabetic medications).
- Hypertriglyceridemia: if plasma concentration ≥ 150 mg/dL or use of lipid-lowering medications.
- Low HDL cholesterol: if HDL ≤ 39 mg/dL in men and ≤ 50 mg/dL in women or use of lipid-lowering medications.
- Hypertension: if ≥ 130/85 mmHg or receiving antihypertensive treatment.

This results in an expansion of the patient population, as NAFLD is included within the definition of MASLD, representing the "purest" form of MASLD since it does not present other concomitant liver diseases. However, in addition to NAFLD, all other causes of liver disease manifesting in patients with hepatic steatosis and at least one of the metabolic syndrome risk factors are also included.

Below is the diagnostic algorithm in cases of detected hepatic steatosis as proposed by the most recent European guidelines for MASLD [6]. As can be observed, once MASLD is suspected, it is necessary to perform a histological examination to distinguish whether this represents an indolent steatosis or a form of steatohepatitis.

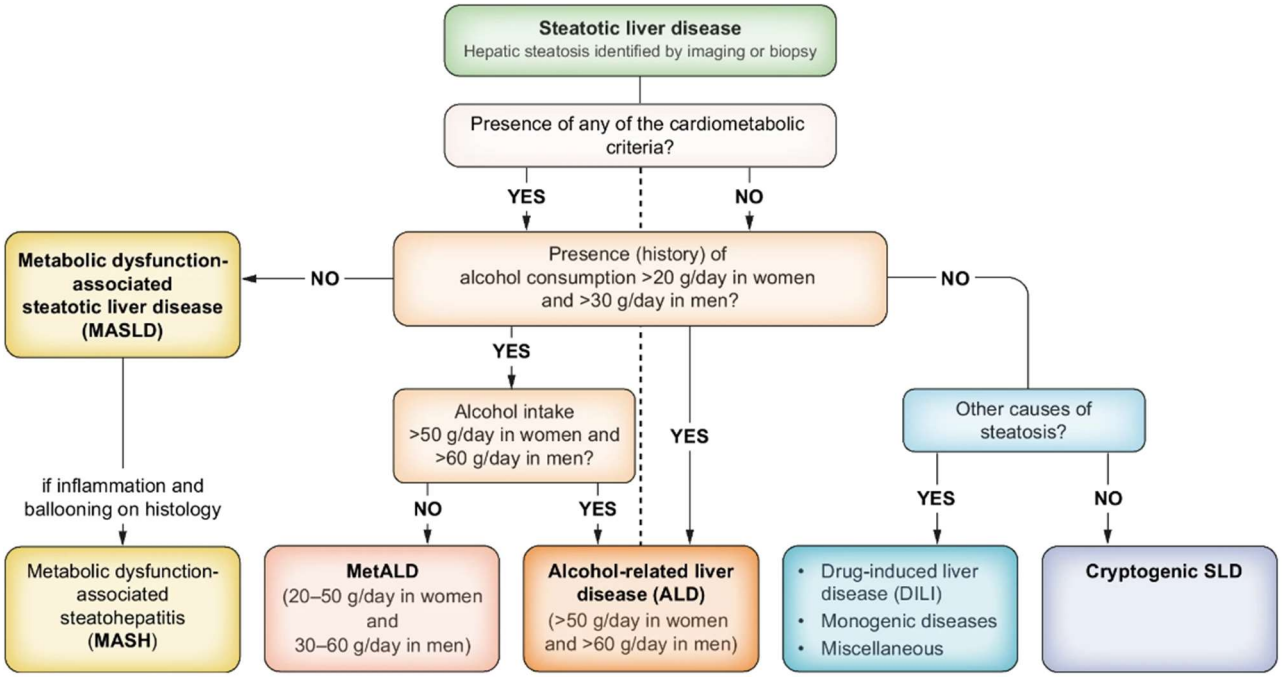


Figure 3. Proposed algorithm for the diagnosis of MASLD. From EASL guidelines [6].

Non-invasive biomarker diagnostic tests

Given the high prevalence of the disease and the invasiveness associated with liver biopsy, which poses significant risks to the patient, it is neither ethical nor economically feasible to subject all patients with hepatic steatosis to liver biopsy. Therefore, efforts have been made to identify non-invasive tests that could more accurately identify patients with steatohepatitis who require liver biopsy. In general, it has been observed that a single marker often lacks specificity and/or sensitivity in determining which patients may require further evaluation, leading to the development of scoring systems that incorporate various tests.

Regarding serum biomarkers, the first ones to be utilized were transaminases, particularly alanine aminotransferase (ALT) and aspartate aminotransferase (AST). ALT levels can be either elevated or normal in patients with MASLD/MASH, and several studies have demonstrated a poor correlation between ALT levels and the degree of fibrosis or lobular inflammation [97,98]. Therefore, the exclusive use of ALT is not

recommended in the context of determining the severity of the disease [97,98]. The same reasoning applies to AST, as these levels can also be elevated in patients with MASH, but they lack specificity. Both transaminases have shown a low area under the curve (AUC) for the diagnosis of MASH, ranging from 0.6 to 0.7 [99,100]. Alkaline phosphatase levels can reach values that are 2-3 times above the normal range; however, this marker is also poorly specific for the diagnosis of NASH [101].

Newer biochemical markers have shown more promising diagnostic power. Cytokeratin-18 (CK-18) reflects the activation of hepatocellular caspase 3 and has demonstrated good specificity, around 77-82%, with lower sensitivity of 66-75%, and a correlation with the histological severity of MASH [102–104]. Some markers related to collagen deposition/degradation have also shown interesting results. In particular, hyaluronic acid (HA) and the amino-terminal pro-peptide of procollagen III (PIIIP) can distinguish simple steatosis from advanced fibrosis [105]. Regarding PIIIP, it originates from the maturation process of collagen in the extracellular matrix, where an aminoprotease is responsible for cleaving this propeptide from collagen fibers [106]; its incomplete removal leads to the creation of non-functional fibrils that are subsequently removed [107].

In this regard, PIIIP can be understood as a dynamic biomarker of collagen deposition, remodelling, and destruction [107]. The measurement of PIIIP generally employs immunofluorescence techniques based on monoclonal or polyclonal antibodies that recognize sequences within the propeptide [108]. The diagnostic accuracy determined by AUROC in distinguishing any degree of fibrosis in the context of steatosis was found to be 0.76 [109]. If the diagnostic cutoff used is 20 ng/mL, the specificity in recognizing advanced fibrosis was found to be 96% [110]. Recently, Tanwar et al. demonstrated interesting results regarding PIIIP. Specifically, PIIIP has excellent diagnostic performance in discriminating F3 fibrosis (AUROC 0.9), distinguishing patients with NASH from those without NASH as assessed histologically (AUROC 0.78-0.83), discriminating patients with more severe forms of NASH, specifically those with a NAFLD activity score greater than 4 (AUROC 0.8-0.88), and with severe lobular inflammation (AUROC 0.86-0.9) [105].

According to the authors, this test would allow for discrimination between patients with simple steatosis and those with NASH and advanced fibrosis, with an AUROC of 0.85-0.87 [105]. As previously described, PIIIP is not specific to the liver, as it reflects collagen turnover to a certain extent. Indeed, elevations in PIIIP can also be observed in inflammatory conditions of the joints, for example [110,111]. Furthermore, since it is related to fibrosis, it is not specific to NASH, but may also be useful in other fibrosing liver diseases, such as cholestatic liver diseases. A study conducted on 137 children with cholestatic liver disease demonstrated that PIIIP is capable of discerning which of these patients had fibrosis; specifically, it showed the best performance when used to differentiate F4 fibrosis (AUC = 0.89), although excellent results were also obtained in distinguishing other degrees of liver fibrosis [112]. In the pediatric context, another study evaluated the diagnostic

performance of PIIIP in discriminating liver fibrosis in patients with NAFLD [113]. This study revealed that PIIIP identifies F2 and F3 fibrosis with AUROCs of 0.92 and 0.99, respectively [113].

Non-invasive radiological test

Radiological examinations have become fundamentally important in the diagnostic work-up of MASLD, as they can serve as the primary catalyst for disease identification and improve the precision in identifying patients who should undergo liver biopsy. The first-line examination is undoubtedly abdominal ultrasound. Ultrasound is an examination that is almost universally available, relatively inexpensive, non-invasive, and does not use ionizing radiation. However, it exhibits sensitivity that is influenced by BMI, with poorer performance observed in severely obese patients. It also demonstrates a certain degree of intra- and inter-observer variability and is capable of detecting hepatic steatosis when it involves at least 20% of hepatocytes, although the best diagnostic performance for detecting steatosis occurs when it involves at least 30% of hepatocytes [114,115]. In reference to this last point, the sensitivity is reported to be 80%, while it drops to 55% if steatosis involves 10-20% of hepatocytes [116]. An advantage of ultrasound is that it provides a comprehensive view of the abdomen and, therefore, can already indicate the morphology of the liver and the presence of alterations in the biliary tract or other organs at an early stage of the diagnostic work-up, allowing for the exclusion of other pathologies. The typical ultrasound characteristic of the liver with steatosis is hyperechogenicity, which is often referred to as a "bright liver" in this context. To define the echogenicity of the liver, it is necessary to compare it with the renal cortex, which generally appears iso-echoic [116].
Abdominal ultrasound can define the severity of steatosis, and this is often based on the degree of attenuation of the ultrasound beam induced by the presence of steatosis. Specifically, there are three different grades [116]:

- Grade 1, mild steatosis: increased echogenicity that does not attenuate the beam.
- Grade 2, moderate steatosis: the ultrasound beam is attenuated, resulting in reduced discrimination of deep structures; however, the diaphragm can still be visualized.
- Grade 3, severe steatosis: the ultrasound beam is attenuated by hepatic steatosis to such an extent that the diaphragm and intrahepatic vessels are no longer visible.

In addition to these visual echographic measurements, software has been introduced to quantify the degree of sound beam attenuation. One of these is the Controlled Attenuation Parameter (CAP) [116]. Among the echographic techniques of greatest relevance in the diagnostic work-up of MASLD, the Fibroscan certainly stands out. This is a cornerstone diagnostic tool as it allows for the assessment of liver stiffness (LS); in fact, diagnostic performance demonstrates excellent sensitivity in discriminating MASLD with advanced fibrosis (85%), while even higher values are obtained when the technique is used to identify MASLD patients with liver cirrhosis (sensitivity 92%) [117]. With reasonable certainty, it is possible to exclude any degree of fibrosis

with a high degree of probability if LS values are below 6 kPa. Conversely, with values above 9.9 kPa, the sensitivity, specificity, and AUROC in identifying liver fibrosis are very robust (97%, 92%, and 0.93, respectively) [118,119]. The measurement of liver stiffness must be performed in fasting patients for at least 6 hours, in the absence of ascites, pregnancy, or acute hepatic inflammatory processes, and by an operator experienced in the procedure. The technique is non-invasive, as it involves the delivery of small elastic waves to the thoracic wall, typically at the sixth intercostal space during expiration.

The gold standard for detecting hepatic steatosis is magnetic resonance imaging, as it can identify hepatic steatosis when it involves as little as 3% of hepatocytes, with a sensitivity of 92%-100% and a specificity of 92%-97% [119]. According to the guidelines, the diagnosis of hepatic steatosis can be made when the histological examination reveals a percentage of steatotic hepatocytes of 5%, or when the percentage of steatosis affecting the liver is equal to or greater than 5.6% when measured using the proton density fat fraction (MR-PDFF) technique. In a recently conducted experimental study by Park et al., the ROC obtained from MR-PDFF in recognizing hepatic steatosis when compared to biopsy was 0.99 [120]. Hepatic MRI is generally not used in clinical practice because it is a less accessible and very expensive examination, but it is more often reserved for experimental contexts.

Diagnostic Scores

As previously mentioned, clinical evaluation, medical history, biomarkers, and radiological investigations have limited diagnostic power when considered individually in identifying MASH, inflammation, and liver fibrosis. To assist clinicians in identifying these characteristics, scores that correlate biomarker, anthropometric, and/or radiological data have been validated. In this section, we will consider the most significant scores for the risk stratification of MASH.

The **FIB-4 index** is a score that was initially validated in cohorts of HCV and HIV patients to determine the risk of advanced liver fibrosis [121]. However, validation studies in patients with NAFLD have shown very promising results. In fact, one of the strengths of this score is its negative predictive value (NPV) of 90% when the obtained value is below the cut-off of 1.3, while the positive predictive value (PPV) for advanced liver fibrosis reaches 83% if the score exceeds 2.67 [122,123]. However, if the score falls between 1.3 and 2.67, an area of uncertainty arises in which it is advisable to use other tests. The FIB-4 index is one of the scores recommended by the European guidelines in the diagnostic work-up of NAFLD/NASH and now MASLD/MASH [6,93].

The variables required to calculate this score are age, AST, platelets (PLT), and ALT. The formula for the calculation is provided below.

$$\frac{\text{Age x AST (U/L)}}{\text{PLT (10}^9\text{/L) x}\sqrt{\text{ALT (U/L)}}} = \text{FIB-4}$$

Another score suggested by the guidelines is the **NAFLD fibrosis score (NFS)**. Unlike the FIB-4, this score was specifically developed to identify liver fibrosis in the NAFLD population. It also has a double cut-off; a lower cut-off of -1.455, below which the probability of advanced fibrosis is low, with a sensitivity of 90% and a specificity of 64%. Conversely, above 0.676, the probability of having advanced fibrosis becomes significantly higher, with a sensitivity of 60% and a specificity of 97% at this value, resulting in a positive predictive value (PPV) of 90% [97].

The variables considered include age, BMI, the presence or absence of diabetes, the AST/ALT ratio, platelet count, and albumin levels. The formula for the calculation is provided below. Utilizing this algorithm allows for the avoidance of 75% of liver biopsies; however, as with the FIB-4, there can be a grey area where approximately 20% to 60% of patients fall, in which the score is not discriminatory [124,125]. Younes et al. conducted a multicenter European study involving over 1,000 patients with NAFLD, of whom nearly 75% had NASH, finding that both the FIB-4 and the NFS demonstrated superior performance compared to other scores (AUC > 0.8) in discriminating fibrosis and that these two scores could also predict liver-related complications such as HCC [126].

$$\text{NAFLD Fibrosis score} = -1.675 + 0.037 \times \text{age (year)} + 0.094 \times \text{BMI (kg/m}^2\text{)} + 1.13 \times \text{IFG/diabetes (yes = 1, no = 0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet count (}\times 10^9\text{/L)} - 0.66 \times \text{albumin (g/dL)}$$

The **AST to platelet ratio index (APRI)** was initially validated for patients with HCV, where it is considered one of the best indicators of fibrosis; it was subsequently validated for patients with NAFLD [127]. The cutoff used in this case is 0.5, above which a sensitivity of 85%, a specificity of 71%, and an AUC of 0.86 were observed [128]. However, inferior performance has been noted in patients with type 2 diabetes mellitus [129]. This score has also been tested in patients with MAFLD, where the NPV was found to be 80% and the PPV was less than 50% [130].

The **BARD score** utilizes a scoring system whereby if the AST/ALT ratio is greater than 0.8, 2 points are assigned; if the BMI is greater than 28 kg/m², another point is awarded, as well as for the presence of diabetes mellitus. Thus, the score can vary from 0 to 4. The NPV of this score is 96%; however, the PPV is only 27% [131]. A validation study of the score demonstrated that the AUC of the BARD score is lower than that of the FIB-4 and NFS, standing at 0.76 [132].

The **FORNS index** is a more complex score to calculate, originally developed to discriminate fibrosis in patients with HCV. The validation study conducted by Forns et al. showed that with a cut-off below 4.21, the NPV was 96%, effectively excluding significant fibrosis, while for values exceeding the cut-off of 6.9, the PPV was 79% [133]. The calculation of the FORNS index relies on platelet count, GGT levels, age, and total cholesterol, as can be seen from the formula below.

$$\text{FORNS index} = 7.811 - 3.131 \times \ln(\text{platelet count}) + 0.781 \times \ln(\text{GGT}) + 3.467 \times \ln(\text{age}) - 0.014 \times (\text{total cholesterol})$$

The **Hepamet Fibrosis Score (HFS)** is one of the most recently validated scores for NAFLD. The validation study included 2,452 patients with NAFLD. In this study, the score demonstrated good performance in discriminating between patients with advanced hepatic fibrosis and those without, exhibiting an AUROC of 0.8, a specificity of 97%, a sensitivity of 74%, an NPV of 92%, and a PPV of 76.3% [134]. De la Tijiera et al. evaluated this score in a NAFLD population with slightly different general characteristics, as the population was younger and there was a higher representation of women; however, these findings were confirmed, with the exception of a significantly lower PPV of 36.7% [135]. The authors concluded that the addition of the HFS to an indeterminate FIB-4 or NFS could reduce the number of patients in this grey area by 10%. The calculation is complex, but online calculators are available to facilitate its execution.

$$1/(1+e[5.390-0.986\times Age[45-64yearsofage]-1.719\times Age[\geq 65yearsofage]+0.875\times Malesex-0.896\times AST[35-69IU/L]-2.126\times AST[\geq 70IU/L]-0.027\times Albumin[4-4.49g/dL]-0.897\times Albumin[<4g/dL]-0.899\times HOMA[2-3.99withnoDiabetesMellitus]-1.497\times HOMA[\geq 4withnoDiabetesMellitus]-2.184\times DiabetesMellitus-0.882\times platelets\times 1.000/\mu L[155-219]-2.233\times platelets\times 1.000/\mu L[<155]])$$

The **Enhanced Liver Fibrosis Test (ELF)** is a biomarker score that considers the levels of HA, tissue inhibitor of metalloproteinase 1, and PIIIP. If the test score exceeds 10.35, the patient may be referred to a hepatologist under suspicion of NAFLD with advanced fibrosis [136,137]. This test is recommended by both the English and European guidelines; however, it is not provided by Italian healthcare systems as it is considered more expensive than the FIB-4 [136,137]. The performance of this score is not well-defined and has been validated in populations with a high prevalence of fibrosis; however, some studies report a specificity of 80%, a sensitivity of 90%, and an AUROC of 0.9 [122]. According to Guha et al., the combination of NFS and ELF could enhance performance in identifying NAFLD with moderate fibrosis (AUROC 0.9) and NAFLD with severe fibrosis (reaching an AUROC of 0.98) [138].

The **AGILE 3+** and **AGILE 4** are two scores based on the measurement of liver stiffness and biomarker data, as well as the presence of diabetes and sex. The formulas for their calculation are provided below. The AGILE 3+ score considers liver stiffness (LS), AST, ALT, platelet count, gender, and age; the latter is not included in the AGILE 4 score. The AGILE 3+ has proven to be an excellent score for identifying F3-F4 fibrosis, whereas AGILE 4 is recommended for detecting F4 fibrosis and hepatic cirrhosis [139]. The score ranges from 0 to 1, and like the previous scores, it incorporates a dual cut-off system. For the AGILE 3+ score, a lower cut-off value of 0.45 is set, below which there is a sensitivity of 83-87%, a specificity of 75-78%, and a negative predictive value (NPV) between 87% and 90% for excluding advanced fibrosis. The upper cut-off is 0.67; above this threshold, the sensitivity ranges from 60-71%, specificity from 87-91%, and positive predictive value (PPV) from 79-81% for advanced liver fibrosis. Approximately 15-20% of patients fall within the interval of 0.45-0.67 [139].

Regarding the AGILE 4 score, as previously mentioned, the aim is to identify patients with NAFLD and F4 fibrosis or cirrhosis. The validation study included 2,700 subjects [139]. An AGILE 4 score lower than the lower cut-off of 0.25 demonstrated a sensitivity of 71-87%, specificity of 82-88%, and a negative predictive value (NPV) of 95-97%. Conversely, a score exceeding the upper cut-off of 0.56 discriminated patients with F4 fibrosis or cirrhosis from others, with a sensitivity of 44-55%, specificity of 95-97%, and a positive predictive value (PPV) ranging from 63% to 72% [139].

Regarding NASH-related cirrhosis, there is currently no score capable of distinguishing patients with NASH and cirrhosis from those without cirrhosis; therefore, AGILE 4+ is the first score that can achieve this. Below are the formulas for calculating AGILE 3+ and AGILE 4.

$$\text{Agile 3+} = \frac{e^{-3.92368+2.29714\times ln(E)-0.00902\times PLT-0.98633\times\frac{ALT}{AST}+1.08636\times Diabete\ status-0.38581\times Gender+0.03018\times Age}}{1+e^{-3.92368+2.29714\times ln(E)-0.00902\times PLT-0.98633\times\frac{ALT}{AST}+1.08636\times Diabete\ status-0.38581\times Gender+0.03018\times Age}}$$

$$\textbf{Agile 4} = \frac{e^{7.50139-15.42498\times\frac{1}{\sqrt{E}}-0.01378\times PLT-1.41149\times\frac{ALT}{AST}-0.53281\times Gender+0.41741\times Diabete\ status}}{1+e^{7.50139-15.42498\times\frac{1}{\sqrt{E}}-0.01378\times PLT-1.41149\times\frac{ALT}{AST}-0.53281\times Gender+0.41741\times Diabete\ status}}$$

Liver biopsy

The first liver biopsy was performed in 1883, and since then, techniques for obtaining liver tissue have significantly improved. Today, several techniques are available; in addition to the classic unguided biopsy, the most commonly used technique employs ultrasound guidance, with tomography being used to a lesser extent. Other techniques include the transjugular approach, as well as laparoscopy and laparotomy [140].

The cornerstone examination for the diagnosis and staging of MASH is liver biopsy [6,93,94]. This is because the information provided by the biopsy is unique, as it is the only method capable of grading hepatic

inflammation and staging hepatic fibrosis. Through liver biopsy, it is possible to confirm the suspicion of MASH-related liver disease, determine its severity, and monitor disease progression or the response to implemented treatments. Furthermore, it is one of the requirements for pharmacological trials involving MASH patients and allows for the exclusion of potential coexisting liver pathologies or the exclusion of a MASH diagnosis. Liver biopsy is an invasive procedure that has shown major complications in 1.4% of patients with MASH, including bleeding with hemoperitoneum or intrahepatic hematoma, as well as infections such as hepatic abscesses and pneumoperitoneum [141].

Approximately 60% of MASH cases are identified through biopsy analysis when patients with suspected MASH undergo a biopsy based on clinical indication; however, this percentage drops to 6.7-29.9% in the absence of such clinical indications [7]. Given the high prevalence of MASLD in the general population, along with the costs and invasiveness of biopsy, it is impractical to use biopsy as a screening investigation to determine which patients with hepatic steatosis also have steatohepatitis. Nonetheless, an adequately identified and treated MASH through an early biopsy increases the likelihood of developing only a moderate form of liver disease or progressing towards less severe forms of MASH [142]. In this context, the demand for liver transplantation is also reduced [142]. The accuracy of the biopsy sample is another important step; indeed, the histological specimen should contain at least 10 portal spaces [143].

As previously noted, fibrosis is the major predictor of both liver-related and extrahepatic mortality, and biopsy allows for precise staging of fibrosis. Four stages of fibrosis are defined. Initially, the deposition of fibrosis occurs in the pericentral region (or acinar zone 3), within the perisinusoidal space; this tends to occur only in NASH and not in other fibrosing liver diseases [144]. In the subsequent stages of fibrosis, the deposition of tissue expands to the portal and periportal zones, ultimately forming fibrotic bridges between portal-portal, central-central, or portal-central regions. The staining technique used for histological examination in NASH is Masson's trichrome. Finally, it should be noted that in the cirrhotic stage, the characteristic alterations of NASH may no longer be present, and the histological examination may reveal findings consistent with cryptogenic cirrhosis. In such cases, the patient's history and negative results for viral and autoimmune serological tests can suggest a diagnosis of cirrhosis related to NASH; however, biopsy may no longer be able to guide the diagnosis [145].

STAGING OF FIBROSIS	
STAGE	**FIBROSIS**
0	none
1	only perisinusoidal in zone 3
2	perisinusoidal and periportal in zone 3
3	bridging fibrosis
4	cirrhosis

Figure 4. Staging of fibrosis adapted from Burnt et al.

Fibrosis F1 can further be subdivided into:

1a: mild, zone 3, perisinusoidal

1b: moderate, zone 3, perisinusoidal

1c: portal/periportal

The images below depict some histological examples of the various stages of fibrosis. The images have been adapted from Brown et al. [146]**.**

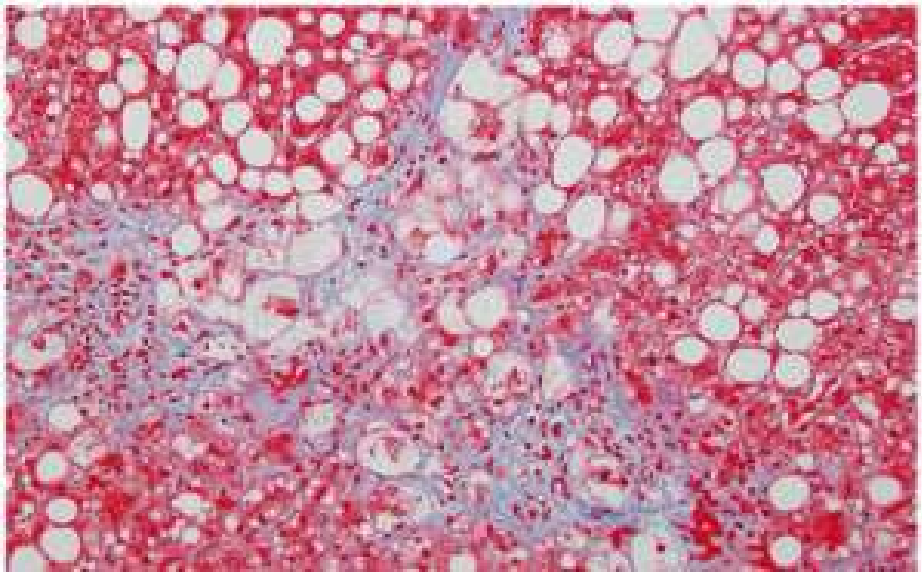


Figure 5. ***Fibrosis F1:*** *Perisinusoidal fibrosis with ballooning degeneration of hepatocytes, magnification 600x. Adapted from Brown et al.*

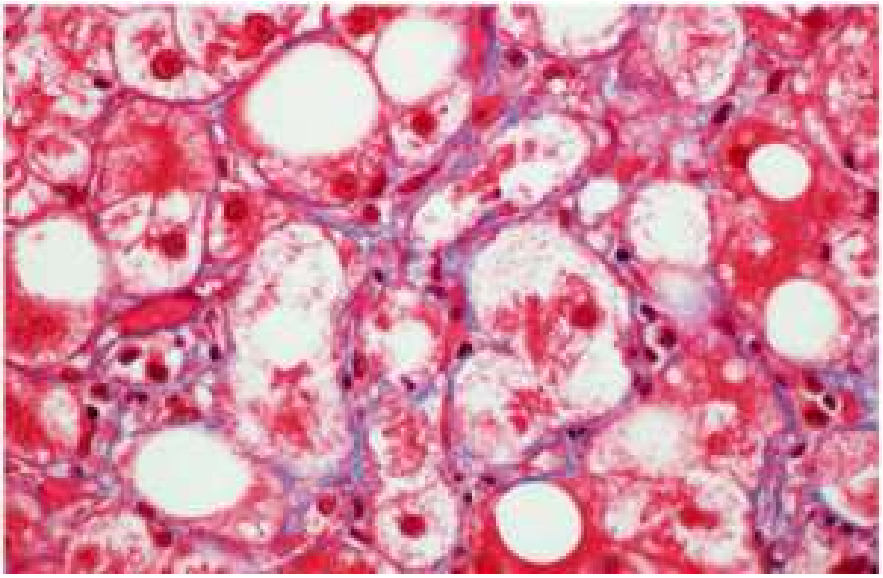


Figure 6. Fibrosis F2: advanced perisinusoidal fibrosis, magnification 200x. Adapted from Brown et al.

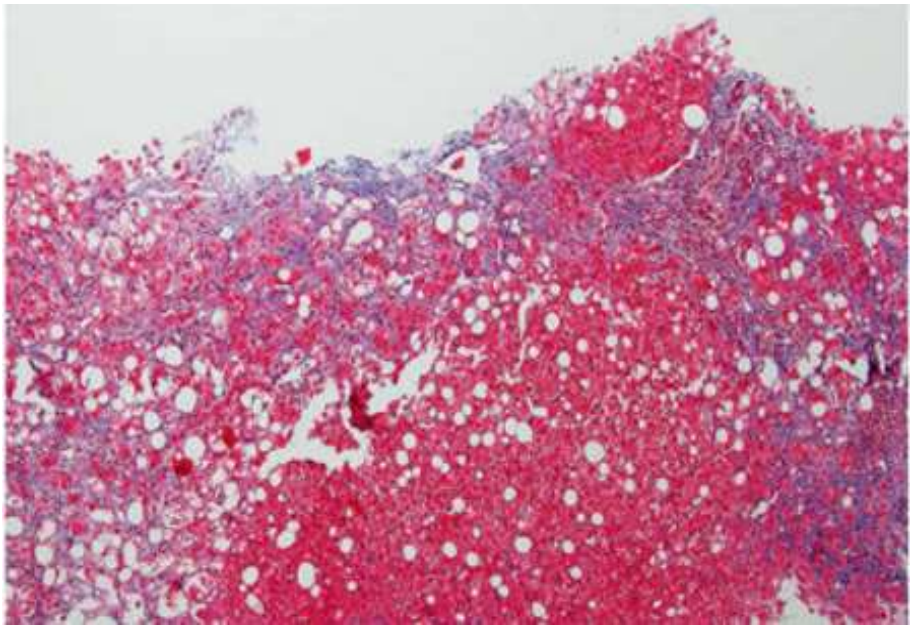


Figure 7. Fibrosis F3: bridging fibrosis with an extensive network of perisinusoidal fibrosis surrounding a regenerative nodule, magnification 100x. Adapted from Brown et al

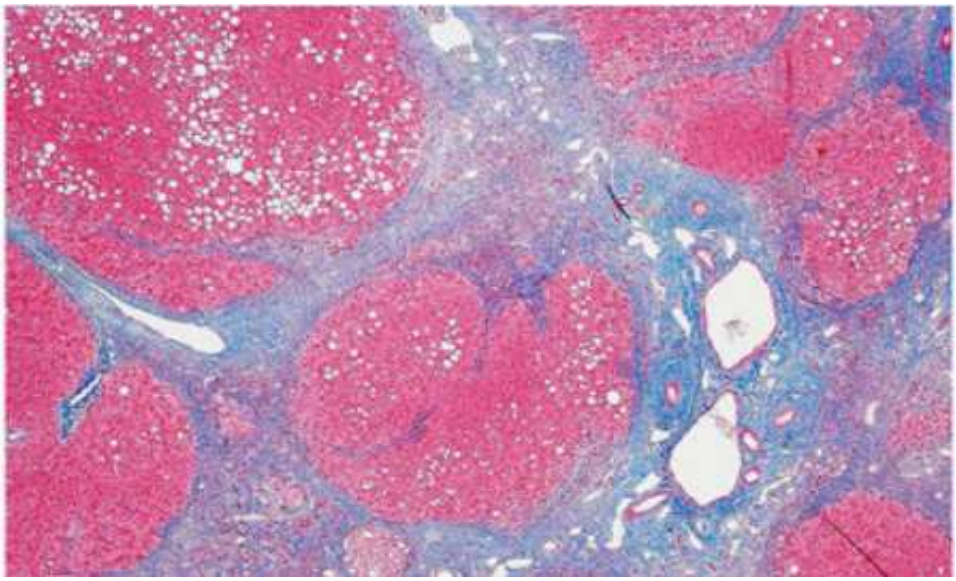


Figure 8. F4 Fibrosis: established cirrhosis, 40x magnification. Adapted from Brown et al

Among the additional information that can be obtained from the biopsy is the presence of steatosis. It is important to clarify that hepatic steatosis is not a pathognomonic feature of MASL as it can also be present in other liver diseases, such as alcoholic hepatitis. However, it is crucial to note that for a diagnosis of MASLD/MASH, steatosis must be present in at least 5% of hepatocytes [147]. More characteristic of hepatocellular damage induced by MASH are ballooning degeneration of hepatocytes, scattered inflammation, apoptotic bodies, and Mallory.

The immune cells commonly observed in histological examination are predominantly CD4+ and CD8+ lymphocytes, along with aggregates of Kupffer cells [148]. Polymorphonuclear cells can also be found near Denk bodies. Portal inflammation often correlates with the stage of fibrosis and, therefore, with disease progression [149]. Mallory-Denk bodies are eosinophilic cytoplasmic inclusions caused by malformed keratin filaments, heat-shock proteins, and chaperones. While these can also be found in other liver diseases, their presence in MASH is associated with a worse prognosis [150].

A key histological feature of MASH is ballooning degeneration. This refers to hepatocytes that are enlarged, with a lighter cytoplasm compared to normal hepatocytes, and fine eosinophilic strands [146]. The nuclei of hepatocytes affected by ballooning degeneration are larger and hyperchromatic compared to their healthy counterparts [146]. The presence of ballooning degeneration correlates with progression towards liver cirrhosis [151].

In the histological examination of MASH, other findings may include megamitochondria, apoptotic bodies, glycogenated nuclei, and iron deposition; however, none of these are specific alterations of MASH. The liver biopsy of a patient with MASLD may also reveal significant fibrosis without signs of hepatocellular damage or inflammation; this is often a classic indicator of disease regression, where MASH was previously active, but,

likely due to improved lifestyle and better management of risk factors, inflammation has subsided. This is typically referred to as "burned-out MASH" [93].

Histological Scores

Since the end of the last century, efforts have been made to identify standardized criteria capable of staging the histological severity of NASH. In 1999, Brunt et al. developed a system that included two main categories: grade and stage. The "grade" refers to the severity of the alterations associated with NASH, such as steatosis, ballooning, lobular and portal inflammation. The "stage," on the other hand, pertains to the distribution of fibrosis and defines five stages, ranging from the least to the most severe. The tables below present the grading and staging systems according to Brunt. One limitation of this system is its inapplicability to pediatric patients.

GRADE OF STEATOHEPATITIS				
GRADE	**STEATOSIS**	**BALLOONING**	**LOBULAR INFLAMMATION**	**PORTAL INFLAMMATION**
MILD	Macrovescicular in up to 66% of the biopsy	Occasional, hepatocytes in zone 3	Diffuse, both mild acute and chronic	None or mild
MODERATE	Any degree	Evident, of hepatocytes in zone 3	Mild, associated with ballooning	From mild to moderate
SEVERE	Tipically in more than 66% of the biopsy	Marked, primarily in hepatocytes of zone 3	From mild to moderate	From mild to moderate

Table 1. Staging of steatohepatitis grade according to Brunt classification. From Brunt et al. [152]

STAGING OF FIBROSIS	
STAGE	**FIBROSIS**
0	none
1	Only perisinusoidal in zone 3
2	Perisinusoidal and periportal in zone 3
3	bridging fibrosis
4	cirrhosis

Table 2. Staging of fibrosis according to the Brunt classification. Adapted from Elizabeth M. Brunt et al. [152]

Another system for determining severity is that proposed by the NASH Clinical Research Network (NASH CRN) [174]. Kleiner et al. aimed to create a system capable of evaluating the histological response to therapy using characteristic and reversible changes of NASH, proposing the NAFLD Activity Score (NAS). The score derived from these evaluations ranges from 0 to 8 and defines the NAS score, while for the staging of fibrosis, the classification system includes criteria comparable to those of the Brunt system [153]. Below is an example of the NAS score classification system.

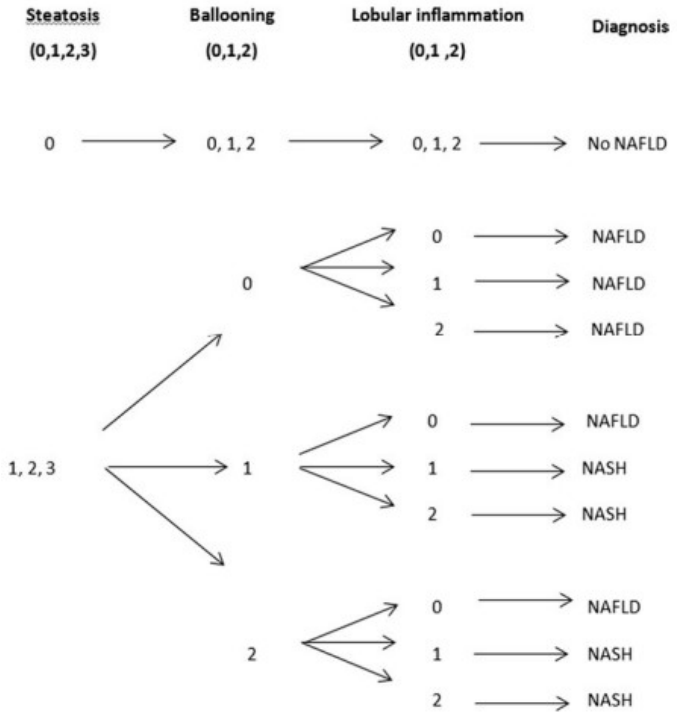
GRADE OR STAGE	BALLOONING	LOBULAR INLAMMATION	PORTAL INFLAMMATION	STEATOSIS	FIBROSIS
0	none	No focus	none	< 5%	Absent
1	mild	< of 2 foci	mild	From 5% to 33%	Perisinusoidal or periportal
2	very	Between 2 and 4 foci	More than mild	From 34% to 66%	Perisinusoidal and periportal
3		> than 4 foci		> 67%	Bridging fibrosis
4					cirrhosis

Table 3. NAFLD Activity Score (NAS). Adapted from Kleiner et al. [153]

Another histological score proposed is the SAF score. The SAF score evaluates steatosis (S), activity (A), and fibrosis (F) and was proposed by Bedossa et al. in 2012 [154]. In this score, steatosis can provide a score ranging from 0 to 3 based on its quantity in the total number of hepatocytes. The grading of steatosis occurs similarly to the NAFLD Activity Score (NAS), with 0 points assigned if steatosis is present in less than 5% of total hepatocytes, 1 point if steatosis is expressed in 5-33% of hepatocytes, 2 points if present in 33-66% of hepatocytes, and 3 points if present in more than 67% of hepatocytes. The activity of NAFLD is assessed both through the quantification of ballooning (from 0 to 2) and by evaluating lobular inflammation (from 0 to 2) to provide an overall score that ranges from 0 to 4 points.

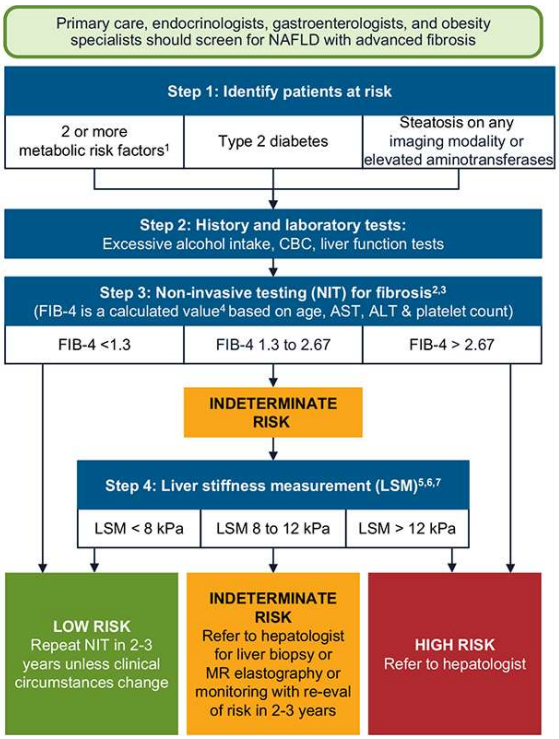
In detail, the ballooning score is characterized based on the severity of the alterations present in the histological examination, specifically: 0 points if the hepatocytes are normal; 1 point if the hepatocytes assume a spherical shape and contain a reticulated cytoplasm while still maintaining a normal size; and finally, 2 points are assigned if, in addition to the previous modifications, the hepatocytes also exhibit dimensions that are twice the normal size [154]. The lobular inflammation is classified based on the presence of two or more inflammatory cells within the lobule, as visualized at a magnification of 20x; this description defines the focus. If more than two foci are present within the lobule, 2 points are awarded, while 1 or 2 foci yield 1 point, and no points are assigned if no foci are present [154]. Based on these determinations, an algorithm has been

formulated, visible in the figure below, allowing for the diagnosis of NASH, NAFLD, or the exclusion of the NAFLD diagnosis [154].



Algorhytm 1. Diagnostic Algorithm for NAFLD/NASH According to the SAF. From Bedossa et al. [154]

Between the guidelines of the American Association for the Study of Liver Diseases (AASLD) from 2017 and 2021 and the guidelines of the European Association for the Study of the Liver (EASL) regarding indications for liver biopsy, significant differences arise. The AASLD emphasizes the utility of liver biopsy in patients at risk for NAFL/NASH and advanced liver fibrosis, particularly in patients with metabolic syndrome and a high risk of advanced fibrosis determined by non-invasive scores such as FIB-4 or NFS, or by elevated liver stiffness. This approach, however, is not mirrored in the EASL guidelines [94,155]. The EASL suggests an algorithm wherein all patients with NAFLD undergo fibrosis scoring using tools such as FIB-4, NFS, ELF, and fibro-test, if possible. Based on these values, liver elastography is performed, and only if a high value is found, is a liver biopsy deemed necessary [93]. Furthermore, the authors of the AASLD guidelines indicate that liver biopsy may be avoided in many cases, as there are currently no FDA-approved pharmacological therapies [155]. Liver biopsy can be repeated based on clinical circumstances; it may be useful for reassessing treatment response, excluding other overlapping liver diseases, or documenting disease progression. The guidelines recommend the possibility of repeating the biopsy after five years [93]. Below is an algorithm for the proper assessment of patients with hepatic steatosis.



Algorhytm 2. Flow chart for the hepatology referral of patients with suspected advanced fibrosis to undergo level III investigations. Adapted from Kanwal F et al., Shubrook JH, Adams LA, et al. [156]

Therapeutic Approaches

To date, there are no drugs specifically licensed for MASLD/MASH, although some molecules indicated for other conditions, such as diabetes, have shown promising results in reducing the activity of MASH at the histological level. The guidelines, however, propose various interventions aimed at least halting the progression of the disease. The approaches are based on maintaining a healthy lifestyle, with the intent of achieving weight reduction where necessary, controlling and improving cardiometabolic risk factors, preventing hepatic and extrahepatic complications, and potentially enrolling patients with MASH in clinical trials for the development of new molecules [157].

The cornerstone of MASH therapy is undoubtedly the improvement of lifestyle. By lifestyle improvement, two main concepts are intended: dietary modifications and physical activity. The guidelines emphasize this point because not only does weight loss led to improved cardiovascular risk, insulin resistance, and the prevention or amelioration of other obesity-related conditions, but it also results in the regression of damage induced by MASH [6,93,157,158]. A reduction of just 5% in body weight has been associated with a decrease in the NAFLD Activity Score (NAS), while a weight loss of 10% can lead to a reduction in MASH, including regression of hepatic fibrosis [159]. Even in non-obese patients, lifestyle improvements resulting in a weight loss of 3-10% have been associated with resolution of MASH [160]. To achieve weight loss, three approaches are recommended, which can be employed in various contexts: improvement of habits, use of pharmacological

therapy, and bariatric surgery. Regarding diet, a hypocaloric diet is advised, with a daily caloric deficit ranging from 500 to 1000 kcal, depending on the guidelines [6,93,94,161].

No specific diet is recommended, although a varied and balanced diet with an adequate intake of macronutrients and the use of unsaturated fatty acids is the most suggested; in this context, the Mediterranean diet has shown the most convincing results in reducing MASH and steatosis [162–164]. Alongside this, it is necessary to avoid the use of alcoholic beverages, discontinue smoking habits, and avoid drinks with high fructose content [6,93,94,161]. Physical activity is the other cornerstone of lifestyle improvement. The guidelines from the World Health Organization (WHO) recommend at least 150 minutes of physical activity per week [165].

The mode of physical activity execution can vary; for example, it is possible to perform continuous aerobic activity or high-intensity interval aerobic activity; however, no evidence has demonstrated the effectiveness between the two [166]. Physical activity leads to an improvement in insulin resistance, reducing hepatic de novo lipogenesis [159], improved lipid control due to increased clearance of VLDL [159], and enhancement of the cardiorespiratory system. All of this results in a modification of disease progression [159].

Bariatric surgery can be undertaken in selected contexts. Among the most commonly used procedures are sleeve gastrectomy, Roux-en-Y gastric bypass, laparoscopic adjustable gastric banding, and duodenal switch. Bariatric surgery induces caloric restriction due to the reduced amount of food that can be ingested and, in some cases, also due to malabsorption [167]. The effects of weight loss induced by bariatric surgery also extend to glycemic profiles, hypertension, dyslipidemia, and obstructive sleep apnea [167]. Interesting data regarding bariatric surgery have emerged from a study by Lee et al., which demonstrated a reduction in steatosis in more than 60% of patients, inflammation in 50%, fibrosis in 40%, and ballooning in 76%, although one in eight patients showed progression of fibrosis or development of MASLD [168].

The decision to proceed with a bariatric intervention must be carefully considered and often relies on psychiatric consultation, as frequent side effects such as malnutrition, diarrhea, malabsorption, and dumping syndrome, along with risks intrinsically related to the surgical procedure, are common [167]. The intervention is recommended in cases of BMI > 35 kg/m² or in diabetic patients with a BMI between 30-35 kg/m² but with poorly controlled diabetes on hypoglycemic therapy or with other cardiovascular risk factors [169].

Pharmacological Therapy Highlights

Currently, there are no approved specific medications for MASLD available in Europe, although in the USA, the FDA recently granted approval for the commercialization of resmetirom [170]. Regarding pharmacological therapy in patients with MASH, one of the objectives is to control cardiometabolic risk factors.

In patients with T2DM without stage IV renal failure, heart failure, and/or advanced lung disease, one of the first-line medications is metformin. It acts by increasing tissue sensitivity to insulin through the regulation of

AMP-activated protein kinase (AMPK). In this patient population, it has demonstrated a reduction in mortality, cardiovascular events, weight loss, and improvement in proteinuria [171]. In patients with MASH who start therapy with metformin, a modest reduction in steatosis and inflammation has been observed, while no differences have been noted regarding fibrosis or in terms of resolution of MASH. The guidelines conclude that there is no evidence to support the use of metformin in MASH, but that it may be continued in patients with T2DM already receiving treatment [6]. Incretin mimetic drugs, such as glucagon-like peptide-1 receptor agonists (GLP1RAs), are approved medications for the treatment of T2DM and obesity. This class includes liraglutide, semaglutide, and tirzepatide, all of which have shown improvements in cardiovascular and renal outcomes [172]. The main effect is an increase in postprandial insulin secretion, leading to appetite inhibition and a sense of satiety responsible for weight loss [173]. In general, these drugs have demonstrated a reduction, up to the resolution of MASH, but they have not improved the stage of fibrosis, likely due to the short duration of the trials, the longest of which lasted 18 months [6,174]. The combination of GLP1RA with glucose-dependent insulinotropic polypeptide (GIP), such as tirzepatide, has shown significant reductions in hepatic and visceral fat, with weight loss comparable to that of bariatric surgery and encouraging results regarding the resolution of MASH [174].

Regarding sodium-glucose cotransporter 2 (SGLT2) inhibitors used in patients with T2DM and renal impairment and/or heart failure, there are currently no pharmacological trials for MASH. They have shown reductions in weight, hepatic and visceral fat, as well as a decrease in ALT, likely due to renal loss of energy created by glucosuria [175–179].

The peroxisome proliferator-activated receptors (PPAR) are nuclear regulatory factors that modulate various systems, including carbohydrate and lipid metabolism, as well as the activation of inflammatory cells and fibrogenesis [180]. There are three subtypes of PPAR receptors: α, β/δ, and γ; the latter, in its isoform 2, is particularly expressed in abdominal and subcutaneous tissue, where it contributes to the redistribution of lipids between subcutaneous and abdominal fat, promoting the accumulation of triglycerides peripherally [180]. This receptor is also present in Kupffer cells. Three molecules are available that act as agonists of PPAR γ: pioglitazone, saroglitazar, and rosiglitazone, but only the first is commercially available. Pioglitazone has demonstrated histological improvement in steatohepatitis in various randomized controlled trials (RCTs), but no regression of fibrosis even after a period of three years [181–183]. However, there are no phase III studies in Europe, and in some European countries, the marketing authorization has been revoked. An enantiomer-R of pioglitazone is under investigation and has shown promising preliminary results regarding hepatic fibrosis but with side effects. The side effects of pioglitazone may include weight gain, hemodilution, and loss of bone mass in postmenopausal women [6].

Lanifibranor is a pan-PPAR, meaning it can activate all types of PPAR receptors. It has demonstrated improvement in steatohepatitis and associated fibrosis in a phase 2 study; however, it was also associated with a 2.5% weight gain, peripheral edema, and mild anemia [184].

One of the alterations that may present in patients with MASH is dyslipidemia; therefore, and due to its potential atherogenic nature, lipid-lowering therapy is often undertaken. The first-line drug is the statin, whose safety in MASH patients is well established. Although there are no randomized controlled trials (RCTs) that have evaluated the improvement of MASH and fibrosis with the use of statins in patients with steatohepatitis, case-control studies have demonstrated that chronic statin use is associated with a reduced risk of MASH, steatosis, hepatic fibrosis, hepatic insufficiency, and HCC [185,186]**.**

Vitamin E is a fat-soluble vitamin with antioxidant, anti-inflammatory, and anti-apoptotic activities. It is capable of reducing hepatic lipid content, and consistently high intake has been associated with reduced mortality in various conditions, such as cardiovascular diseases, cerebrovascular diseases, and cancers [187]. Patients with MASH and advanced hepatic fibrosis or cirrhosis chronically exposed to vitamin E have shown a lower incidence of hepatic failure and a reduced need for liver transplantation [188]. The largest RCT on the use of vitamin E in patients with MASH demonstrated a reduction in steatosis, enzymatic activity, and disease activity; however, the reduction of hepatic fibrosis was not assessed [188,189].

Obeticholic acid is a farnesoid X receptor agonist. It is approved for both primary biliary cholangitis and MASH, and studies conducted in phase 2 and phase 3 have shown improvement in fibrosis and liver enzymes after 18 months of treatment [190–192]. Despite improvements in ballooning degeneration and lobular inflammation, it does not lead to the resolution of MASH; moreover, it is associated with side effects such as itching and an increase in LDL cholesterol, the impact of which on cardiovascular risk is still unknown.

Finally, interesting data have recently emerged regarding liver-direct thyroid hormone receptor agonists. In patients with MASLD or MASH, it has been found that in cases of both clinical and subclinical hypothyroidism, there is an association with poorer outcomes [193]. This appears to be linked to the ability of thyroid hormones to reduce hepatic steatosis [193]. A specific subtype of thyroid hormone receptor, β, is expressed in the liver, and a molecule capable of acting as an agonist, resmetirom, has been evaluated [194]. Data from phase III studies have shown resolution of steatohepatitis with improvement in fibrosis stage and reduced progression of fibrosis compared to the control arm one year after the start of therapy [195].

The molecule received accelerated approval in March 2024; however, studies with outcomes based on a duration longer than 1 year are not yet available.

CHAPTER 3

STUDY DESIGN

Materials and methods

Between October 2021 and May 2024, a prospective observational study was conducted, enrolling outpatient patients from the participating Operative Units (Internal Medicine with a metabolic focus at the Baggiovara Civil Hospital (OCB) and Internal Medicine at the Pavullo nel Frignano Hospital). These patients were referred by endocrinology, obesity, and diabetes specialists from the Baggiovara Civil Hospital. The U.O. of Internal Medicine at the Pavullo Hospital mainly served a coordinating role, being able to enroll patients but then referring them to the U.O. of Internal Medicine at the Baggiovara Hospital. Therefore, the main site for patient enrollment, diagnostic investigations, and follow-up was the U.O. of Internal Medicine with a metabolic focus at the Baggiovara Civil Hospital.

The following Operating Units of the Azienda Ospedaliero-Universitaria Policlinico di Modena were involved:

- CORELAB Analysis Laboratory
- Radiology Operating Unit for performing some liver biopsies
- Pathological Anatomy for the analysis of biopsy specimens

Inclusions criteria

Adult patients aged between 18 and 75 years were enrolled, who were referred for specialized evaluation by obesity specialists, diabetologists, or endocrinologists from the Endocrinology Unit of the OCB with a diagnosis of MASLD and clinical, laboratory, or radiological suspicion of MASH and/or significant fibrosis, for whom a diagnostic liver biopsy was indicated. The diagnosis of MASL was made by excluding other liver diseases and with radiological evidence of hepatic steatosis, while the suspicion of MASH with significant fibrosis was based on the presence of multiple metabolic risk factors and their duration over time and/or alterations in liver enzyme levels and/or non-invasive test (NIT) results compatible with significant fibrosis, for which a liver biopsy was indicated [93]. The liver biopsy was performed under ultrasound guidance and after obtaining adequate informed consent. A conditio sine qua non for enrollment was the performance of the liver biopsy.

Exclusions criteria

Patients who did not consent to undergo liver biopsy, those under 18 years of age or over 75 years, pregnant women, patients with severe comorbidities that limited their participation in the study, patients with a reduced life expectancy, and those with evidence of secondary causes of liver disease (positive for HBsAg,

HCV RNA, HIVAb, autoimmune liver diseases, cholestatic liver diseases, genetic liver diseases, and hemochromatosis) were excluded from the study.

Study design

Patients enrolled based on the inclusion criteria underwent several assessments:

- A detailed medical history regarding dietary habits and physical activity through the completion of a 3-day food diary [196] to evaluate dietary habits and a standardized questionnaire on physical activity [197].
- Measurement of anthropometric indices including weight, height, waist circumference, and blood pressure.
- Biochemical tests including: complete blood count with differential, blood glucose, glycated hemoglobin, fasting insulin, creatinine, AST, ALT, GGT, alkaline phosphatase, total and fractionated bilirubin, albumin, protein electrophoresis, total cholesterol, LDL, HDL, and triglycerides.
- Biomarker assays for direct measurement of fibrosis markers such as collagen IV, laminin, hyaluronic acid, and the N-terminal fragment of procollagen III (PIIIP).
- Abdominal ultrasound with elastography (FibroScan) to measure liver stiffness.
- Liver biopsy with histological examination for diagnosis, grading, and staging using validated NAFLD scores (Brunt, NAS, and SAF).

Through biochemical tests, anthropometric data, and medical history information, values of indirect complex scores such as FIB-4, NFS, Bard index, Hepamet, AST/ALT ratio, AST/PLT ratio (APRI), and FORNS index were calculated, as presented in the previous chapter. Metabolic syndrome was defined by the presence of at least three of the harmonized criteria, namely: elevated triglycerides (≥ 150 mg/dl), low HDL-c levels (< 50 mg/dl for females, < 40 mg/dl for males), elevated waist circumference (≥ 80 cm for females, ≥ 94 cm for males in the Caucasian population), elevated blood pressure (systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg), and elevated blood glucose (≥ 100 mg/dl) [198].

During the initial visit, a clinical assessment was conducted, including measurements of blood pressure, waist circumference, weight, and height. Questionnaires related to dietary habits and physical activity were administered. Biochemical samples were taken for the determination of the lipid profile, glycemic levels, liver function, and direct fibrosis markers. Additionally, an abdominal ultrasound with hepatic elastography was performed to measure liver stiffness (LS). Subsequently, a liver biopsy was scheduled. Following the enrollment phase, patients were periodically evaluated, both instrumentally and clinically, according to individualized follow-ups based on comorbidities, disease severity, and the risk of progression of extrahepatic and cardiovascular disease.

Statistical analysis

For the statistical analysis, SPSS 21 software [199] was used. The comparison between continuous variables was performed using medians with the Mann-Whitney test, while categorical variables were analyzed using the chi-square test or Fisher's exact test when categories contained fewer than 6 elements. The correlation analysis among histological, anthropometric/metabolic, and ultrasonographic variables was conducted using Spearman's rho. Severe MASH was defined as the presence of MASH on histological examination, NAS ≥ 4 (with at least 1 point in ballooning and lobular inflammation), and a fibrosis stage of at least F3. The assessment of the diagnostic performance of non-invasive tests (NITs) was conducted using Receiver Operating Characteristic (ROC) curves, evaluating the diagnostic performance of various NITs for the histological outcomes of NASH, fibrosis of at least F2, fibrosis of at least F3, fibrotic MASH (defined as MASH with NAS ≥ 4 and fibrosis of at least F2), and severe MASH. Based on the coordinate values of the points describing the ROC curve, the Youden index was utilized to identify cut-off values with optimal sensitivity and specificity for detecting the histological outcome of severe MASH. Once these values were obtained, sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy were calculated. These threshold values were then used to create diagnostic algorithms, for which the percentage of liver biopsy referrals was calculated.

CHAPTER 4

RESULTS

GENERAL CHARACTERISTICS OF THE POPULATION

Between October 2021 and May 2024, 84 patients were enrolled in our Unit. The general characteristics are presented in the table below.

Variable	Absolute Number (% of Total) or Median [Min-Max]
ANTHROPOMETRIC-METABOLIC VARIABLES	
MALES	59 (70.2%)
AGE AT BIOPSY (years)	52.4 [21.6-69.4]
BMI > 30 (kg/m²)	55 (65.5%)
MEDIAN BMI (kg/m²)	31.8 [22.2-54.0]
BMI CATEGORIES **NORMAL** **OVERWEIGHT** **OBESE**	 7 (8.3%) 22 (26.2%) 55 (65.5%)
ARTERIAL HYPERTENSION	65 (77.4%)
IFG OR DIABETES	52 (61.7%)
DIABETES	33 (39.3%)
ANTIDIABETIC THERAPY	31 (36.9%)
INSULIN (µUI/mL)	16.6 [3.8-277.9]
GLYCEMIA (mg/dL)	99.5 [51-334]
HOMA	4.08 [1-61]
HbA1c (mmol/mol)	37 [25-96]
METABOLIC SYNDROME	59 (70.2%)
LOW HDL ACCORDING TO IDF METABOLIC SYNDROME	38 (45.2%)
TRIGLYCERIDES> 150 mg/dl	34 (40.5%)
TOTAL CHOLESTEROL (mg/dL)	177.5 [90-365]
LDL CHOLESTEROL (mg/dL)	118 [48-229]
HDL CHOLESTEROL (mg/dL)	45 [27-90]
TRIGLYCERIDES (mg/dL)	128 [50-1429]
BIOCHEMICAL VARIABLES	
PLATELETS (n/mm³)	216.500 [67.000-331.000]
GOT (U/L)	35 [13-148]
GPT (U/L)	47 [9-214]
GGT(U/L)	44.5 [9-949]
ALBUMIN (g/dL)	4.4 [3.5-5.1]
VARIABILI ECOGRAFICHE	
LIVER STIFFNESS (kPa)	9.2 [3.3-53.1]

Table 4. General Characteristics of the Population. ***HbA1c*** *= Glycated Hemoglobin,* ***HOMA*** *= Homeostatic Model Assessment,* ***kPa*** *= KiloPascal*

Considering the general characteristics of the population, it can be observed that there is a predominance of the male sex (70%), with a median age at the time of liver biopsy of 52.4 years, and that the factors of metabolic syndrome were often present individually. In fact, 77% of patients presented with hypertension according to harmonized criteria, 90% were overweight/obese, 61.7% had impaired fasting glucose or type 2 diabetes mellitus, 40% had hypertriglyceridemia, and the median HOMA index for determining insulin resistance was 4.08. In light of these significant metabolic alterations, no substantial changes were noted in the median values of GOT, GPT, GGT, albumin, and platelets, which showed values slightly above the threshold limits. Liver stiffness (LS) was found to be increased.

Regarding the characteristics of the histological variables, as shown in the table below, a high prevalence of patients with MASLD (90%) was noted, with steatohepatitis (72.6%) and advanced degrees of fibrosis.

Variable	Absolute Number (% of Total)
MASLD	76 (90.5%)
MASH	61 (72.6%)
FIBROSIS ≥ 2	44 (52.4%)
FIBROSIS≥ 3	34 (40.5%)
FIBROTIC MASH	27 (32%)
SEVERE MASH	20 (23.8%)

Table 5. Histological Variables Related to MASLD

Among the other histological findings, hepatic steatosis was present in 90.5% of the patients, with more than 50% having a NAS of 4 or higher. Additionally, 75% had portal inflammation, and 75% of patients presented with hepatic fibrosis exceeding the first stage. The tables below provide detailed information on the various histological characteristics.

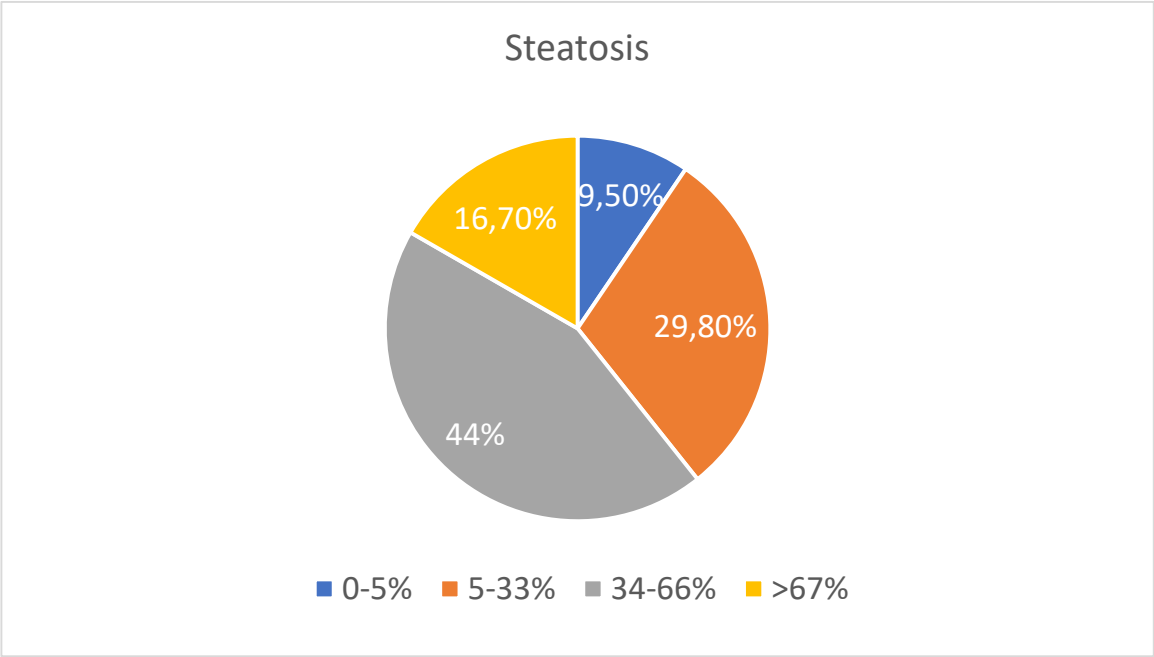


Figure 9. Distribution of the various grades of steatosis in the examined population.

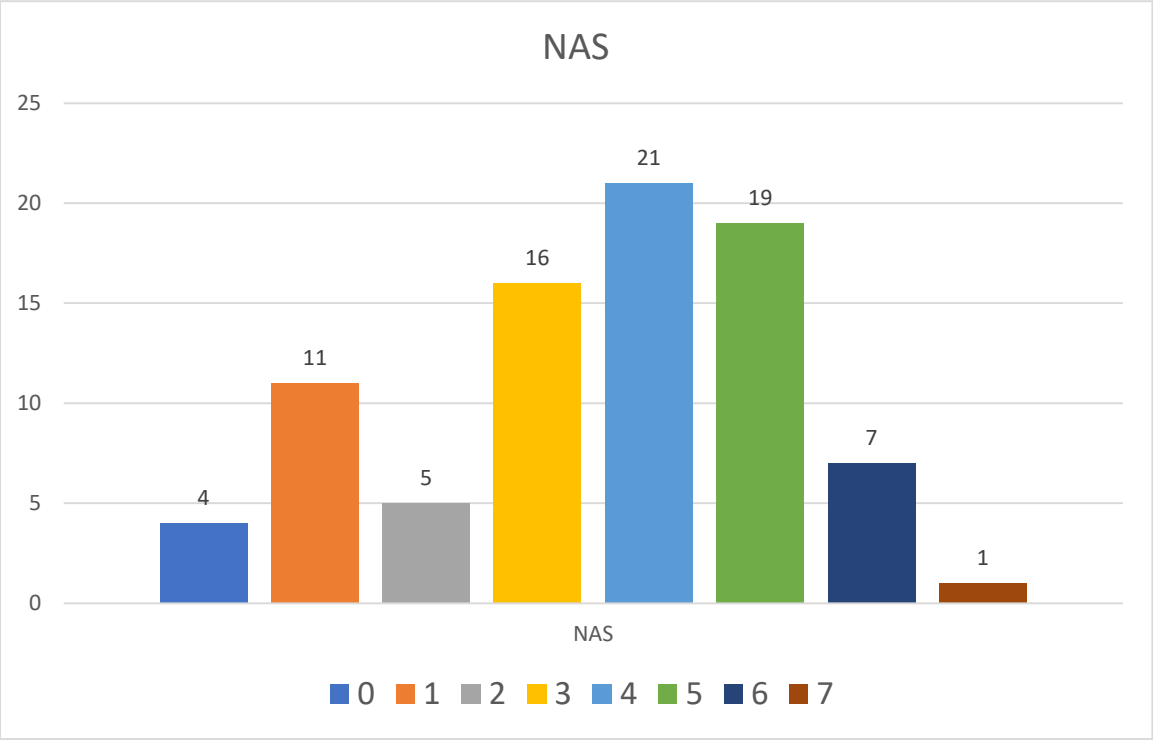


Figure 10. Distribution of the different grades of NAS in the examined population.

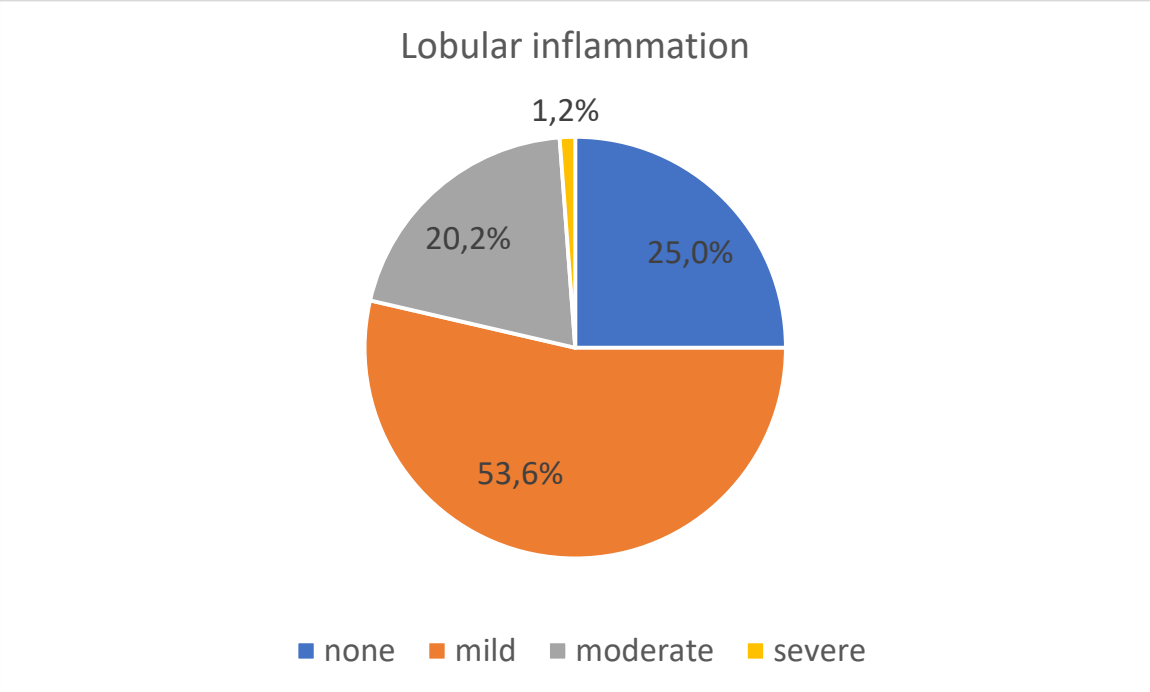


Figure 11. Distribution of the degree of lobular inflammation in the examined population

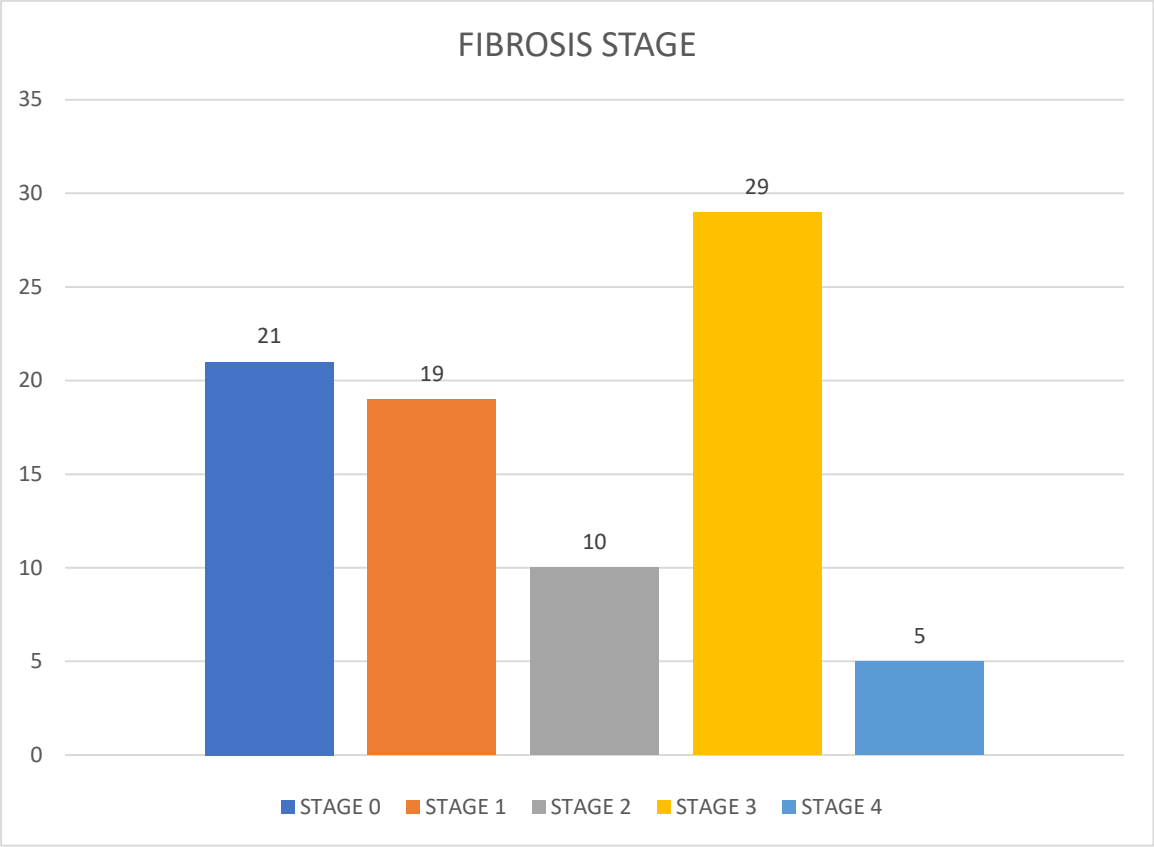


Figure 12. Fibrosis stage in the examined population

Below are the median values of the direct fibrosis markers and the simple and complex indirect scores.

Variable	Median [min-max]
COLLAGEN IV (ng/mL)	13.8 [4.8-160]
PIIIP (ng/mL)	25.95 [3.1-304.0]
COLYGLYCIN (µg/mL)	0.23 [0.025-7.72]
HYALURONIC ACID (ng/mL)	56.5 [28.4-459]
LAMININ (ng/mL)	26.1 [7.3-718]

Table 6. Median Values of Direct Fibrosis Markers in the General Population

Variable	Median [min-max]
GOT/GPT	0.79 [0.41-3.33]
APRI	0.50 [0.1-2.5]
BARD	2 [0-4]
FIB4	1.25 [0.36-7.10]
NAFLD FIBROSIS SCORE	-0.6 [-5.0--3.0]
FORNS INDEX	5.03 [2.00-11.00]
HEPAMET	0.09 [0.00-0.72]
AGILE 3+	0.45 [0.01-0.99]
AGILE 4	0.08 [0.00-0.93]

Table 7. Median Values of Simple and Complex Indirect Scores in the Examined Population

Performance of NITs

This section will evaluate the diagnostic performance of various NITs in identifying different histological outcomes. Specifically, the diagnostic capabilities will be assessed in distinguishing patients with MASH, patients with at least F2 liver fibrosis, at least F3 liver fibrosis, fibrotic MASH (i.e., MASH, NAS ≥ 4 and at least F2 liver fibrosis), and severe MASH (i.e., MASH, NAS ≥ 4 and at least F3 liver fibrosis). For each histological outcome, the performance will be evaluated using Receiver Operating Characteristic (ROC) curves for simple indirect biomarkers (GOT, GPT, albumin, platelets, AST/ALT ratio, and APRI), complex indirect scores (FIB-4, NFS, Hepamet, BARD, FORNS index), direct biomarkers of fibrosis (collagen IV, PIIIP, coliglycin, hyaluronic acid, and laminin), and ultrasound tests (liver stiffness, AGILE 3+, and AGILE).

Comparison of NIT Performance in Patients With and Without MASH

The comparative analysis between the population with MASH and the population without MASH highlighted that the anthropometric and biochemical variables associated with metabolic syndrome exhibited a statistically higher distribution in patients with MASH. These data are presented in Table 8.

Variable	Without MASH	With MASH	p-value
ANTHROPOMETRIC-METABOLIC VARIABLES			
MEN	**19** (82,6%)	**40** (65,6%)	0,18
AGE AT BIOPSY (YY)	**49,1** [29,6-65,8]	**53,7** [21,6-69,4]	0,13
BMI (Kg/m^2)	**28,4** [22,2-54]	**32,0** [24,6-53,6]	0,26
BMI > 30 Kg/m^2	**11** (20%)	**44** (80%)	**0,037**
HYPERGLICEMIA (MetS definition)	**10** (19,2%)	**42** (80%)	**0,033**
HYPERTRIGLYCERIDEMIA (MetS definition)	**4** (11,8%)	**30** (88,2%)	**0,012**
METABOLIC SYNDROME	**10** (16,9%)	**49** (83,1%)	**0,001**
BIOCHEMICAL VARIABLES			
GLUCOSE (mg/dL)	**94** [77-122]	**102** [51-334]	**0,012**
GLYCATED HEMOGLOBIN (mmol/mol)	**34** [27-51]	**38** [25-96]	**0,005**
INSULIN (µUI/mL)			**<0,001**
HOMA INDEX	**2,19** [1-15]	**5,18** [1-61]	**<0,001**
URIC ACID (mg/dL)	**5** [2,9-8,0]	**6,3** [1,2-10,4]	**0,002**
TRIGLYCERIDEMIA (mg/dL)	**107** [50-257]	**141** [167-400]	**0,009**
DIRECT FIBROSIS MARKER			
COLLAGEN IV (ng/mL)	**10,7** [4,8-147,0]	**14,8** [5,0-160,0]	**0,024**
LAMININ (ng/mL)	**22,6** [7,3-41,1]	**29,3** [11,3-718,0]	**0,018**
COMPLEX INDIRECT SCORES			
FIB-4	**0,9** [0,46-6,45]	**1,33** [0,36-7,1]	**0,004**
HEPAMET	**0,03** [0,0-0,7]	**0,09** [0,0-0,69]	**0,007**

Table 8. Comparison of Characteristics Between the MASH Subpopulation and Non-MASH Patients. Categorical variables are expressed as absolute numbers (% of the total); continuous numerical variables are expressed as median (min-max). Fisher's exact test was used for nominal variables, while the Mann-Whitney U test was employed for continuous variables

In the images below, the ROC curve of simple indirect biomarkers is presented for identifying patients with MASH compared to those without, along with the corresponding AUROC values, 95% confidence intervals, and respective p-values.

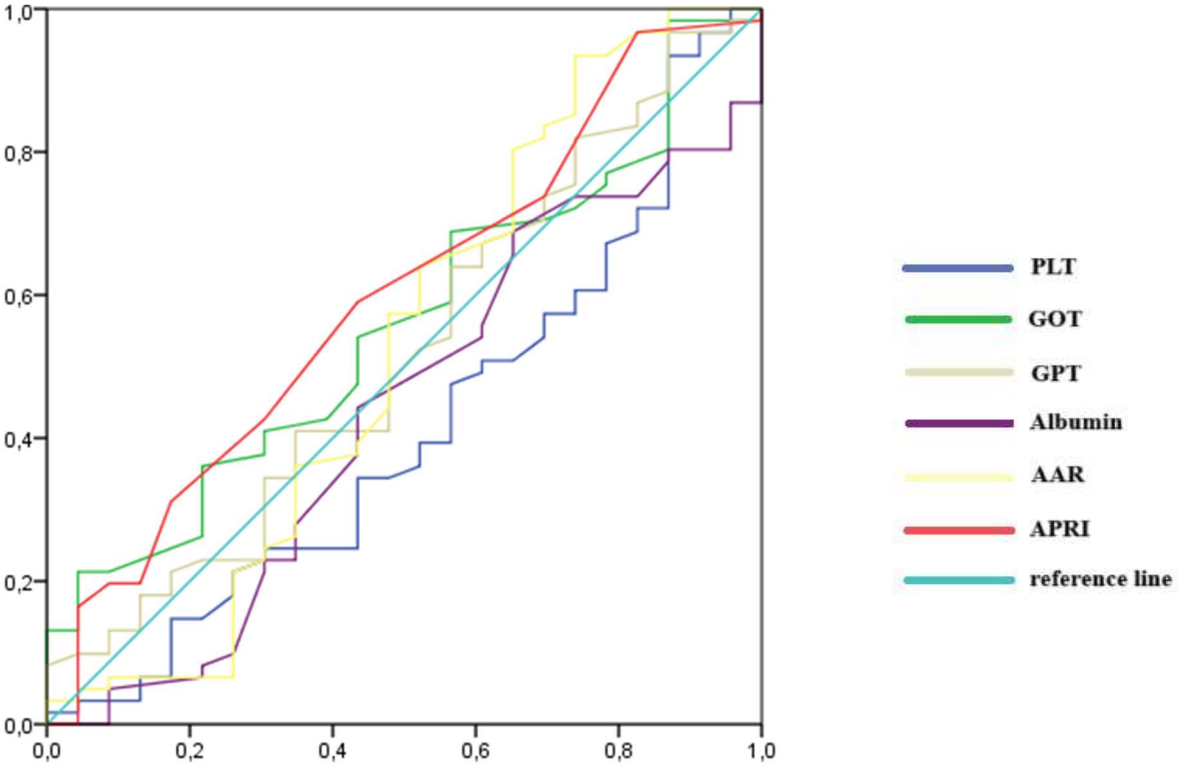


Figure 13. ROC curve of simple indirect biomarkers in discriminating patients with MASH from those without MASH. The Y-axis represents sensitivity, while the X-axis represents 1-specificity.

Variable	AUROC	CI 95%	p-value
PLT	0.41	0.27-0.55	0.22
GOT	0.56	0.43-0.69	0.41
GPT	0.52	0.38-0.66	0.76
ALBUMIN	0.44	0.30-0.58	0.36
AAR	0.52	0.37-0.68	0.73
APRI	0.59	0.46-0.73	0.18

Table 9. AUROC of simple indirect markers in identifying MASH. The performance of simple indirect markers in identifying patients with MASH was low, with AUROC values that were not statistically significant.

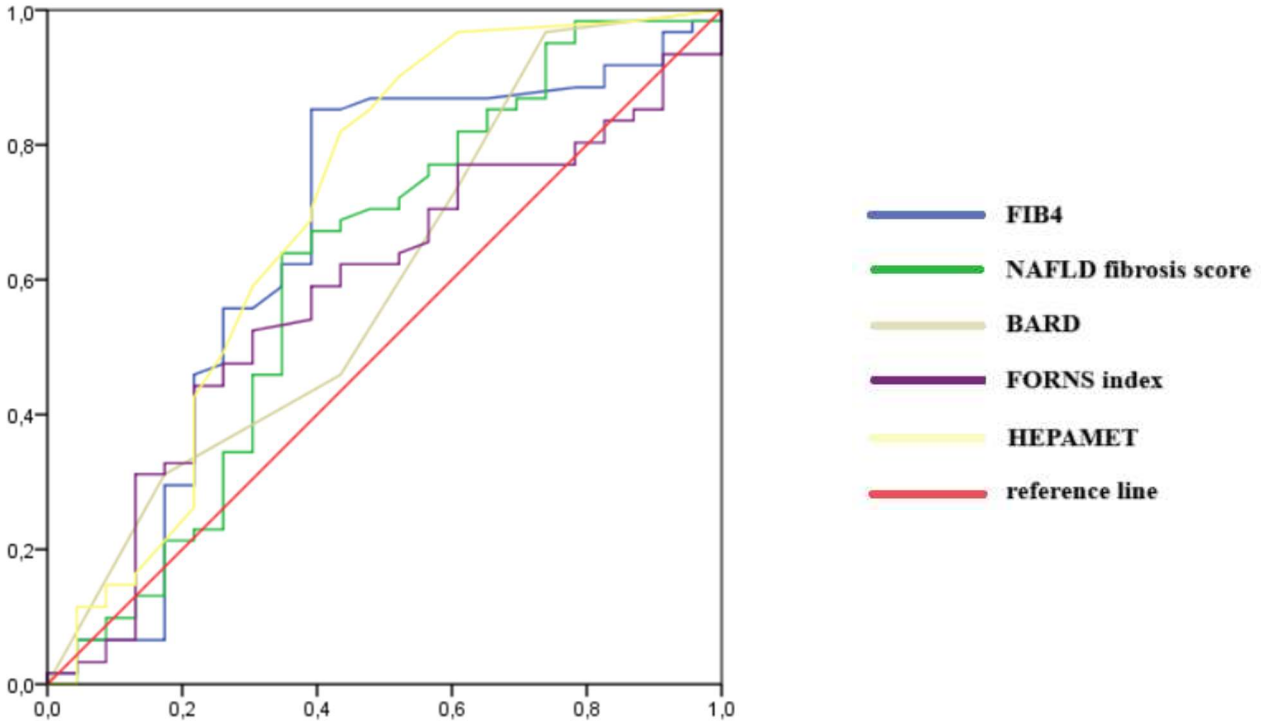


Figure 14. ROC Curve of Complex Indirect Scores in Identifying Patients with MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the variable of 1-specificity.

Variable	AUROC	CI 95%	p-value
FIB4	0.66	0.51-0.80	**0.02**
NFS	0.62	0.47-0.77	0.10
BARD	0.60	0.46-0.74	0.16
FORNS	0.58	0.45-0.72	0.25
HEPAMET	0.70	0.56-0.85	**0.01**

Table 10. AUROC of Complex Indirect Scores in Identifying MASH. This table presents the Area Under the Receiver Operating Characteristic (AUROC) values for various complex indirect scores utilized to identify patients MASH

The complex indirect scores, as reported in Figure 14 and Table 10, generally did not show statistically significant results in identifying MASH, except for FIB4 with a moderate AUROC (AUC 0.66) and Hepamet (AUC 0.70).

The figures below illustrate the diagnostic performance of direct fibrosis markers in distinguishing patients with MASH from those without.

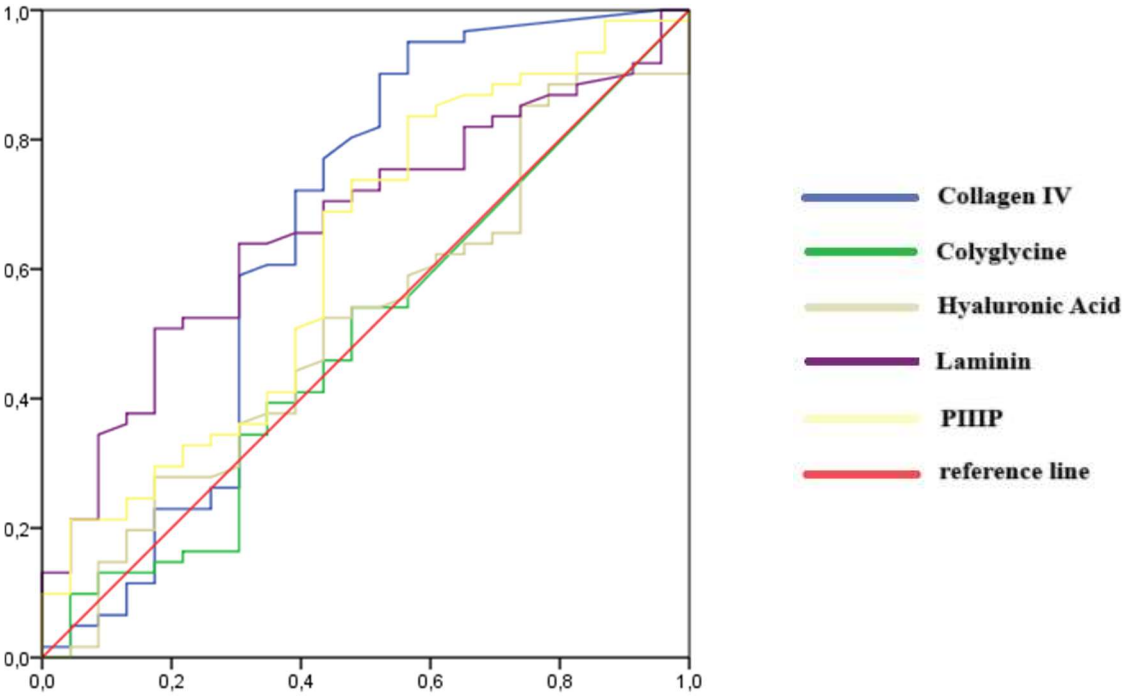


Figure 15. ROC curve of direct fibrosis markers in identifying patients with MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the variable 1-specificity.

Variable	AUROC	CI 95%	p-value
CIV	0.66	0.51-0.82	**0.02**
CG	0.49	0.35-0.63	0.91
HA	0.52	0.38-0.66	0.82
LM	0.67	0.55-0.79	**0.02**
PIIIP	0.62	0.48-0.76	0.08

Table 11. AUROC of direct fibrosis markers in identifying MASH. CIV = collagen IV; CG = coliglycine; HA = hyaluronic acid; LM = laminin.

Among the direct fibrosis markers, only laminin and collagen IV were able to statistically significantly distinguish patients with MASH from those without, although the AUROC values were moderate (0.66 and 0.67, respectively).

In the figures below, the diagnostic performances of LS and the related scores in distinguishing patients with MASH are presented.

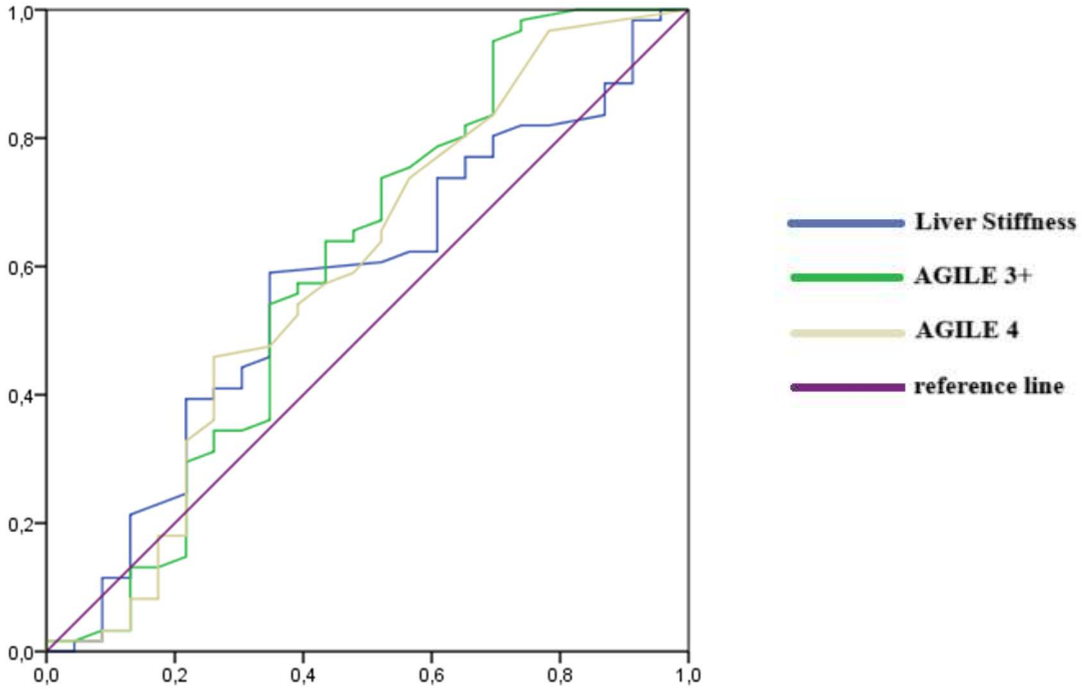


Figure 16. ROC curve of the radiological scores in identifying patients with MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the variable 1-specificity.

Variable	AUROC	CI 95%	p-value
LS	0.57	0.43-0.71	0.32
AGILE 3+	0.60	0.45-0.76	0.15
AGILE 4	0.59	0.44-0.74	0.19

Table 12. AUROC of the radiological scores in identifying MASH

In Figure 16 and Table 12, the diagnostic performances of radiological tests are presented, indicating that these tests are not able to discriminate patients with MASH.

Overall, the NITs have not proven to be tests capable of significantly discerning patients with MASH with optimal AUROC values. Exceptions include FIB4, Hepamet, laminin, and collagen IV, which, however, exhibited moderate AUROC values despite being statistically significant.

Performance of NITs in identifying patients with fibrosis of at least F2

In this paragraph, the diagnostic performance of various NITs in distinguishing patients with fibrosis of at least F2 from those with F3 and F4 fibrosis will be analyzed. Table 13 presents the salient characteristics that differed between patients with fibrosis of at least F2 and those without. As can be seen, although there are no statistically significant differences in age at biopsy, sex, and BMI, patients with fibrosis of at least F2 exhibited higher values for anthropometric and biochemical characteristics related to metabolic syndrome.

Variable	Without F2	With AT LEAST F2	p-value
ANTHROPOMETRIC-METABOLIC VARIABLES			
MEN	**30** (75%)	**29** (65,9%)	0,36
AGE AT BIOPSY (YY)	**51,1** [21,6-67,1]	**54,3** [25,1-69,4]	0,23
BMI (Kg/m^2)	**30,6** [22,2-52,6]	**33,6** [24,2-54,0]	0,36
BMI > 30 Kg/m^2	**22** (55%)	**33** (75%)	**0,05**
BLOOD HYPERTENSION (MetS definition)	**15** (38,5%)	**29** (65,9%)	**0,01**
HYPERGLYCEMIA or IFG (MetS definition)	**19** (47,5%)	**33** (75%)	**0,01**
METABOLIC SYNDROME	**23** (57,5%)	**36** (81,8%)	**0,02**
BIOCHEMICAL VARIABLES			
GLUCOSE (mg/dL)	**95,5** [51-173]	**104** [74-334]	**0,01**
GLYCATED HEMOGLOBIN (mmol/mol)	**35** [25-55]	**38,5** [28-96]	**<0,001**
TRIGLYCERIDEMIA (mg/dL)	**114,5** [50-1429]	**141,5** [61-902]	**0,013**
DIRECT FIBROSIS MARKER			
COLLAGEN IV (ng/mL)	**11,8** [5,0-64,1]	**17,2** [4,8-160]	**0,04**
COLYGLYCINE (µg/mL)	**0,025** [0,025-7,72]	**0,40** [0,03-1,66]	**0,03**
SIMPLE AND COMPLEX INDIRECT SCORES			
APRI	**0,4** [0,1-1,5]	**0,5** [0,2-2,5]	**0,048**
BARD	**2** [0-4]	**3** [0-4]	**0,004**
FIB-4	**1,1** [0,6-3,59]	**1,28** [0,46-7,1]	**0,03**
NFS	- **0,94** [-5,0-2,0]	-**0,41** [-3,0-3,0]	**0,02**
ULTRASOUND SCORES			
LIVER STIFNESS (kPa)	**7,9** [3,3-53,1]	**11,7** [5,1-37,4]	**0,001**
AGILE 3+	**0,21** [0,01-0,96]	**0,65** [0,08-0,99]	**0,001**
AGILE 4	**0,03** [0,0-0,64]	**0,14** [0,01-0,93]	**0,001**

Table 13. Comparison of characteristics between the subpopulation with fibrosis of at least F2 and patients without fibrosis of at least F2. Categorical variables are expressed as absolute numbers (% of the total); continuous numerical variables are expressed as median (min-max). The Fisher's exact test was used for nominal variables, while the Mann-Whitney U test was applied for continuous variables.

In the images below, the diagnostic performance of indirect biochemical markers is presented.

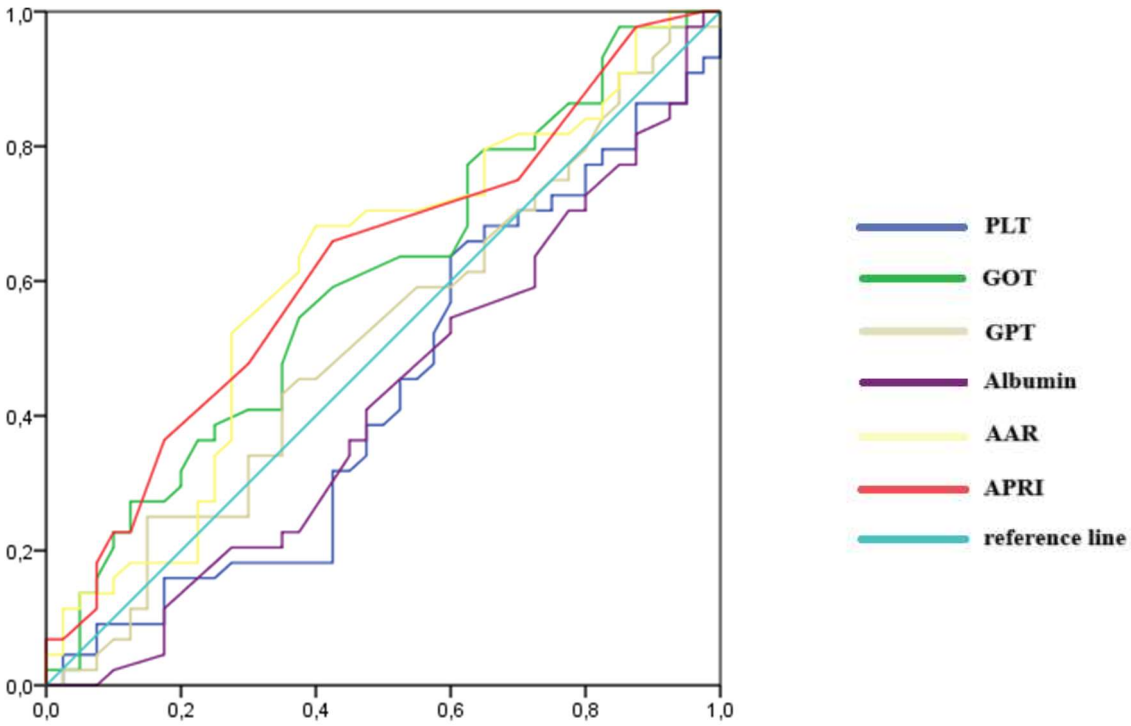


Figure 17. ROC curve of simple indirect biomarkers in identifying patients with at least F2 fibrosis. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
PLT	0.41	0.27-0.55	0.22
GOT	0.59	0.48-0.72	0.13
GPT	0.52	0.39-0.64	0.81
ALBUMIN	0.42	0.30-0.54	0.21
AAR	0.61	0.49-0.73	0.08
APRI	0.62	0.50-0.74	**0.05**

Table 14. AUROC of simple indirect markers in identifying patients with at least F2 fibrosis.

As can be seen from Figure 17 and Table 14, the simple indirect markers did not show statistically significant AUROC values in identifying patients with at least F2 fibrosis. An exception is the case of APRI, which, although statistically significant, does not provide good diagnostic performance.

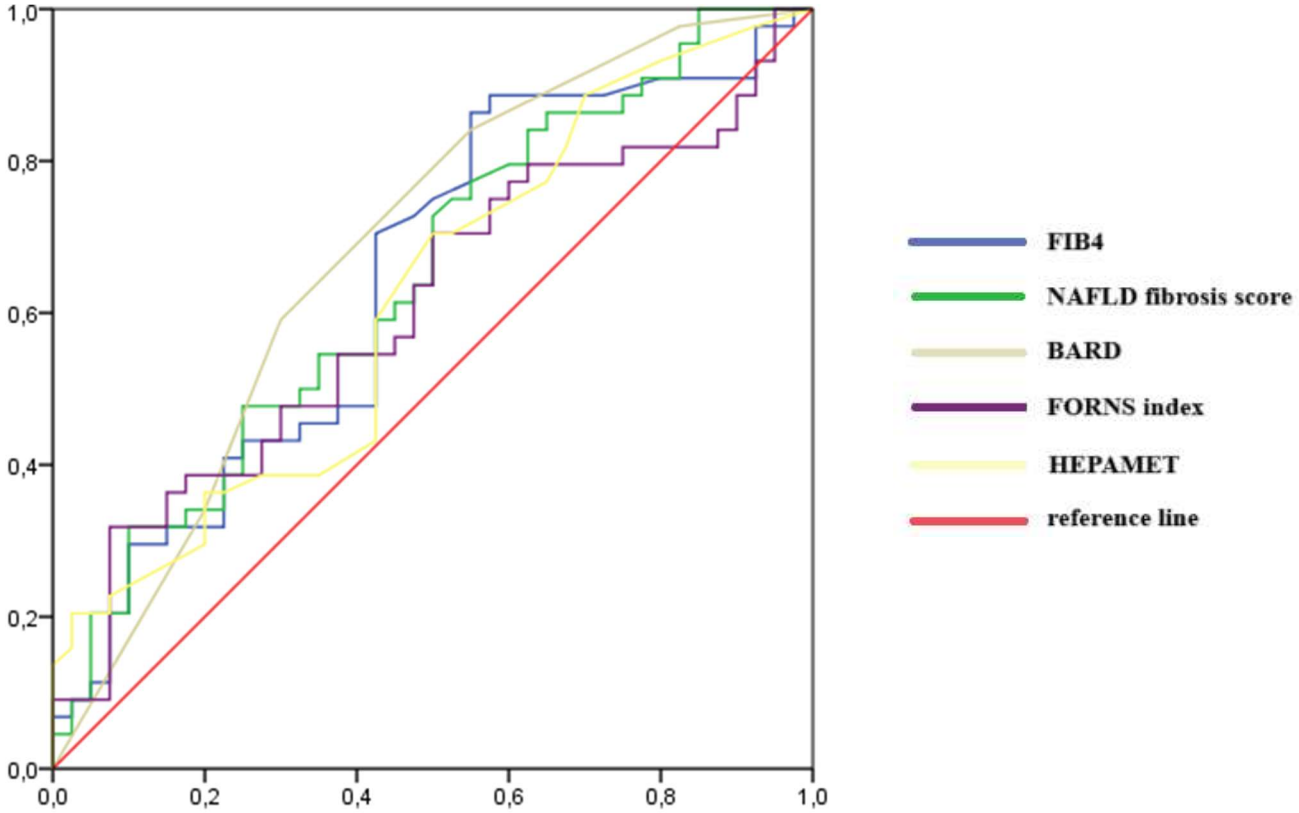


Figure 18. Curve ROC of the complex indirect scores in identifying patients with at least F2 fibrosis. The Y-axis displays the sensitivity variable, while the X-axis shows the variable 1-specificity.

Variable	AUROC	CI 95%	p-value
FIB4	0.64	0.52-0.76	**0.03**
NFS	0.65	0.53-0.76	**0.02**
BARD	0.68	0.57-0.80	**<0,01**
FORNS	0.60	0.48-0.73	0.10
HEPAMET	0.62	0.50-0.74	0.06

Table 15. AUROC of the complex indirect scores in identifying at least F2 fibrosis.

As can be seen from Figure 18 and Table 15, the complex indirect scores begin to show statistically significant p-values in discriminating patients with at least F2 fibrosis. FIB4, NFS, and BARD are able to discriminate patients with at least F2 fibrosis, albeit with moderate AUROC values.

The diagnostic performances of the direct fibrosis markers in identifying at least F2 fibrosis are presented in the figures below.

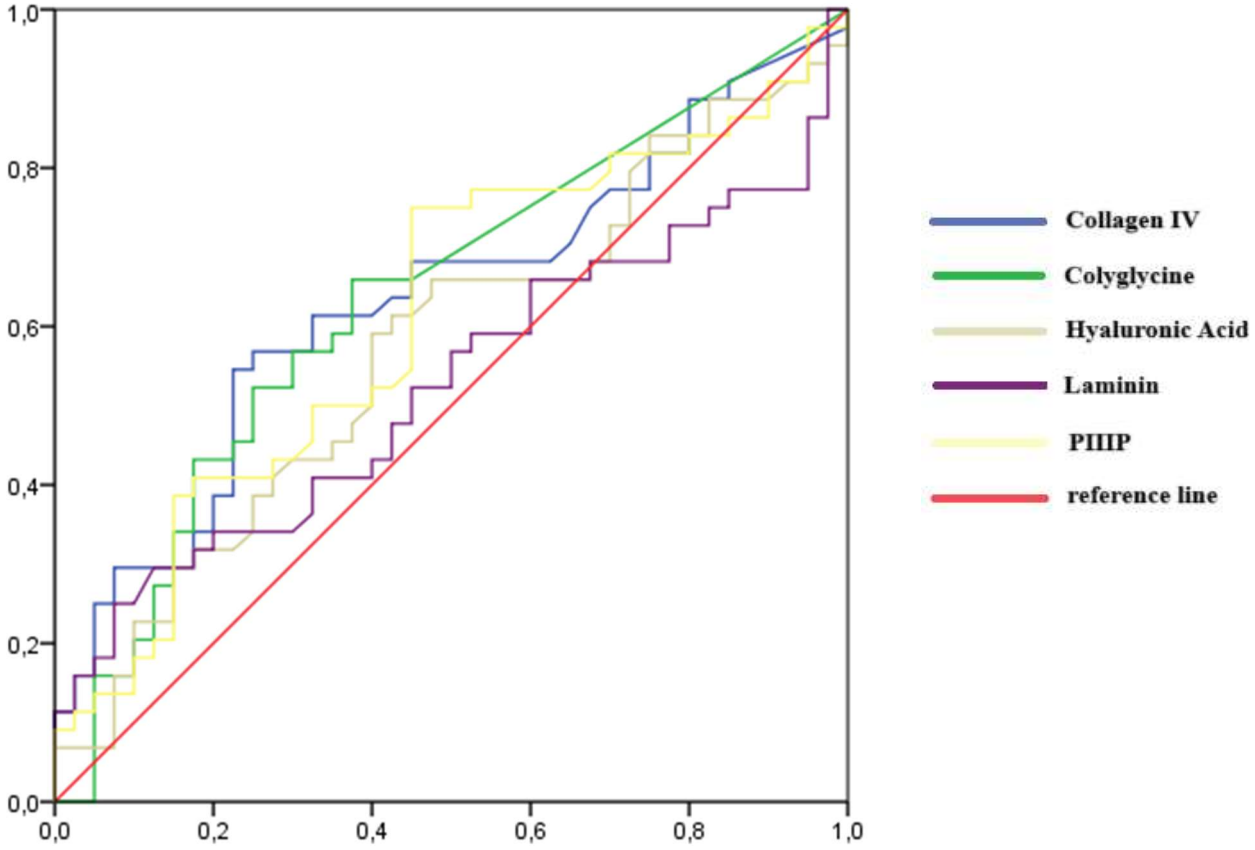


Figure 19. ROC curve of direct fibrosis markers in identifying patients with at least F2 fibrosis. The Y-axis displays the sensitivity variable, while the X-axis represents the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
CIV	0.63	0.51-0.75	**0.04**
CG	0.64	0.52-0.76	**0.03**
HA	0.57	0.45-0.70	0.26
LAMININ	0.53	0.40-0.65	0.70
PIIIP	0.61	0.49-0.73	0.08

Table 16. AUROC of direct fibrosis markers in identifying at least F2 fibrosis

From Figure 15 and Table 22, it can be observed that only collagen IV and colyglycine are capable of distinguishing patients with at least F2 fibrosis from the rest of the patients. The AUROC values are also modest, at 0.63 and 0.64, respectively.

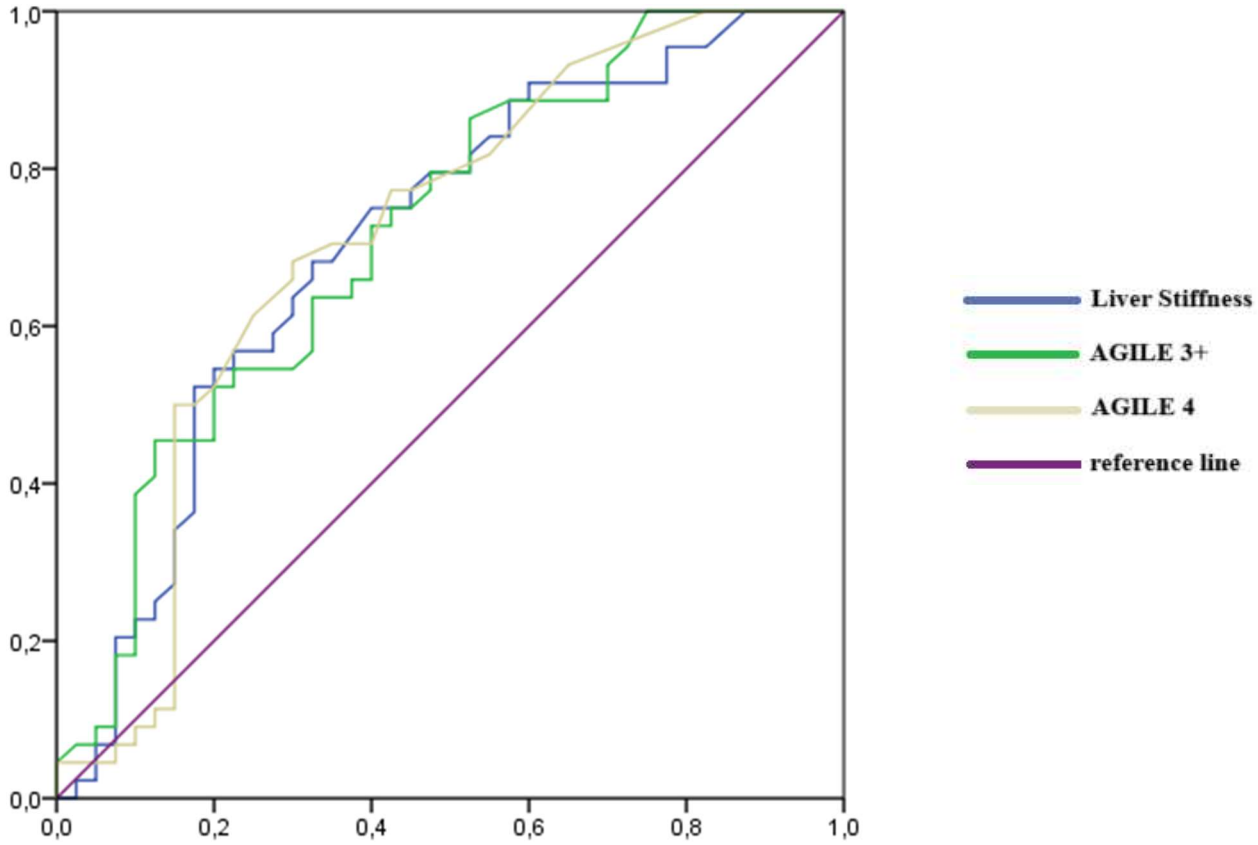


Figure 20. ROC curve of radiological scores in identifying patients with at least F2 fibrosis. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable

Variable	AUROC	CI 95%	p-value
LS	0.71	0.60-0.82	**<0,01**
AGILE 3+	0.72	0.61-0.83	**<0,01**
AGILE 4	0.71	0.60-0.83	**<0,01**

Table 17. AUROC of radiological markers in identifying at least F2 fibrosis.

As shown in Figure 20 and Table 17, the radiological markers can significantly discriminate patients with at least F2 fibrosis, with AUROCs reaching up to 0.72, as seen with AGILE 3+.

Performance of NITs in Identifying Patients with At Least F3 Fibrosis

In this section, the diagnostic performance of various NITs in discriminating patients with at least F3 fibrosis from those with F2 and F4 fibrosis will be analyzed. Table 18 presents the salient characteristics that differed between patients with at least F3 fibrosis and those without. Although there are no statistically significant differences in age at biopsy, sex, and BMI, patients with at least F3 fibrosis exhibited higher values in anthropometric and biochemical characteristics related to metabolic syndrome.

Variable	Without F3	With AT LEAST F3	p-value
ANTHROPOMETRIC-METABOLIC VARIABLES			
MEN	**34** (68%)	**25** (73,5%)	0,59
AGE AT BIOPSY (YY)	**50,2** [21,56-67,1]	**56,1** [25,1-69,4]	
BLOOD HYPERTENSION (MetS definition)	**26** (52%)	**30** (88,2%)	**0,001**
HYPERGLYCEMIA or IFG (MetS definition)	**25** (50%)	**27** (79,4%)	**0,006**
HYPERTRIGLYCERIDEMIA (MetS definition)	**19** (38%)	**15** (44,1%)	0,58
METABOLIC SYNDROME	**30** (60%)	**29** (85,3%)	**0,01**
BIOCHEMICAL VARIABLES			
GLUCOSE (mg/dL)	**95,5** [51-173]	**104,5** [74-334]	**0,02**
GLYCATED HEMOGLOBIN (mmol/mol)	**35** [25-69]	**40** [28-96]	**0,001**
HOMA INDEX	**3,86** [1-61]	**5,36** [1-20]	**0,05**
TRIGLYCERIDEMIA (mg/dL)	**124,5** [50-1429]	**138,5** [66-902]	0,24
DIRECT FIBROSIS MARKER			
COLLAGEN IV (ng/mL)	**11,9** [5,0-64,1]	**18,3** [4,8-160,0]	**0,006**
COLYGLYCINE (µg/mL)	**0,03** [0,03-7,72]	**0,41** [0,03-1,23]	**0,01**
HYALURONIC ACID (ng/mL)	**53,1** [28,4-98,3]	**64,9** [40,9-459,0]	**0,005**
PIIIP (ng/mL)	**19,7** [3,1-170,0]	**43,9** [5,9-304,0]	**0,01**
SIMPLE AND COMPLEX INDIRECT SCORES			
GOT (U/L)	**34** [13-124]	**39** [15-148]	**0,03**
GOT/GPT	**0,73** [0,41-1,39]	**0,84** [0,44-3,33]	**0,01**
APRI	**0,4** [0,1-1,5]	**0,6** [0,2-2,5]	**0,01**
BARD	**2** [0-4]	**3** [0-4]	**0,01**
FIB-4	**1,09** [0,36-3,59]	**1,43** [0,46-7,1]	**0,003**
NFS	**-0,78** [-5,0-2,0]	**-0,37** [-3,0-3,0]	**0,02**
FORNS	**4,6** [2,0-8,0]	**5,3** [2,0-11,0]	**0,03**
HEPAMET	**0,07** [0,0-0,7]	**0,09** [0,00-0,72]	**0,04**
ULTRASOUND SCORES			
LIVER STIFNESS (kPa)	**7,9** [3,3-53,1]	**11,9** [5,1-37,4]	**<0,001**
AGILE 3+	**0,23** [0,01-0,96]	**0,69** [0,08-0,99]	**<0,001**

AGILE 4	**0,03** [0,0-0,64]	**0,15** [0,02-0,93]	**0,001**

Table 18. Comparison of Characteristics Between the Subpopulation with At Least F3 Fibrosis and Patients Without At Least F3 Fibrosis. Categorical variables are expressed as absolute numbers (% of total); continuous numerical variables are expressed as median (min-max). For nominal variables, Fisher's exact test was used, while the Mann-Whitney U test was applied for continuous variables.

The following figures display the AUROC of various NITs in identifying patients with at least F3 fibrosis. Figure 21 and Table 19 present the data regarding the diagnostic performance of simple indirect markers.

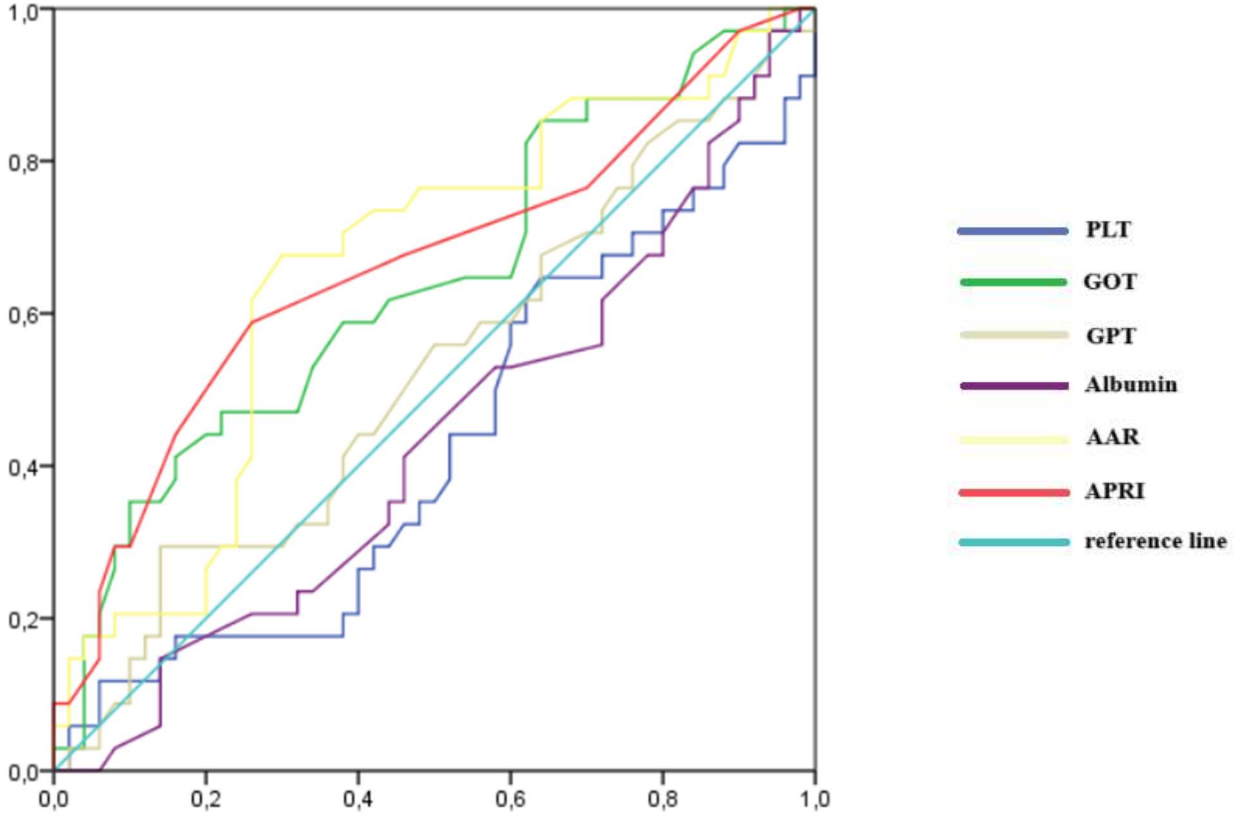


Figure 21. ROC curve of simple indirect biomarkers in identifying patients with at least F3 fibrosis. The Y-axis displays the sensitivity variable, while the X-axis shows the variable 1-specificity.

Variable	AUROC	CI 95%	p-value
PLT	0.43	0.30-0.55	0.25
GOT	0.64	0.52-0.76	**0.03**
GPT	0.52	0.40-0.65	0.73
ALBUMIN	0.43	0.31-0.56	0.31
AAR	0.66	0.54-0.78	**0.01**
APRI	0.66	0.54-0.79	**0,01**

Table 19. AUROC of simple indirect biomarkers in identifying patients with at least F3 fibrosis

Among the simple indirect biomarkers, GOT, AAR, and APRI are the NITs that significantly distinguish patients with at least F3 fibrosis; however, even in this context, the diagnostic performance is moderate. It is noteworthy that simple indirect markers such as platelets and GPT completely fail to provide useful insights regarding at least F3 fibrosis. Below, the performance of complex indirect biomarkers in discriminating patients with at least F3 fibrosis is analyzed.

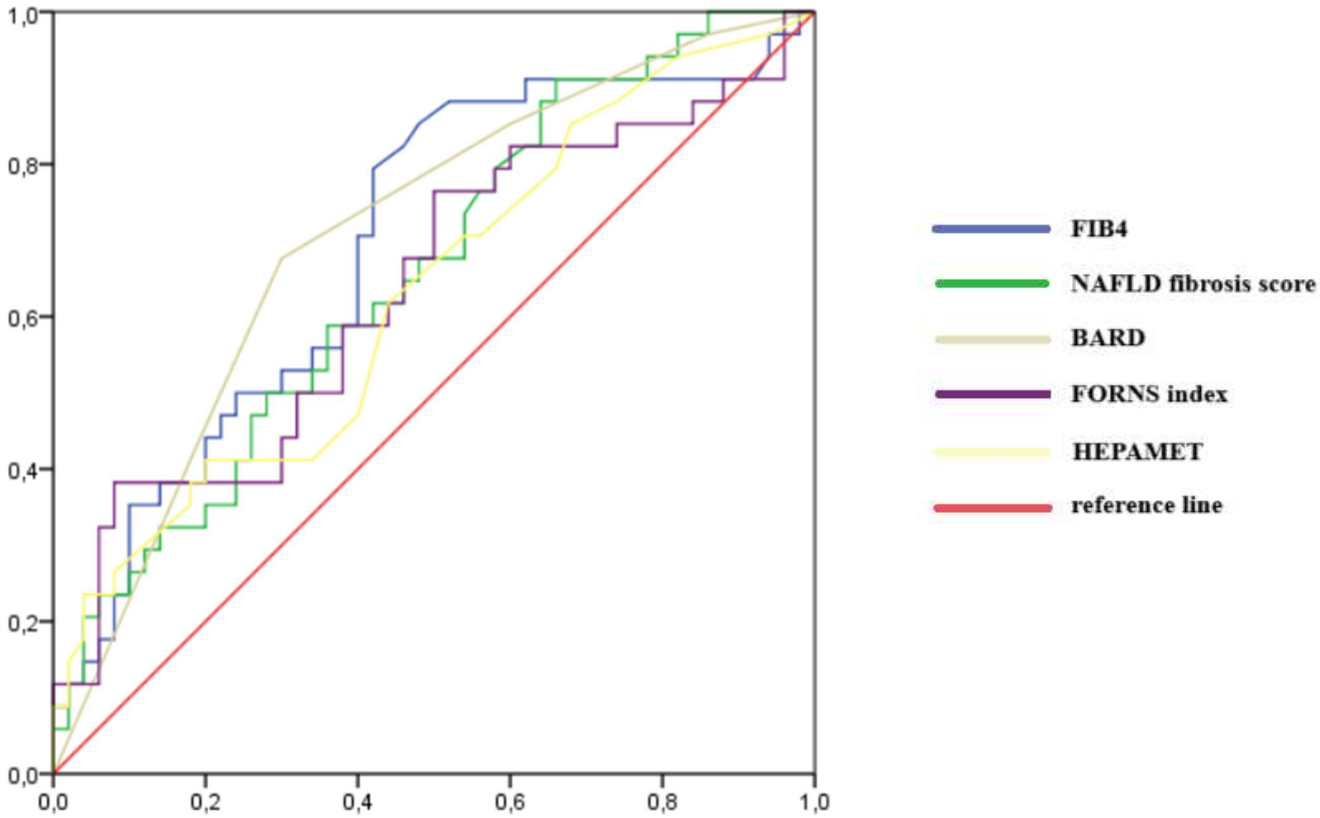


Figure 22. ROC curve of complex indirect scores in identifying patients with at least F3 fibrosis. The Y-axis displays the sensitivity variable, while the X-axis displays the variable 1-specificity.

Variable	AUROC	CI 95%	p-value
FIB4	0.69	0.58-0.81	**<0,01**
NFS	0.66	0.54-0.77	**0.02**
BARD	0.71	0.59-0.82	**<0,01**
FORNS	0.64	0.51-0.76	**0.03**
HEPAMET	0.63	0.51-0.75	**0.05**

Table 20. AUROC of complex indirect scores in identifying patients with at least F3 fibrosis

From Figure 22 and Table 20, it can be inferred that all complex indirect scores can significantly distinguish patients with advanced fibrosis, some with moderate AUROC, such as FIB4 and BARD. The following figures show the diagnostic performances of direct fibrosis biomarkers in identifying patients with at least F3 fibrosis.

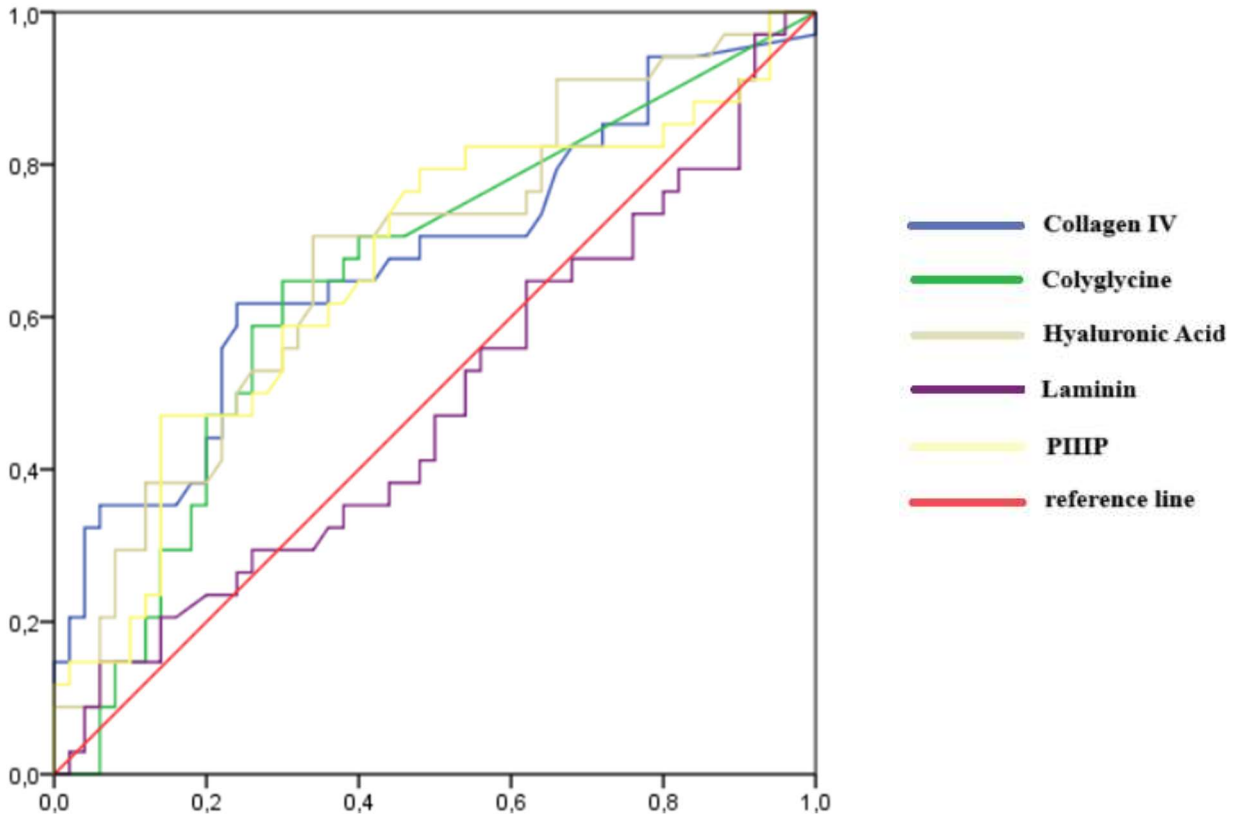


Figure 23. ROC curve of direct fibrosis markers in identifying patients with at least F3 fibrosis. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
CIV	0.68	0.55-0.80	**0.01**
CG	0.65	0.53-0.77	**0.02**
HA	0.68	0.56-0.80	**0.01**
LAMININ	0.48	0.35-0.61	0.80
PIIIP	0.66	0.54-0.79	**0.01**

Table 21. AUROC of direct fibrosis markers in identifying patients with at least F3 fibrosis.

As can be seen from Figure 23 and Table 21, direct fibrosis markers are able to statistically significantly distinguish patients with at least F3 fibrosis, except for laminin. In fact, CIV, CG, HA, and PIIIP showed p-values less than 0.05; however, the AUROC values remain moderate.

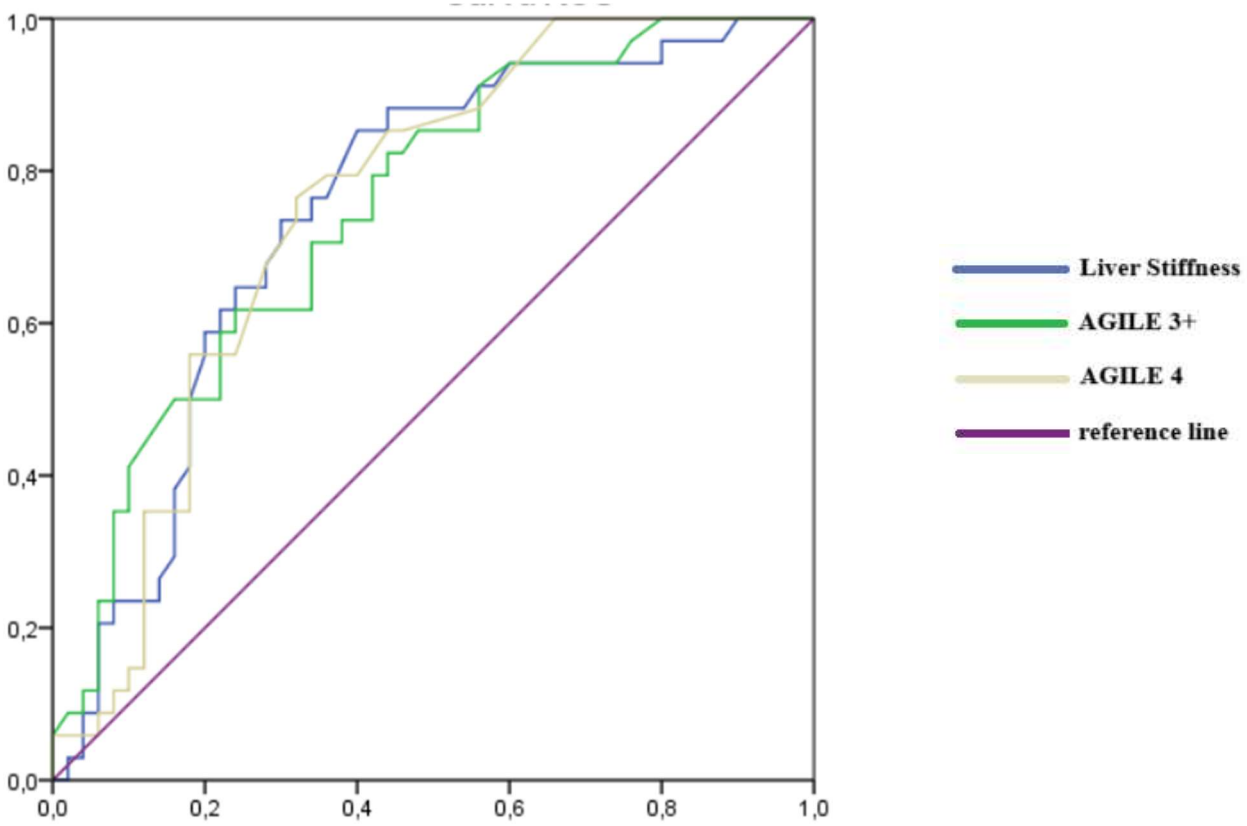


Figure 24. ROC curve of non-invasive radiological tests for identifying patients with at least F3 fibrosis. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
LS	0.75	0.64-0.85	**<0,01**
AGILE 3+	0.75	0.65-0.85	**<0,01**
AGILE 4	0.75	0.69-0.86	**<0,01**

Table 22. AUROC of radiological scores in identifying patients with at least F3 fibrosis.

From Figure 24 and Table 22, it can be observed that among all the previously analyzed NITs, the radiological scores exhibit the best AUROC in identifying patients with advanced fibrosis, achieving good AUROCs of 0.75

Performance of NITs in Identifying Patients with Fibrotic MASH

This section will evaluate the diagnostic performance of various NITs in identifying fibrotic MASH, which is characterized by the presence of a NAS ≥ 4 and at least F2 fibrosis.

Table below presents a comparison of the characteristics of the subpopulation of patients with fibrotic MASH versus those without fibrotic MASH. In this case, the variables related to metabolic syndrome, which are usually more prevalent in patients with the histological outcome, are reduced. It is noteworthy that laminin is significantly expressed in patients with fibrotic MASH, and there is no difference between the two groups concerning radiological scores.

Variable	Without FIBROTIC MASH	With FIBROTIC MASH	p-value
ANTHROPOMETRIC-METABOLIC VARIABLES			
MEN	**44** (77,2%)	**15** (55,6%)	**0,04**
AGE AT BIOPSY (YY)	**51,6** [21,6-67,1]	**53,2** [25,1-69,4]	0,48
BIOCHEMICAL VARIABLES			
GLUCOSE (mg/dL)	**97** [51-183]	**106** [74-334]	**0,03**
GLYCATED HEMOGLOBIN (mmol/mol)	**36** [25-58]	**40** [28-96]	**0,001**
TRIGLYCERIDEMIA (mg/dL)	**120** [50-1429]	**166** [61-902]	**0,01**
DIRECT FIBROSIS MARKER			
LAMININ (ng/mL)	**24,1** [7,34-49,9]	**31,7** [14,2-718]	**0,02**
PIIIP (ng/mL)	**21,6** [3,5-304,0]	**28,9** [3,1-179,0]	**0,04**
SIMPLE AND COMPLEX INDIRECT SCORES			
GOT	**34** [13-124]	**41** [20-148]	**0,01**
GGT	**39** [15-292]	**62** [12-949]	**0,03**
APRI	**0,4** [0,1-2,5]	**0,6** [0,3-1,6]	**0,01**
ULTRASOUND SCORES			
LS (kPa)	**8,7** [3,3-53,1]	**11,8** [5,2-23,4]	0,08

Table 23. Comparison of the Characteristics of the Subpopulation with Fibrotic MASH and Patients without Fibrotic MASH. Categorical variables are expressed as absolute numbers (% of total); continuous numerical variables are expressed as median (min-max). Fisher's t-test was used for nominal variables, while the Mann-Whitney U test was applied for continuous variables.

The diagnostic performances of simple indirect biochemical tests are presented in the images below.

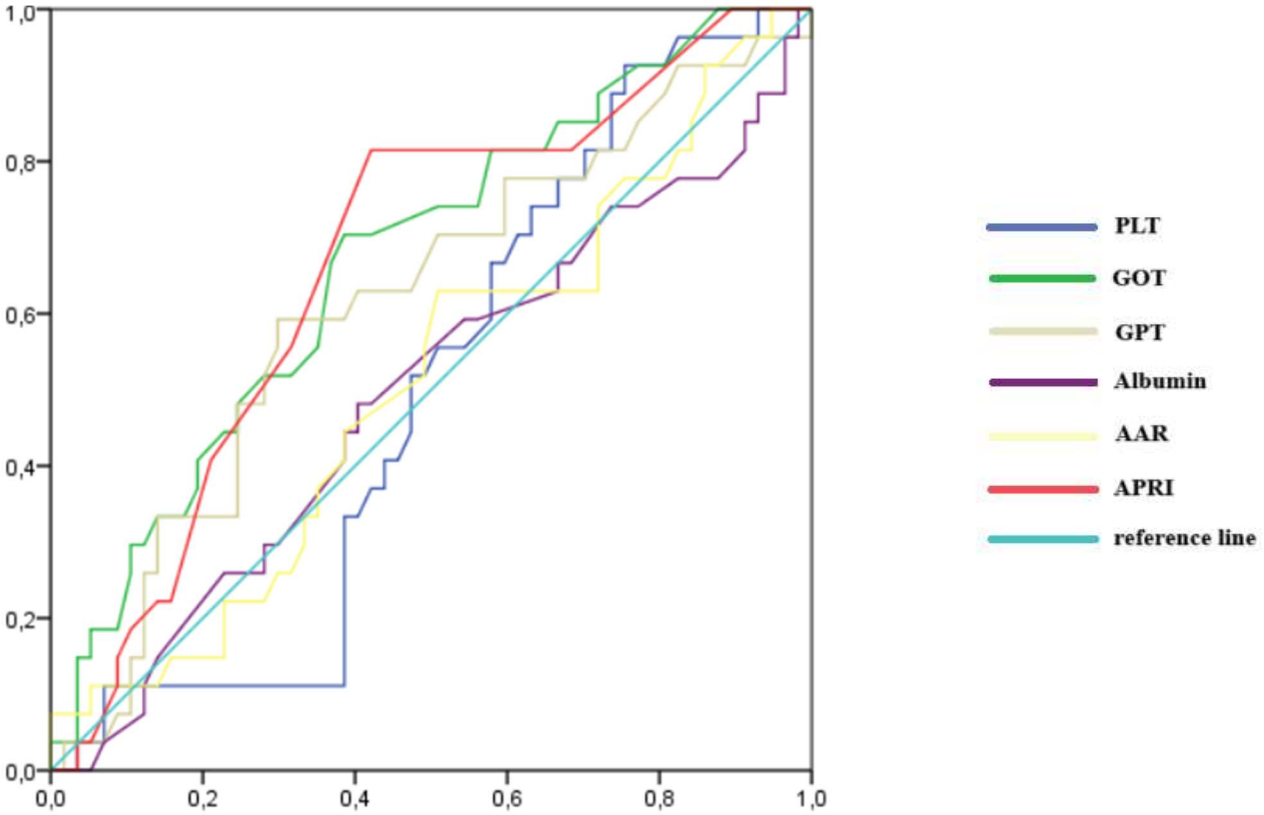


Figure 25. ROC curve of simple indirect biomarkers in identifying patients with fibrotic MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
PLT	0.50	0.37-0.62	0.98
GOT	0.67	0.55-0.79	**0.01**
GPT	0.62	0.49-0.75	0.09
ALBUMIN	0.49	0.39-0.63	0.93
AAR	0.51	0.38-0.64	0.86
APRI	0.67	0.55-0.79	**0.01**

Table 24. AUROC of simple indirect markers in identifying patients with fibrotic MASH.

From Figure 25 and Table 24, it can be inferred that simple indirect markers generally do not significantly discriminate between patients with fibrotic MASH. An exception to this is found with GOT and APRI, both of which show AUROC values of 0.67 and statistically significant p-values ($p<0.01$).

The diagnostic performance of complex indirect markers in identifying fibrotic MASH is presented in the images below.

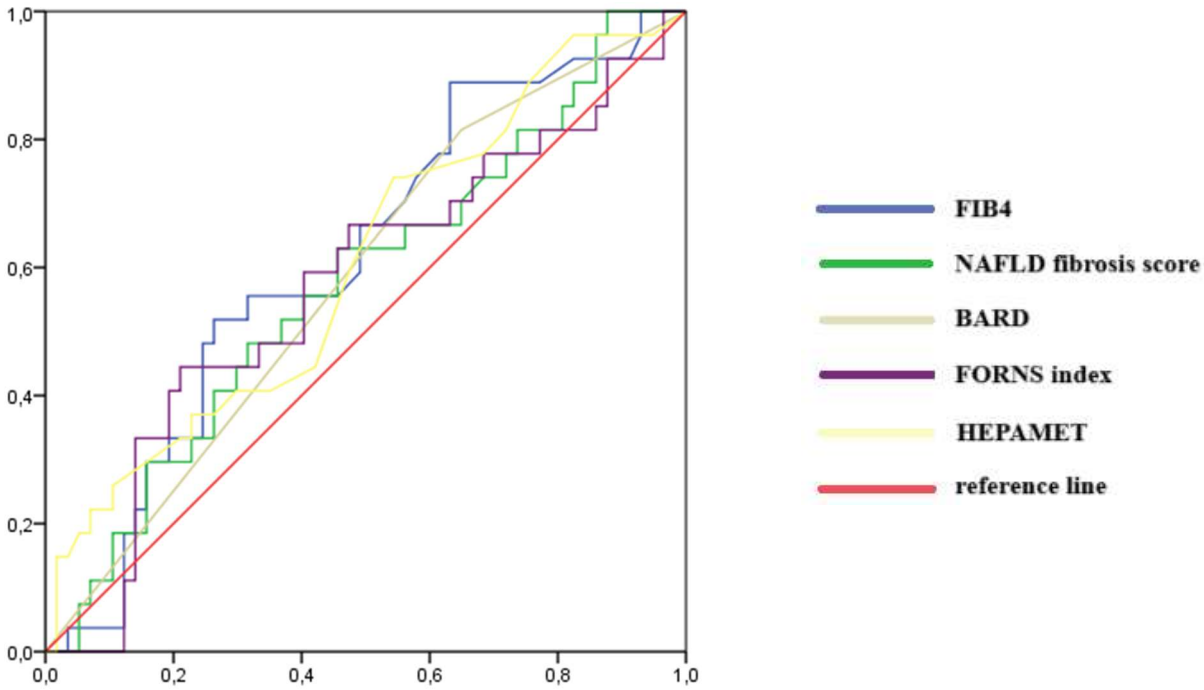


Figure 26. ROC curve of complex indirect scores in identifying patients with fibrotic MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
FIB4	0.61	0.49-0.74	0.10
NFS	0.58	0.44-0.71	0.27
BARD	0.59	0.47-0.72	0.18
FORNS	0.57	0.44-0.71	0.29
HEPAMET	0.61	0.48-0.73	0.12

Table 25. AUROC of complex indirect scores in identifying patients with fibrotic MASH

From the previous figures, it is evident that none of the complex indirect markers are able to statistically significantly identify patients with fibrotic MASH. In the following images, the diagnostic performance of direct fibrosis markers in identifying patients with fibrotic MASH will be evaluated.

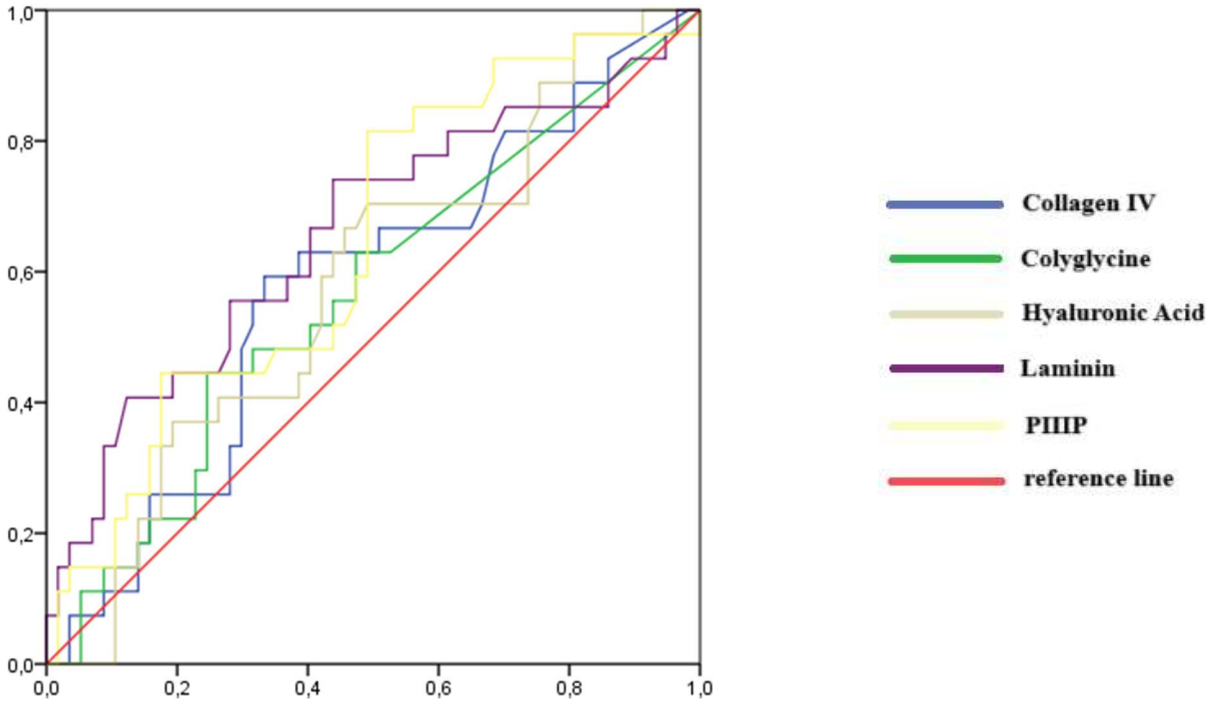


Figure 27. ROC curve of direct fibrosis markers in identifying patients with fibrotic MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
CIV	0.58	0.45-0.71	0.25
CG	0.57	0.43-0.70	0.33
HA	0.58	0.45-0.71	0.24
LAMININ	0.66	0.53-0.79	**0.02**
PIIIP	0.64	0.52-0.77	**0.04**

Table 26. AUROC of direct fibrosis markers in identifying patients with fibrotic MASH.

As can be seen from Figure 27 and Table 26, only laminin and PIIIP can statistically significantly discriminate patients with fibrotic MASH, with modest AUROCs of 0.66 and 0.64, respectively. The remaining direct fibrosis markers were unable to distinguish between the two groups. In the following images, the diagnostic performance of radiological markers in identifying patients with fibrotic MASH will be evaluated.

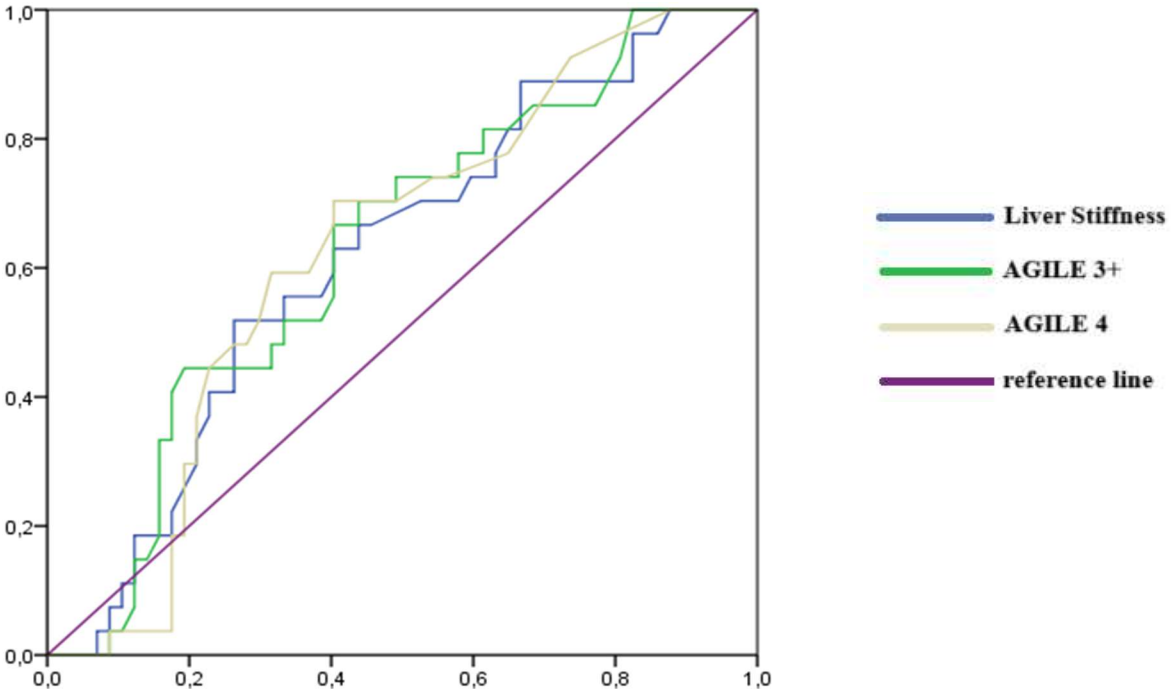


Figure 28. ROC curve of radiological markers in identifying patients with fibrotic MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
LS	0.62	0.50-0.74	0.08
AGILE 3+	0.63	0.51-0.75	0.06
AGILE 4	0.63	0.51-0.75	0.06

Table 27. AUROC of radiological markers in identifying patients with fibrotic MASH

As can be seen from Figure 28 and Table 27, the radiological tests are not able to statistically significantly discriminate patients with fibrotic MASH; however, they show a trend toward significance.

Performance of NITs in Identifying Patients with Severe MASH

In this section, the ROC curves of non-invasive tests for identifying patients with severe MASH, specifically those with NASH, NAS ≥ 4, and F3 fibrosis, will be analyzed.

Regarding the comparison of characteristics between the subgroup of patients with severe MASH and those without, hypertension, metabolic syndrome, glycated hemoglobin, and HOMA index were more significantly represented in the former population compared to the latter. Additionally, in this population, the values of direct fibrosis markers, except for laminin and radiological scores, were significantly increased.

Variable	Without SEVERE MASH	With SEVERE MASH	p-value
ANTHROPOMETRIC-METABOLIC VARIABLES			
MEN	**46** (71,9%)	**13** (65,0%)	0,56
AGE AT BIOPSY (YY)	**51,3** [21,6-67,1]	**55,5** [25,1-69,4]	0,38
BLOOD HYPERTENSION	**38** (59,4%)	**18** (90%)	**0,01**
METABOLIC SYNDROME	**41** (64,1%)	**18** (90%)	**0,03**
BIOCHEMICAL VARIABLES			
GLYCATED HEMOGLOBIN (mmol/mol)	**37** [25-69]	**44** [28-96]	**0,004**
HOMA INDEX	**3,98** [1-61]	**6,23** [2-20]	**0,031**
DIRECT FIBROSIS MARKER			
COLLAGNE IV (ng/mL)	**12** [4,8-160,0]	**18,3** [8,5-135,0]	**0,04**
COLYGLYCINE (µg/mL)	**0,05** [0,03-7,72]	**0,43** [0,03-1,23]	**0,02**
HYALURONIC ACID (ng/mL)	**53,5** [28,4-459,0]	**65** [44,9-93,9]	**0,02**
PIIIP (ng/mL)	**21,7** [3,1-304,0]	**68,3** [12,1-179,0]	**0,001**
SIMPLE AND COMPLEX INDIRECT SCORES			
GOT	**34** [13-124]	**46,5** [22-148]	**0,002**
APRI	**0,4** [0,1-2,5]	**0,7** [0,3-1,6]	**0,008**
FIB-4	**1,19** [0,36-7,10]	**1,53** [0,61-6,00]	**0,03**
ULTRASOUND SCORES			
LS (kPa)	**8,6** [3,3-53,1]	**12,0** [5,5-23,4]	**0,01**
AGILE 3+	**0,3** [0,01-0,99]	**0,68** [0,08-0,94]	**0,02**
AGILE 4	**0,05** [0,00-0,93]	**0,16** [0,02-0,47]	**0,01**

Table 28. Comparison of Characteristics between the Subgroup with Severe MASH and Non-Severe MASH Patients. Categorical variables are expressed as absolute numbers (% of the total); continuous numerical variables are expressed as median (min-max). For nominal variables, Fisher's exact test was used, while the Mann-Whitney U test was applied for continuous variables.

In the images below, the performance of simple indirect biomarkers is evaluated.

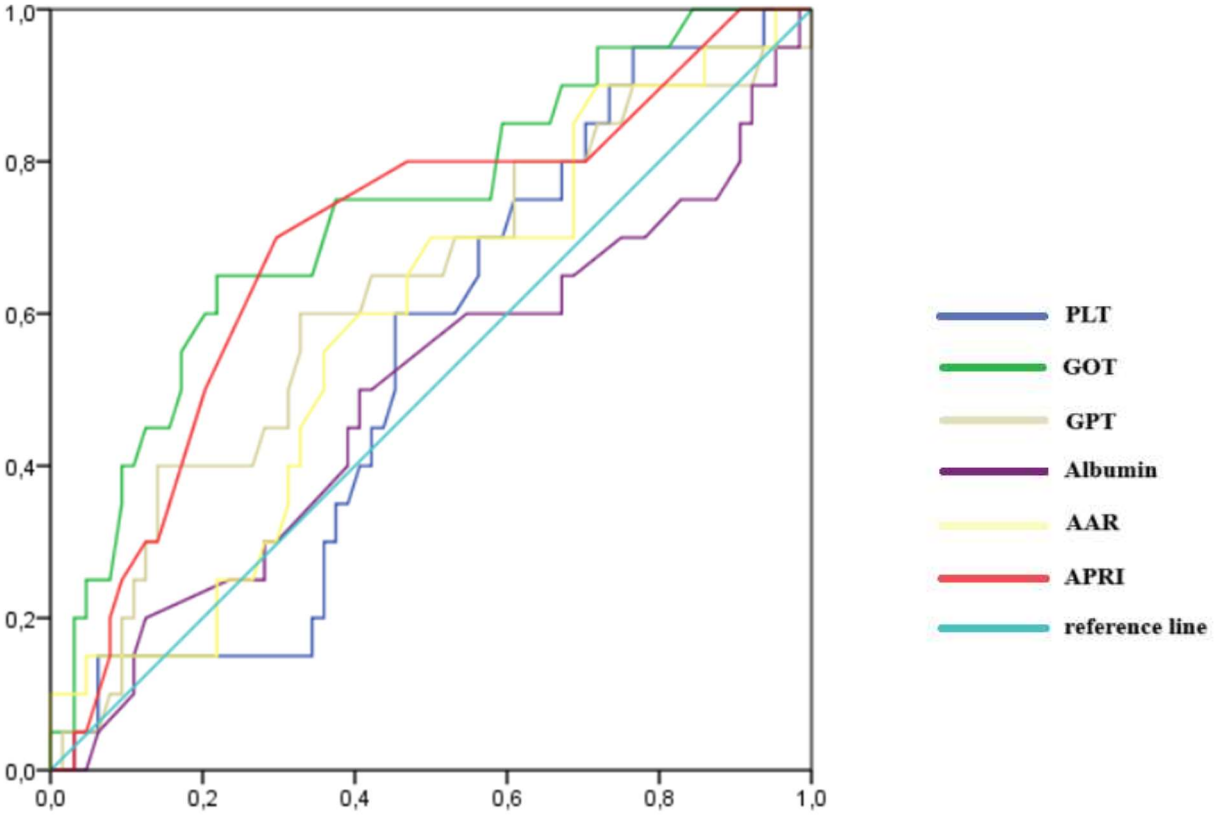


Figure 29. ROC Curve of Simple Indirect Biomarkers in Identifying Patients with Severe MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
PLT	0.54	0.40-0.67	0.63
GOT	0.73	0.60-0.86	**<0,01**
GPT	0.62	0.48-0.77	0.10
ALBUMIN	0.49	0.34-0.65	0.92
AAR	0.58	0.44-0.72	0,07
APRI	0.70	0.56-0.83	**0,01**

Table 29. AUROC of Simple Indirect Biomarkers in Identifying Patients with Severe MASH

As noted in Figure 29 and Table 29, simple indirect biomarkers do not significantly discriminate patients with severe MASH, except for GOT and APRI, which demonstrated moderate AUROC values of 0.73 and 0.70, respectively.

In the following images, the diagnostic performances of complex indirect biomarkers in identifying patients with severe MASH will be analyzed.

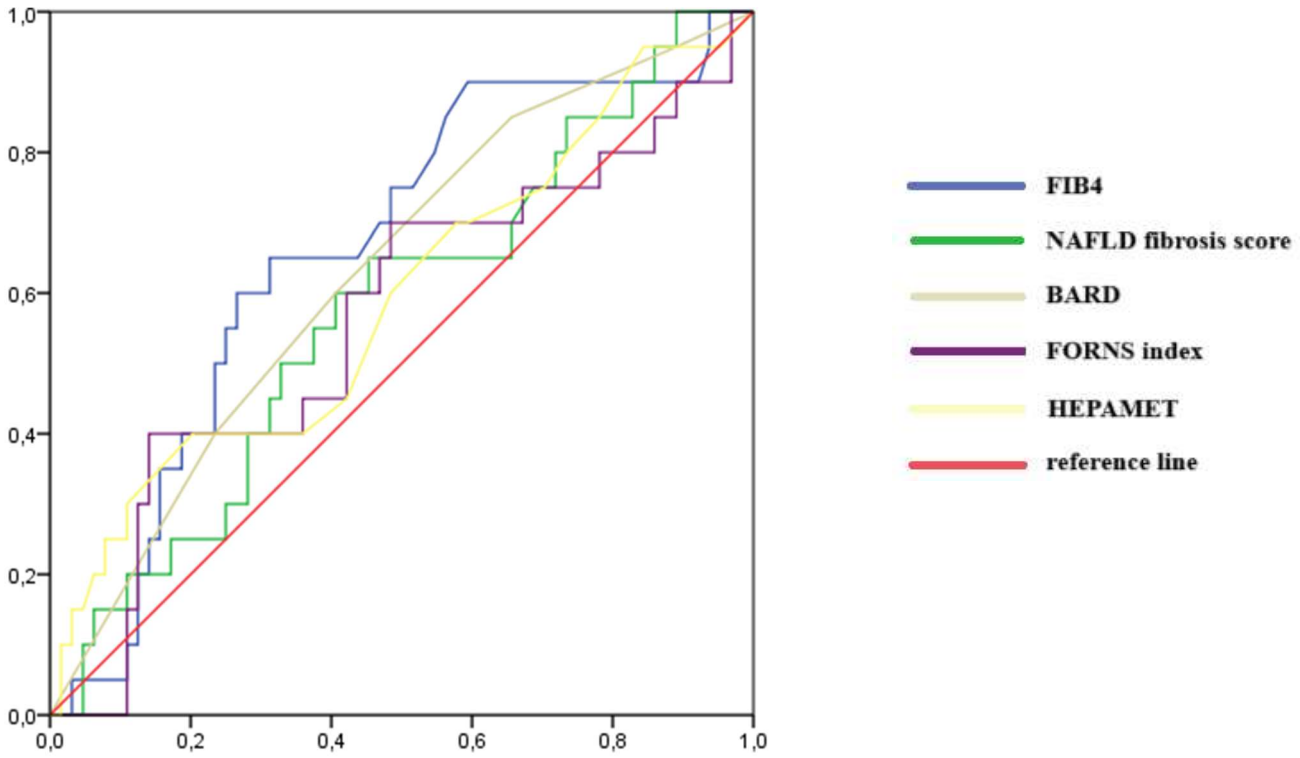


Figure 30. ROC curve of complex indirect scores in identifying patients with severe MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
FIB4	0.66	0.53-0.79	**0.03**
NFS	0.58	0.43-0.72	0.31
BARD	0.63	0.50-0.77	0.08
FORNS	0.57	0.41-0.72	0.38
HEPAMET	0.59	0.44-0.74	0.22

Table 30. AUROC of complex indirect scores in identifying patients with severe MASH

As can be seen from Figure 30 and Table 30, the complex indirect scores did not demonstrate good diagnostic performance in identifying patients with severe MASH. The only exception is represented by FIB4, which achieved an AUROC of 0.66. In the following figures, the diagnostic performances of direct fibrosis markers in identifying patients with severe MASH are reported.

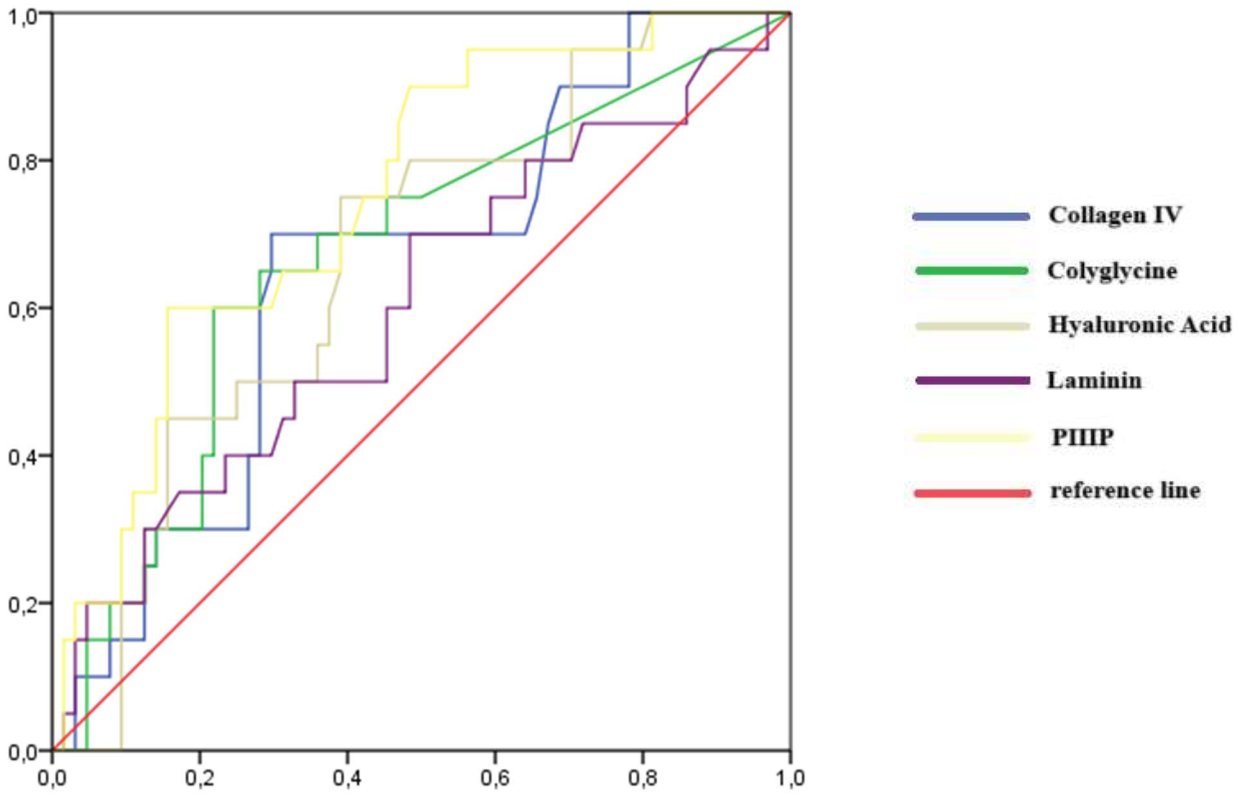


Figure 31. ROC curve of direct fibrosis markers in identifying patients with severe MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
CIV	0.65	0.52-0.78	**0.04**
CG	0.67	0.53-0.81	**0.02**
HA	0.67	0.54-0.80	**0.02**
LAMININ	0.60	0.46-0.75	0.16
PIIIP	0.75	0.63-0.87	**<0,01**

Table 31. AUROC of direct fibrosis markers in identifying patients with severe MASH

Direct fibrosis markers have shown the ability to statistically significantly discriminate patients with severe MASH, with the exception of laminin. PIIIP demonstrated an excellent AUROC, reaching 0.75. In the images below, the diagnostic performances of the radiological scores in identifying patients with severe MASH are presented.

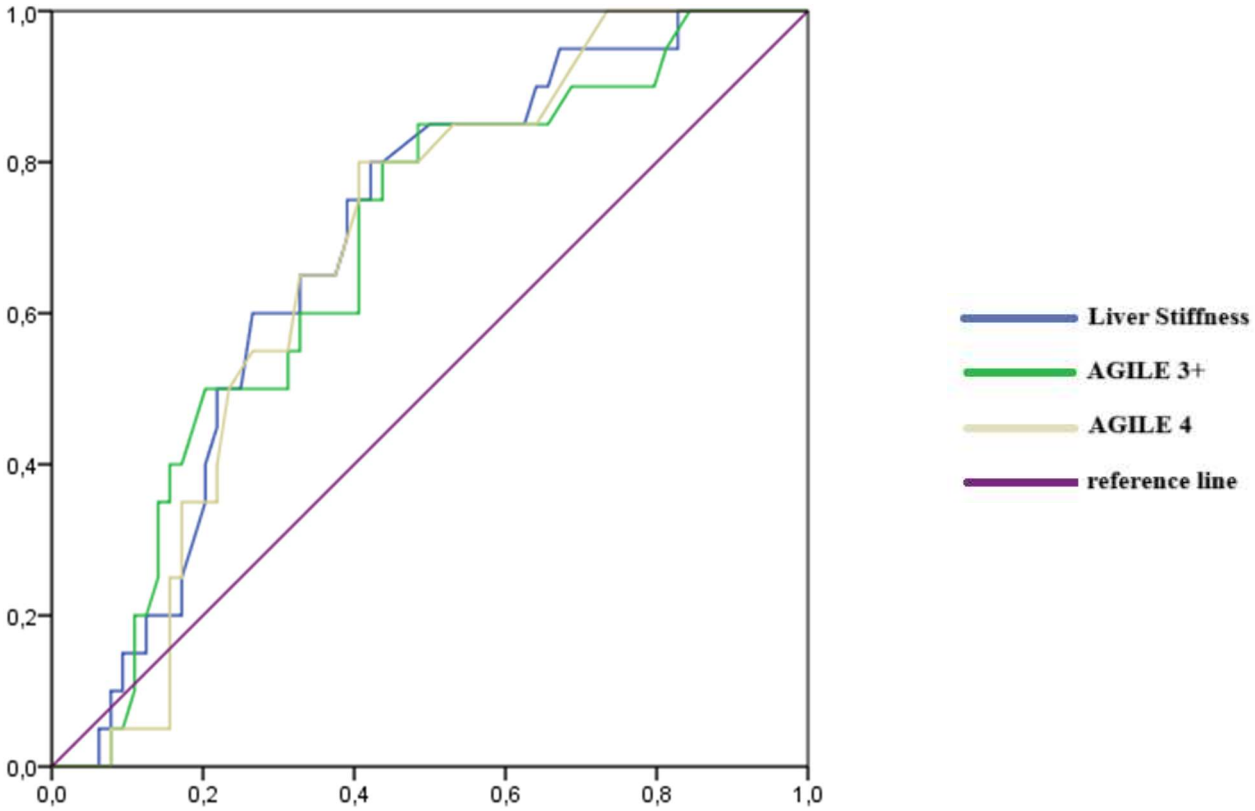


Figure 32. ROC curve of radiological scores in identifying patients with severe MASH. The Y-axis displays the sensitivity variable, while the X-axis shows the 1-specificity variable.

Variable	AUROC	CI 95%	p-value
LS	0.69	0.57-0.81	**0.01**
AGILE 3+	0.68	0.55-0.81	**0.02**
AGILE 4	0.68	0.56-0.80	**0.02**

Table 32. AUROC of radiological scores markers in identifying patients with severe MASH

As can be seen from Figure 32 and Table 32, the radiological scores demonstrated modest but statistically significant performance in identifying patients with severe MASH.

Analysis of Patients with Severe MASH

Patients with severe MASH are those at the highest risk of disease progression to end-stage liver cirrhosis, as they exhibit elevated disease activity, specifically a NAS of at least 4, and advanced fibrosis, at least F3. Therefore, identifying these patients is crucial. It is particularly important to minimize the number of patients who undergo liver biopsy to confirm this diagnosis. This approach aims to reduce the number of patients subjected to an invasive procedure that carries potentially severe complications and to decrease the number of patients with less severe forms who undergo liver biopsy.

In this regard, the current scientific literature presents various algorithms and non-invasive scores designed to identify severe forms of MASH. In the examined population, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of the most recommended scores in the literature were evaluated. Therefore, the values for the cut-offs recommended by the guidelines and authors for the FIB4, NFS, BARD, FORNS, and Hepamet scores were evaluated. For the FIB4, the two cut-offs recommended by the EASL guidelines [6,93] of 1.3 and 2.67 were used. For the NFS, the cut-off was set at 0.676; for the BARD, the cut-off was 2; for Hepamet, the cut-off was 0.47; and for the FORNS score, the cut-off was 6.9.

Additionally, using the Youden test, cut-offs were identified that provided the highest sum of sensitivity and specificity for collagen IV, coliglycin, laminin, PIIIP, hyaluronic acid, liver stiffness, AGILE 3+, and AGILE 4 in identifying patients with severe MASH. Table 33 presents the data related to this analysis.

Variable	SENSIBILITY (%)	SPECIFICITY(%)	PPV	NPV	ACCURACY
BARD ≥ 2	85%	34%	29%	88%	46%
FIB4 ≥ 1,3	65%	61%	34%	85%	62%
FIB 4 ≥ 2,67	5%	91%	14%	75%	70%
NFS ≥ 0,676	20%	83%	27%	77%	68%
FORNS ≥ 6,9	20%	88%	33%	78%	71%
HEPAMET>0,47	20%	92%	44%	79%	75%
CIV>16,5 ng/mL	70%	70%	42%	88%	70%
CG>0,425 µg/mL	75%	50%	32%	86%	56%
HA>57,8 ng/mL	80%	42%	30%	87%	51%
LM>25 ng/mL	70%	52%	31%	85%	56%
PIIIP>61,5 ng/mL	65%	84%	60%	87%	**79%**
LS≥ 9 kPa	80%	58%	37%	90%	63%
AGILE 3+ > 0,32	85%	52%	35%	92%	60%
AGILE 4 > 0,08	80%	59%	38%	90%	64%

Table 33. Performance of Complex Indirect Scores with Cut-Offs Proposed by Guidelines and Authors and Performance of Direct Fibrosis Markers and Radiological Scores Calculated with the Youden's J Test

As can be seen from table 33, the cut-offs proposed in the literature for complex indirect scores present excellent negative predictive values; however, they are burdened by low positive predictive values. This results in a high rate of false negatives and a relatively low diagnostic accuracy. The best results were obtained with Hepamet, achieving a diagnostic accuracy of 75%. As for direct fibrosis markers, they also demonstrated an excellent NPV, generally exceeding the previous scores. Again, the PPV remained limited. Nevertheless, an exception is represented by PIIIP, which at the cut-off of 61.5 ng/mL showed a PPV of 60% with a diagnostic accuracy of 79%. Regarding ultrasound scores, despite demonstrating an extremely high negative predictive value, reaching 90%, the diagnostic accuracy remains around 60%.

Performance of diagnostic algorithms in identifying patients with severe MASH

Following the recommendations provided by the European EASL guidelines and the guidelines of the Italian Association for the Study of the Liver (AISF) [6,93,200], it is recommended that in cases of FIB-4 > 1.30 and LS ≥ 8 kPa, further evaluation should be conducted. Moreover, the AISF guidelines suggest using the NFS as an alternative to FIB-4, with a cut-off of -1.455 and subsequent measurement of liver elastometry with the same cut-off. In the following figures, the diagnostic performance of these two algorithms will be assessed in the population under examination, calculating true positives, true negatives, false positives, and false negatives for each algorithm.

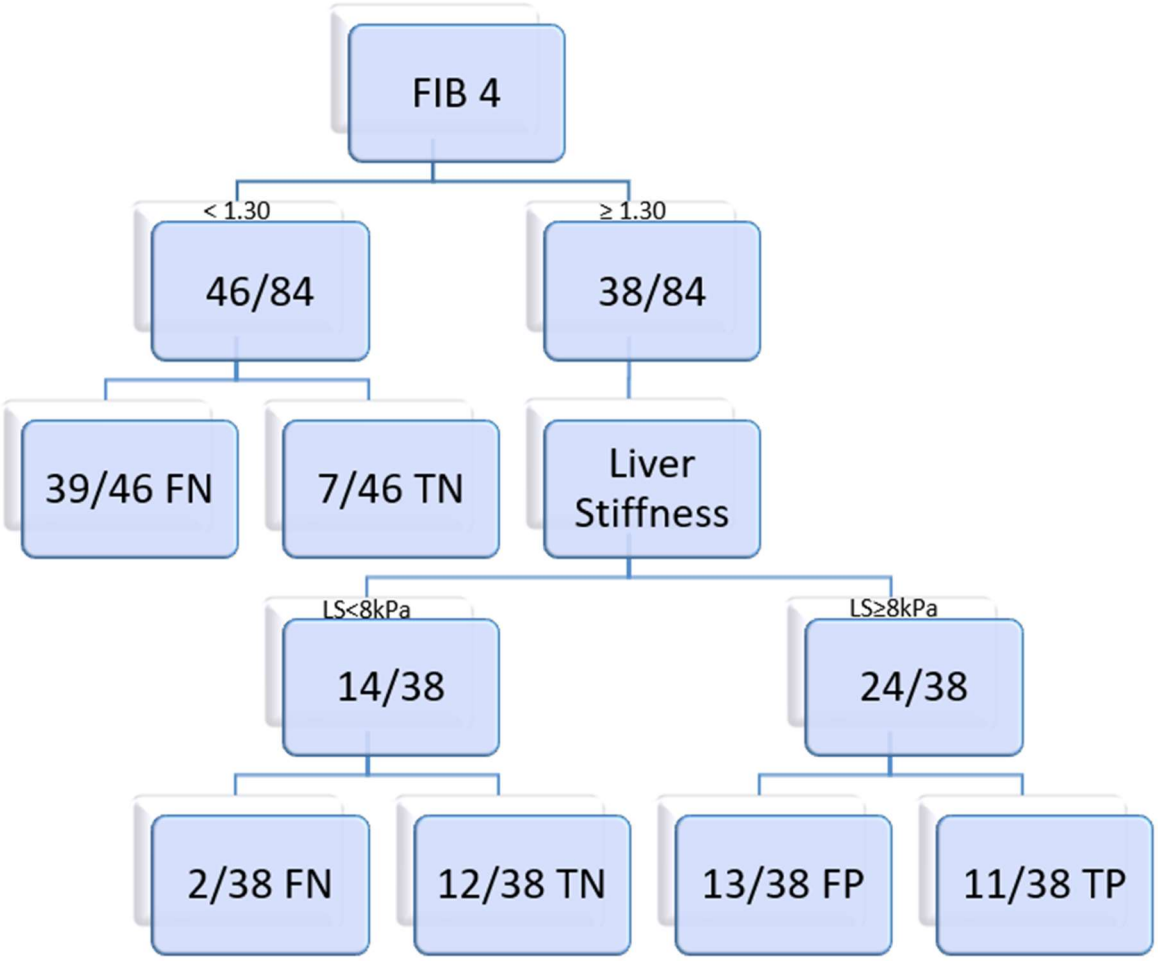


Figure 33. Diagnostic flowchart with execution of FIB4 followed by liver elastometry with a cut-off of 8 kPa in identifying patients with severe MASH. Abbreviations: FN = false negative, TN = true negative, FP = false positive, TP = true positive.

Variable	TN (%)	FN (%)	FP (%)	TP (%)
N. of PATIENTS	51 (60.7%)	9 (10.7%)	13 (15.5%)	11 (13.1%)

Using this algorithm, there would be 9/84 (10.7%) false-negative patients and 13 (15.5%) false-positive patients. A liver biopsy would be performed in 24 (28.6%) patients, and the diagnostic accuracy of the algorithm in identifying patients with severe MASH is approximately 73%.

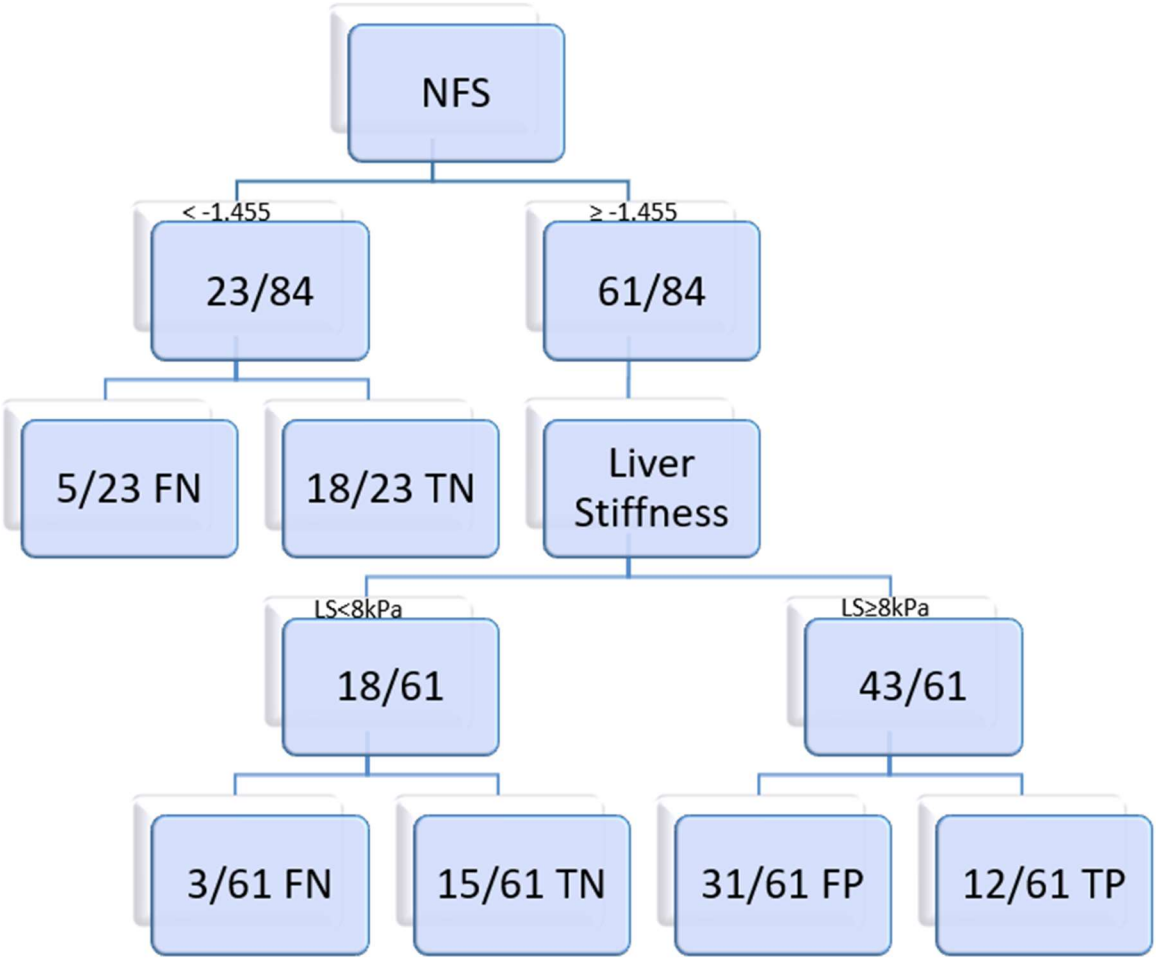


Figure 34. Diagnostic flow chart with the application of NFS using a cut-off of -1.455, followed by liver elastography with a cut-off of 8 kPa in identifying patients with severe MASH. Abbreviations: FN = false negative, TN = true negative, FP = false positive, TP = true positive, NFS = NAFLD fibrosis score.

Variable	TN (%)	FN (%)	FP (%)	TP (%)
N. of PATIENTS	33 (39.2%)	8 (9.5%)	31 (36.9%)	12 (14.4%)

Using this algorithm, 8/84 (10.7%) patients would be false negatives and 31/84 (36.9%) would be false positives. Liver biopsy would be performed in 43 (51.1%) patients, and the diagnostic accuracy of the algorithm in identifying patients with severe MASH is 53.6%.

By applying the cut-offs obtained through the Youden test for LS and PIIIP in the study population—9 kPa and 61.5 ng/mL, respectively—the results presented in figure 35 would be obtained.

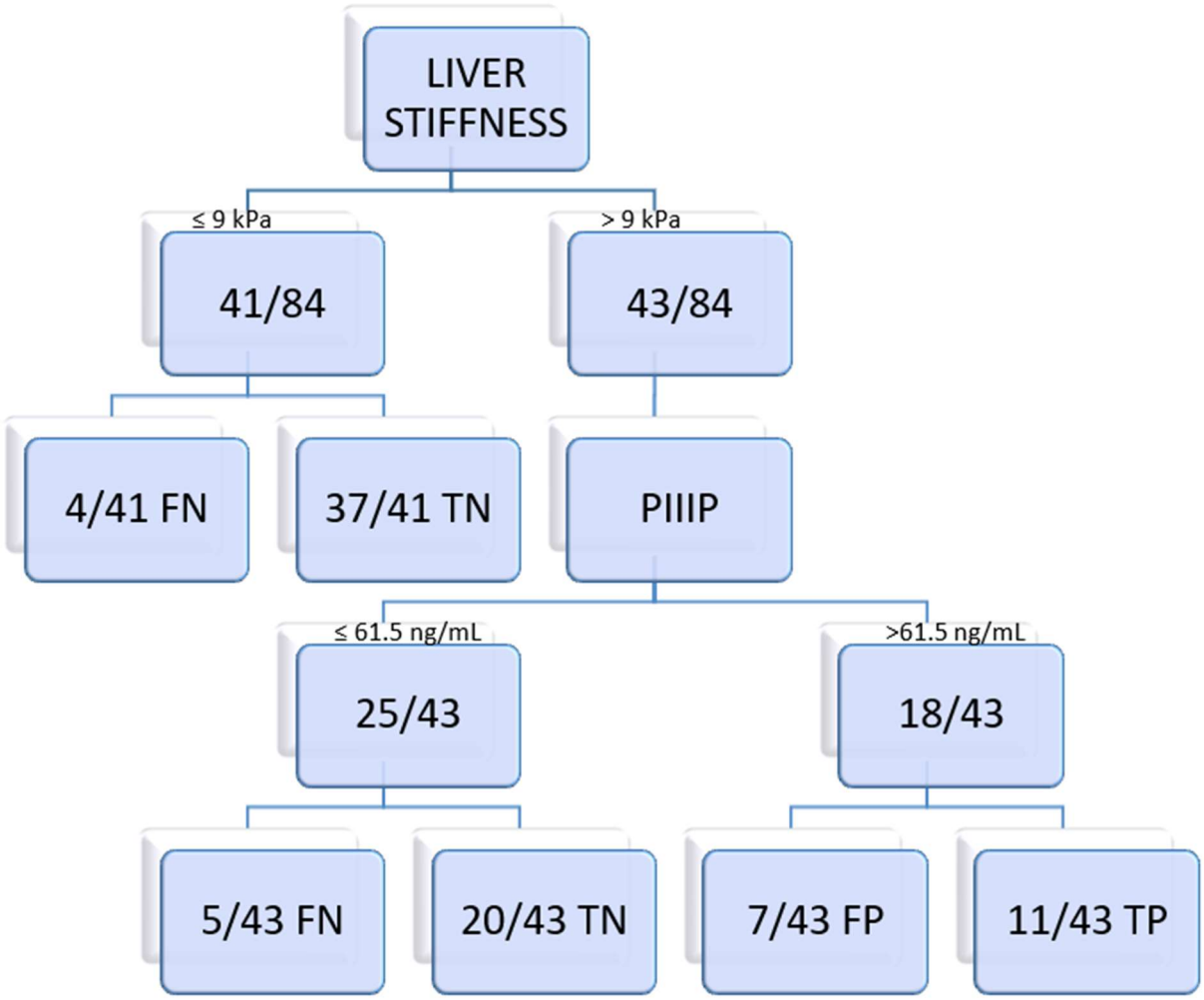


Figure 35. Diagnostic flow chart with liver elastography followed by PIIIP testing in patients with abnormal values, using a cut-off greater than 61.5 ng/mL. Abbreviations: FN = false negative, TN = true negative, FP = false positive, TP = true positive.

Variable	TN (%)	FN (%)	FP (%)	TP (%)
N. of PATIENTS	57 (67.9%)	9 (10.7%)	7 (8.3%)	11 (13.1%)

Using this algorithm, 9/84 (10.7%) patients would be false negatives and 7/84 (8.3%) would be false positives. Liver biopsy would be performed in 18 (21.4%) patients, and the diagnostic accuracy of the algorithm in identifying patients with severe MASH would be 81%.

CHAPTER 5

DISCUSSION

Indirect Simple Biomarkers

The utility of indirect biochemical markers such as GOT, GPT, platelets, and albumin is very limited and not significant in identifying patients with MASH compared to non-MASH patients (figure 13 and table 9), in identifying patients with liver fibrosis of at least F2 compared to those with fibrosis F0 and F1 (figure 17 and table 14), and in identifying patients with fibrosis of at least F3 compared to those with fibrosis F1-F2-F3 (figure 21 and table 19). Only APRI demonstrated statistical significance in identifying patients with fibrosis of at least F3 (AUROC 0.62 CI95% 0.50-0.74, p=0.05), but with modest results. Regarding the discrimination of patients with fibrosis of at least F3, only APRI (AUROC 0.66 CI95% 0.55-0.79, p=0.01), GOT (AUROC 0.64 CI95% 0.52-0.76, p=0.03), and AAR (0.66 CI95% 0.54-0.79, p=0.01) demonstrated statistical significance; however, as can be seen from figure 21 and table 19, the results remain modest.

In the identification of patients with fibrotic MASH, only GOT and APRI proved to be statistically significant (AUROC 0.67 CI95% 0.55-0.79, p=0.01 and AUROC 0.67 CI95% 0.55-0.79, p=0.01, respectively), and they also showed good performance in identifying patients with severe MASH (AUROC 0.73 CI95% 0.60-0.86, p<0.01 and AUROC 0.70 CI95% 0.56-0.83, p=0.01). In general, indirect simple biomarkers such as GPT, albumin, and platelets have not proven to be useful tools in identifying the various histological outcomes, whereas GOT and APRI improved their diagnostic performance with the increasing severity of the histological findings.

The study aligns with the known literature, confirming that simple indirect markers are unreliable in distinguishing between mild and advanced fibrosis and in identifying patients with MASH [97–100]. In a study conducted by Harrison et al., the AUROCs obtained from GOT and GPT in identifying patients with liver fibrosis of at least F2 were 0.63 and 0.55, respectively, while the AUROCs for identifying patients with liver fibrosis of at least F3 were 0.66 and 0.58, respectively—data that are comparable to those found in the population of the present study [201]. Both AAR and APRI were shown to significantly distinguish patients with fibrosis of at least F3, a result also reported by Nielsen et al. with AUROCs of 0.68 for both biomarkers, which are consistent with those obtained in this study [109]. Regarding APRI, the diagnostic performance of the score in identifying patients with fibrosis of at least F3 has been reported in the literature with AUROCs ranging between 0.68 and 0.86, higher than those obtained in the study population [129,202–205]. A recent study conducted by Pennisi et al. analyzed the ability of APRI to identify patients with fibrotic MASH and type 2 diabetes mellitus, finding an AUROC similar to that observed in the population of the present study (AUROC 0.70) [206].

Complex Indirect Biomarkers

Complex indirect biomarkers have shown variable results depending on the histological outcomes. In general, their performance improves when used to distinguish patients with liver fibrosis, with a trend of increasing accuracy as fibrosis stage advances. As shown in figure 18 and table 15, both FIB4, NFS, and the BARD score demonstrated statistical significance in identifying patients with at least F2 fibrosis (AUROC 0.64, 0.65, and 0.67, respectively). The performance of all direct fibrosis biomarkers improved when identifying patients with at least F3 fibrosis (figure 20 and table 20), though with modest AUROCs ranging from 0.63 to 0.71.

In identifying patients with MASH, only FIB4 and Hepamet demonstrated statistically significant discrimination, with AUROCs of 0.66 and 0.70, respectively. None of these items were able to discriminate patients with fibrotic MASH, and only FIB4 was shown to significantly identify patients with severe MASH, as seen in figure 30 and table 30 (AUROC 0.66, CI95% 0.53-0.79, p=0.03).

Regarding NFS, the literature reports diagnostic performance in discriminating patients with at least F2 fibrosis that varies between 0.60 and 0.68, aligning with the results obtained in this study [201,204,207]. However, its performance in identifying F3 fibrosis is significantly better in other studies, with ROC values ranging from 0.81 to 0.84 [203,208], although statistical significance is still observed in the study population (AUROC 0.65, CI95% 0.53-0.76, p=0.02).

NFS did not significantly discriminate patients with MASH, fibrotic MASH, or severe MASH in this study. Pennisi et al. reported an AUROC of 0.71 for NFS in identifying fibrotic MASH in a diabetic patient population, a result not far from what was observed in this study population [206].

The FIB-4 has demonstrated strong performance in several studies for distinguishing patients with at least F2 fibrosis (AUROC around 0.70), with performance increasing as hepatic fibrosis progresses [201,207]. In this study, FIB-4 showed significant performance in identifying patients with at least F2 or F3 fibrosis, though with moderate AUROC values. Various authors have reported that this score tends to perform worse in populations with diabetes mellitus.

Chung et al. conducted a study on 267 patients, demonstrating that in patients with diabetes mellitus, the performance of FIB-4 was lower compared to those without diabetes in identifying patients with at least F3 fibrosis (AUROC 0.653 vs. 0.826, respectively) [209]. Pennisi et al. found an AUROC of 0.65 and 0.71 in identifying patients with MASH and fibrotic MASH in diabetic populations, which aligns with the results found in the population examined in this study [206].

The low performance of FIB-4 observed in this study could be related to the high prevalence of subjects with T2 diabetes mellitus or fasting glucose intolerance. Additionally, FIB-4 was the only complex indirect marker able to significantly discriminate patients with severe MASH, although its diagnostic capabilities were limited (see figure 30 and table 30). Similar AUROC values for the identification of MASH by FIB-4 have also been reported by Nielsen et al. [109].

In the studies conducted by Balakrishan et al. [202] and McPherson et al. [121], the BARD score demonstrated AUROCs of 0.76–0.81 in distinguishing significant and advanced fibrosis. However, this finding was not confirmed in our study, where the BARD score showed significant but reduced diagnostic performance in identifying significant and advanced fibrosis (AUROCs of 0.68 and 0.71, with p-values <0.01 for both).

The FORNS index only proved effective in discriminating patients with at least F3 fibrosis (AUROC 0.64, p = 0.03). Ballestri et al. found poor performance of the FORNS index in distinguishing significant fibrosis in patients with NAFLD (AUROC 0.62), similar to what was observed in this population [210]. In the same study, excellent capability was found in distinguishing advanced fibrosis (AUROC 0.92), but the index failed to distinguish patients with NASH from those without, a finding also mirrored in this study [210].

Regarding HEPAMET, the results from this study highlighted that this score was not significant in identifying at least F2 fibrosis, or in distinguishing patients with fibrotic MASH and severe MASH. However, the score demonstrated an AUROC of 0.70 in identifying patients with MASH (p=0.01, figure 14 and table 10). In a study conducted on a Latin American population, HEPAMET showed statistical significance in identifying at least F2 fibrosis in patients with NAFLD (AUROC 0.725, p=0.002), but not for advanced fibrosis [211].

Another study conducted on 200 patients with NAFLD demonstrated an AUROC of 0.68 for HEPAMET in distinguishing patients with advanced fibrosis [135]. The same authors concluded that the predictive power of this score is lower than expected, a finding that is confirmed in our study [135]. In fact, in the validation study of HEPAMET, the score had shown an AUROC of 0.85 in discriminating advanced fibrosis.

Direct fibrosis biomarkers

Direct fibrosis markers have demonstrated variable performance depending on the histological outcome analyzed. CIV showed statistical significance for all histological outcomes, with the exception of fibrotic MASH. LM was able to distinguish only patients with MASH or fibrotic MASH, losing significance as hepatic fibrosis progressed, including in the case of significant, advanced fibrosis, and severe MASH. Conversely, HA, PIIIP, and CG significantly distinguished patients with at least stage F3 hepatic fibrosis and those with severe MASH. In particular, PIIIP demonstrated a good discriminatory ability in identifying patients with severe MASH (AUROC 0.75, p<0.01, figure 31 and table 31).

A study conducted by Stefano et al. on 126 patients with biopsy-proven NAFLD showed an AUROC of 0.718 for discriminating significant fibrosis and 0.79 for advanced fibrosis [212]. In the same study, the diagnostic performances of HA, CG, and LN were also evaluated in distinguishing significant fibrosis, reporting AUROCs of 0.57, 0.59, and 0.578, respectively, and in distinguishing advanced fibrosis, where the AUROCs were 0.63 for all three markers, similar to those found in the examined population [212].

Regarding PIIIP, Nielsen et al. reported an AUROC of 0.70 in recognizing significant fibrosis forms of NAFLD, while Bril et al. found an AUROC of 0.90 with a cut-off of 20 ng/ml in discriminating patients with at least F3

hepatic fibrosis [109,129]. Tanwar et al. reported an AUROC of 0.86 for PIIIP in identifying patients with severe MASH, a value significantly higher than that observed in the examined population, although PIIIP was the marker that demonstrated the best diagnostic performance [105].

PIIIP has demonstrated excellent diagnostic performance even in children affected by NASH, as shown by Mosca et al., discriminating patients with at least F2 fibrosis with an AUROC of 0.92 and patients with at least F3 fibrosis with an AUROC of 0.99 [113]. These data were not confirmed in our population, although PIIIP was significantly different in patients with at least F3 fibrosis, showing a more modest AUROC of 0.66.

HA has proven capable of significantly distinguishing patients with at least F3 fibrosis (AUROC 0.68, p=0.01, figure 23 and table 21), although the literature contains studies demonstrating better performance, such as that by Suzuki et al. (AUROC 0.89) [213].

US Elastographic tests

Ultra-sound Elastographic tests have demonstrated good results in identifying almost all histological outcomes. However, they have not shown significant discriminatory capacity in patients with MASH and exhibited a trend toward significance in recognizing patients with fibrotic MASH, which could potentially reach significance with an increase in the study population.

Specifically, LS has shown improved performance with increasing fibrosis; as highlighted in figures 20 and 24 and tables 17 and 22, the AUROC for discriminating significant and advanced fibrosis are 0.71 and 0.75, respectively. In the literature, regarding these outcomes, the results are more favourable. Kumar et al. found an AUROC of 0.85 for at least F2 fibrosis and an AUROC of 0.94 for discriminating advanced fibrosis [214]. A recent meta-analysis reported slightly lower AUROC values, namely 0.83 for significant hepatic fibrosis and 0.85 for advanced fibrosis [215]. A study conducted by Won Lee et al. found that LS could discriminate patients with NASH from those without NASH, a finding not confirmed in the present study [216]. Additionally, Pennisi demonstrated better results regarding the discrimination of patients with MASH and fibrotic MASH by LS, with AUROC values of 0.71 and 0.79, respectively, in a population of patients with T2DM and NAFLD [206].

AGILE 3+ and AGILE 4 have demonstrated results similar to LS, of which they are surrogates. Indeed, both have not been able to significantly distinguish patients with MASH from those with fibrotic MASH; however, they showed interesting AUROC values in discriminating patients with at least F2 and F3 fibrosis, as well as those with severe MASH. The diagnostic performances obtained in patients with at least F3 fibrosis are lower than those reported in the literature. Pennisi et al. demonstrated that AGILE 3+ has an AUROC of 0.88 for advanced fibrosis [206]. In the same study, AUROC values of 0.69 and 0.77 were reported for identifying patients with NAFLD and fibrotic NAFLD using AGILE 3+ [206].

AGILE 4 is a score designed to identify patients with F4 fibrosis. In the study in question, it demonstrated the ability to distinguish patients with at least F2 fibrosis, at least F3 fibrosis, and severe MASH. Compared to validation studies, the AUROC for discriminating advanced fibrosis is lower, with reported values ranging from 0.89 to 0.93; however, in our study, patients with F3 and F4 fibrosis were evaluated, not solely those with F4 fibrosis [139]. Among all non-invasive tests, elastosonographic tests have shown superior performance, particularly as the degree of hepatic fibrosis increases.

Severe MASH

An important subpopulation of this study consists of patients with severe MASH, as these individuals exhibit high disease activity (NAS ≥ 4) and advanced fibrosis (at least F3). Consequently, they are at risk for disease progression to liver cirrhosis and the potential development of HCC, as well as an increased CV risk. Therefore, identifying these patients through liver biopsy is crucial.

Analyzing Table 28, it is evident that this subgroup is predominantly male (65%) with a median age at liver biopsy of 55 years [25-96]. Compared to patients without severe MASH, those with severe MASH present a higher prevalence of metabolic profile alterations, including hypertension (90% vs 59%), insulin resistance (median HOMA 6.23 vs 3.98, p=0.03), and a higher median glycated hemoglobin (44 mmol/mol vs 37 mmol/mol, p=0.004). Additionally, they have a greater overall number of diagnoses of metabolic syndrome (90% vs 64%, p=0.03).

Direct fibrosis markers are significantly higher in patients with severe MASH, along with ultrasound scores. Specifically, the median LS is 12 kPa compared to 8.6 kPa in patients without severe MASH (p=0.01).

The PIIIP was significantly higher in patients with severe MASH (68.3 ng/mL vs 21.7 ng/mL, p<0.01). These differences between the two groups in the values of LS and PIIIP were utilized to assess a sequential diagnostic algorithm considering the identification of patients with severe MASH as the outcome, evaluated using the algorithm proposed by the Italian Society of Hepatology AISF [200].

Through the Youden test, the ideal cut-off for sensitivity and specificity for LS and PIIIP in identifying patients with severe MASH was determined, and for each value, sensitivity, specificity, PPV, and NPV were identified [table 33]. From table 33, it can be observed the performance in terms of sensitivity, specificity, PPV, NPV, and diagnostic accuracy of the various indirect complex biomarkers at the cut-offs proposed by the AISF and EASL guidelines [6,93,200], as well as the cut-offs identified using the Youden test for direct fibrosis markers and ultrasound scores.

In general, the indirect complex markers are deficient in sensitivity, with the exception of BARD, while demonstrating good specificity and PPV values. The diagnostic accuracy ranges from 46% for BARD to 75% for HEPAMET. The direct fibrosis markers exhibit good sensitivity and excellent negative predictive value, with the best diagnostic accuracy for identifying patients with severe MASH reported by PIIIP, achieving nearly

80%. Finally, regarding the ultrasound scores, it can be noted that they possess excellent sensitivity with an outstanding NPV (greater than 90%), but a diagnostic accuracy around 60%.

Based on the threshold values provided by the AISF guidelines for FIB-4, NFS, and LS, the classification of patients was evaluated according to the algorithm (figures 33 and 34). Figure 33 illustrates an algorithm that initially employs FIB-4 with a cut-off of 1.30; if patients have a result above this value, hepatic elastometry is assessed, and if the value exceeds 8 kPa, the patient should undergo liver biopsy. According to this algorithm, the study patients would exhibit 107% of false negatives, 15.5% of false positives who would undergo liver biopsy, 60.7% of true negatives, and 13.1% of true positives. The diagnostic accuracy would be 73%, and the referral for liver biopsy would occur in 28.6% of patients.
In contrast, figure 34 presents the scenario where NFS is utilized in the same manner as FIB-4, with a cut-off of -1.455. In this case, 39.2% of patients would be true negatives, 9.5% false negatives, 36.9% false positives, and 14.4% true positives. The diagnostic accuracy of the algorithm is slightly above 50%, and the referral for liver biopsy would occur in 51% of patients.

Proposal for an Alternative Algorithm

Based on the results obtained with the cut-off values identified through the Youden test, it was possible to create an alternative algorithm where patients with suspected severe MASH would first undergo the measurement of LS. If this value exceeds 9 kPa, the plasma concentration of PIIIP would be measured. If this concentration is greater than 61.5 ng/mL, patients would be referred for liver biopsy (figure 35).
Following this algorithm, the examined population would yield 67.9% true negatives, 10.7% false negatives, 8.3% false positives, and 13.1% true positives. In this scenario, liver biopsy would be performed in 21.4% of patients, and the diagnostic accuracy would be 81%. These performance metrics are superior to those of the algorithms proposed by the EASL and AISF guidelines [6,93,200].

Limitations of the Study

A limitation of the study is undoubtedly determined by the small sample size. Indeed, it can be observed that in several cases, there was a tendency toward significance for some biomarkers or ultrasound scores that could potentially have been significant if the sample size had been larger. A selection bias in the study, due to the high prevalence of patients with partial or complete manifestations of metabolic syndrome, particularly those with T2DM, may have resulted in reduced performance of complex indirect markers, a known issue in the literature. However, it is important to emphasize that this aspect is highly representative of real-life cases,

as the prevalence of diabetes and metabolic syndrome is very high among patients with NAFLD. In specific categories such as obese patients or those with T2DM, the prevalence of MAFLD is significantly greater than in the general population.

Strengths

One of the main strengths of the study was the opportunity to evaluate the diagnostic performance of direct fibrosis markers and their potential use in combination with LS to identify a diagnostic algorithm aimed at more accurately identifying patients with severe MASH who should undergo liver biopsy. In particular, there are no studies in the literature assessing the diagnostic performance of these markers in identifying patients with a more advanced form of the disease, such as those with severe MASH. Equally important is the fact that the study is based on data obtained through histological examination of liver biopsies from all patients.

CHAPTER 6

CONCLUSIONS

MASLD is a condition that encompasses a broad spectrum of phenotypic manifestations, ranging from indolent forms (MASL) to forms of hepatitis (MASH), which can ultimately culminate in cirrhosis and liver tumors. Advanced forms of the disease are not only burdened by liver-related manifestations but also by comorbidities and increased cardiovascular and extrahepatic cancer mortality. MASLD has experienced a progressive increase in prevalence over recent decades. Currently, there are no non-invasive tests (NITs) available to identify patients with severe forms of MASH, as the gold standard remains liver biopsy. However, liver biopsy is an invasive test associated with potential complications, even severe ones, and incurs costs for the healthcare system. Therefore, research in this field is focused on identifying a non-invasive strategy to pre-select patients with advanced disease, thereby reducing the number of liver biopsies and increasing diagnostic accuracy. A problem is that individual non-invasive tests (NITs) often create "grey areas" where severe forms of the disease cannot be conclusively excluded or strongly suspected. This study has determined that in specific high-risk populations for MASH, such as obese, diabetic, hypertensive patients, or those with metabolic syndrome, the prevalence of MASH is high, reaching up to 72%; advanced fibrosis is present in 40% of patients, fibrotic MASH in 32%, and severe MASH in 23.8%. Moreover, there is a correlation between metabolic variables such as hypertension, lipid profile abnormalities, and glucose alterations and the histological outcomes of disease severity.

The study demonstrated that simple indirect biomarkers do not correlate with the presence of MASH, and their performance is relatively limited in identifying forms of hepatic fibrosis. Complex biochemical markers have proven to be of little use in determining histological outcomes, although in some cases, they have shown statistical significance, with AUROC values reaching, at best, 0.70.

Another interesting finding from the study is that fibrosis markers, particularly PIIIP, can serve as a valuable tool, especially when used in conjunction with elastometric data to identify patients with severe forms of MASH. The sequential use of LS and PIIIP would result in a 7% reduction in the number of biopsies performed, alongside an improvement in diagnostic accuracy of nearly 10%. However, given the initial findings and the small sample size, further studies should be conducted in this direction.

CHAPTER 7

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