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Risk of Liver-Related Events in Metabolic Dysfunction-Associated Steatohepatitis (MASH) Patients with Fibrosis: A Comparative Analysis of various risk stratification criteria

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Manuel Romero-Gomez consults, advises, and received grants from AbbVie, Gilead, and Novo Nordisk. He advises Alfa-Sigma, Madrigal, and Pfizer. He received grants from Echosens, Siemens, and Theratechnologies. Elisabetta Bugianesi consults for Boehringer Ingelheim, Bristol Myers Squibb, MSD, Novo Nordisk, and Pfizer. She received grants from Gilead. Vincent Wai-Sun Wong consults, advises, and is on the speakers' bureau for AbbVie, Gilead, and Novo Nordisk. He consults and advises Boehringer Ingelheim, Echosens, Intercept, Inventiva, Pfizer, Sagimet, and TARGET. He is on the speakers' bureau for AbbVie and Unilab. He owns stock in Illuminatio Medical Technology. Victor de Ledinghen consults and is on the speakers' bureau for Escopics and Giled. He consults for Bristol Myers Squibb and Novo Nordisk. He is on the speakers' bureau for

AbbVie, GlaxoSmithKline and Janssen. Jacob George consults, advises, and is on the speakers' bureau for Novo Nordisk. He advises and is on the speakers' bureau for Eisai. He advises Histoindex, Pfizer, and Roche. Annalisa Berzigotti consults for Boehringer Ingelheim. She is on the speakers' bureau for General Electric and Hologic. Giada Sebastiani consults, advises, and is on the speakers' bureau for Novo Nordisk. She consults and advises Intercept. She advises and is on the speakers' bureau for AbbVie, Gilead, Merck, and Pfizer. She received grants from Theratechnologies. Roberto Cannella received grants from Bayer, Bracco, and Siemens Healthcare. Salvatore Petta advises and is on the speakers' bureau for AbbVie, Echosens, Gilead, MSD, Novo Nordisk, and Pfizer. The remaining authors have no conflicts to report.

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Graphical Abstract

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ABSTRACT

Background: International regulatory agencies recommend testing drug therapy for patients with noncirrhotic high-risk metabolic dysfunction-associated steatohepatitis (MASH) because they are at risk of liver-related events(LRE). We aimed to compare the risk of LRE in MASLD patients stratified for F2-F4 fibrosis and MASH.

Methods:1938 consecutive patients with biopsy-proven MASLD were enrolled. High-risk MASH was defined as MASH with F2-F4 fibrosis. LSM was measured by transient elastography. LRE were recorded during follow-up. Cox multivariate models were used to assess the association between high-risk MASH or F2-F4 fibrosis without MASH, of LSM(≥8 or ≥10 Kpa) and of AGILE 3+ with LRE. The diagnostic performance for the prediction of LRE was assessed using the area under the receiver operating characteristic(AUROC) curves.

Results: The observed 5-year actuarial rate of LRE was 0.4%,0.2%,5.1% and 6.6% in patients with F0-F1 fibrosis without MASH, F0-F1 fibrosis with MASH, F2-F4 fibrosis without MASH, and high-risk MASH, respectively. At multivariate Cox regression analysis using F0-F1 fibrosis without MASH as reference, both F2-F4 fibrosis without MASH (adjusted hazard ratio[aHR] 9.96) and high-risk MASH (aHR 10.14) were associated with LRE. In the 1074 patients with available LSM, LSM≥10 KPa(aHR 6.31) or AGILE 3+ >0.67 (aHR 27.45) independently predicted the development of LRE and had similarly acceptable 5-year AUROC to high-risk MASH and F2-F4 fibrosis(0.772,0.818,0.739, and 0.780, respectively).

Conclusions: The risk of LRE is similar in patients with high-risk MASH and with F2-F4 fibrosis without MASH. The use of LSM≥10 KPa or AGILE 3+ >0.67 could be an accurate option to identify MASLD patients worthy to be included in clinical trials.

Keywords: MASLD, MASH, Fibrosis, High-risk MASH, liver stiffness measurement.

INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) affects about 30% of general population worldwide with an estimated prevalence of nonalcoholic steatohepatitis of about 5% [1]. MASLD is a growing cause of chronic liver diseases, leading to both liver-related –liver decompensation (LD) and hepatocellular carcinoma (HCC) - and extrahepatic–cardiovascular events (CVE) and extrahepatic cancer (EHC) – complications, as well as to an increase in direct and indirect costs for national health systems [2-4].

Currently in clinical practice, lifestyle intervention remains the backbone of MASLD therapy, even though many promising drugs -like resmetirom, lanifibranor, semaglutide, pegozafermin, etc- with different mechanisms of action against metabolic dysfunction-associated steatohepatitis (MASH) and/or fibrosis are under investigation [5-9]. Some of them showed positive preliminary results in clinical trials [5-9] and are currently under review by international regulatory agencies.

In this rapidly evolving landscape, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) encourage focusing drug development in phase IIb and III clinical trials “on the area of greatest need and potential effect on health, i.e., noncirrhotic patients with MASH -included in presence of a NASH activity score (NAS) greater than or equal to 4 with at least 1 point each in inflammation and ballooning- and with liver fibrosis”- namely high-risk MASH (MASH + F2-F4 fibrosis)-, or, in separate trials on patients with compensated MASLD-related cirrhosis” [10-12].

These recommendations are an outcome of evidence showing that MASH patients have double the rapidity of fibrosis progression compared to those with simple fatty liver [13], and longitudinal data reporting that the severity of liver fibrosis is the main determinant of both hepatic

and extrahepatic events in MASLD patients [4,14,15]. However, when looking at natural history studies in MASLD, some reports have also observed fibrosis progression also in patients without MASH [16], supporting a role for fibrosis, regardless of the presence of steatohepatitis, in driving liver-related and extrahepatic outcomes [14]. This suggests that testing new drugs only in the setting of patients with high-risk MASH could miss a slice of those with clinically relevant fibrosis who are also at risk of adverse outcomes.

The aim of our study was to compare the risk for the occurrence of hepatic and extrahepatic complications (LRE, CVE and EHC) in a large biopsy-confirmed cohort of MASLD patients stratified for significant fibrosis and the presence of steatohepatitis. Our secondary aim was to compare the accuracy of histological defined high-risk MASH with liver stiffness measurement (LSM) and with AGILE 3+ score, for the prediction of hepatic and extrahepatic outcomes.

METHODS

Patient selection

We retrospectively analyzed data from a multicentric cohort of MASLD patients with histological diagnosis of MASLD, prospectively recruited at the Gastrointestinal and Liver Unit of the Palermo University Hospital, at Centre d'Investigation de la Fibrose Hépatique of the Bordeaux University Hospital, at Hepatology Unit of Ospedale San Giuseppe University of Milan, at Hospital Universitario Virgen del Rocío de Sevilla, at Department of Medicine and Therapeutics of the Chinese University of Hong Kong, at the Swiss Liver Center, at Division of Gastroenterology, Department of Medical Sciences of University of Torino, at Department of Pathophysiology and Transplantation, Ca' Granda IRCCS Foundation of Policlinico Hospital of University of Milan, at the Division of Gastroenterology and Hepatology, McGill University Health Centre of Montreal QC and at Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney. Patients were included if they had follow-up and a survival of at least 6

months. Other causes of liver disease were ruled out, including alcohol intake (>20 g/day) as evaluated by a questionnaire, viral (hepatitis B surface antigen, anti–hepatitis C virus, and anti–human immunodeficiency virus negativity) and autoimmune hepatitis, hereditary hemochromatosis, and alpha-1 antitrypsin deficiency.

The study was carried out in accordance with the principles of the Helsinki Declaration and with local and national laws. Approval was obtained from the AOUP "Paolo Giaccone" of Palermo, and locally approved also from all the other centres.

Histological assessment of MASLD

Slides were coded and read at each clinical center by one expert pathologist who was unaware of the patients' identity and history. A minimum 15mm-length of the biopsy specimen or the presence of at least 10 complete portal tracts was required [17]. The inter-observer agreement of pathologists between centers was acceptable -as previously tested in Palermo, Turin and Milan cohorts- [18] and similar to that reported in literature [19]. Kleiner scoring system [19] was used to compute steatosis, ballooning, and lobular inflammation, and to stage fibrosis from 0 to 4. MASH was defined by concomitantly presence of steatosis, lobular inflammation and ballooning. Patients were categorized in 4 groups according to F2-F4 fibrosis (significant fibrosis) and MASH: 1) F0-F1 fibrosis without MASH; 2) F0-F1 fibrosis with MASH; 3) F2-F4 fibrosis without MASH; 4) F2-F4 fibrosis with MASH (High-risk MASH).

Patient evaluation

Clinical and metabolic data were collected at the time of enrollment. Body mass index (BMI) was calculated in kilograms for weight and in meters for height. Obesity was defined as BMI ≥ 30 Kg/m^2. The diagnosis of type 2 diabetes (T2D) was made according to the American Diabetes Association [20], using a value of fasting blood glucose ≥ 126 mg/dl. In patients with a previous diagnosis of T2D, current medications and their changes were documented and used for making

diagnosis of T2D. Arterial hypertension was defined by systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, or use of blood-pressure-lowering agents [21].

A 12-hours overnight fasting blood sample was drawn to determine serum levels of AST, ALT, platelets (PLT), albumin, INR, total cholesterol and triglycerides.

LSM was used to noninvasively assess fibrosis. LSM was evaluated with transient elastography by FibroScan (Echosens, Paris, France) after overnight fasting, using the M and the XL probe when appropriate. We classified all LSM examinations into three reliability categories: "very reliable" (IQR/M ≤0.10), "reliable" (0.10< IQR/M ≤0.30, or IQR/M >0.30 with LSM median <7.1 kPa), and "poorly reliable" (IQR/M >0.30 with LSM median ≥7.1 kPa) [22]. Only patients with 10 valid measurements were included, and “poorly reliable” results were excluded from the analysis. Based on LSM, patients were classified as at low (LSM<8 KPa) or intermediate-high (LSM≥8 KPa) risk of advanced fibrosis [23], or with/without MASLD-related compensated advanced chronic liver disease (cACLD) (LSM≥10 KPa or LSM<10 KPa, respectively) [24]. For the definition of cACLD we also used the recent proposed LSM cut-off of 12 KPa [25].

The AGILE 3+ score (comprising age, sex, AST, ALT, PLT, T2D status, and LSM) was calculated using the original reported formula, and patients were classified as at low (AGILE 3+ <=0.67) or high risk of advanced fibrosis (AGILE 3+ > 0.67) [26, 27].

Liver-related Events (LRE) and Extra- Hepatic Events (EHC) assessment

LRE and extrahepatic events were recorded during the entire follow-up. LRE were defined as LD - occurrence of ascites and/or bleeding varices and/or encephalopathy and/or jaundice- or development of HCC. Extrahepatic events were defined as CVE –stroke, transient ischemic attack, myocardial infarction, unstable angina– or EHC.

Ultrasound examination was carried out yearly in patients with F0-F2 fibrosis and, as stricter surveillance for HCC, every 6 months in patients with F3 fibrosis or cirrhosis, according to international guidelines [28].

In the presence of cirrhosis, esophageal gastroscopy was performed at baseline and repeated as recommended by clinical guidelines [24]. Patients with progression to medium or large (F2 or F3) esophageal varices were treated with beta-blockers or underwent elastic banding, whereas no prophylaxis was scheduled for patients with small (F1) varices. After the publication of the PREDESCI trial in 2019 [29] some centres started using carvedilol beyond the grade of varices, in an individualized fashion, for patients with documented or highly suspected clinically significant portal hypertension.

Patients developing LRE during follow-up were evaluated for available therapies and/or for liver transplantation, if appropriate. Evidence of extrahepatic events was provided by clinical charts from emergency areas and/or hospitalization.

Patients were censored at the last available visit or in case of death.

Statistical Analysis

Data for continuous variables was expressed as mean and standard deviation (SD) or median and interquartile ranges (IQR), and data for categorical variables were expressed as frequency and percentage. Differences between continuous data were assessed by Student t test or by Mann Whitney U test. Differences between categorical variables were assessed by χ^2 test.

Multivariate Cox models were used to assess the impact of high-risk MASH and of F2-F4 fibrosis without MASH -having F0-F1 fibrosis without MASH as reference- on the risk of developing LRE, CVE and EHC. Sub-group analyses were done in patients without cirrhosis, and in patients with availability of LSM, and the independent association of LSM ≥8 KPa, LSM ≥10 KPa, and AGILE 3+ >0.67 with LRE, CVE and EHC was also tested. Variables to be included in the multivariate model (Male gender, age≥50 years, BMI≥30 Kg/m^2 and type 2 diabetes) were chosen

based on clinical relevance, and all models were adjusted for enrolling center. Variables in the final models with a P value of <0.05 were considered statistically significant. The results are expressed as adjusted hazard ratios (aHR) and their 95% confidence intervals (CI). The accuracy of high-risk MASH, F2-F4 fibrosis without MASH, F2-F4 fibrosis, LSM ≥8 KPa, LSM ≥10 KPa and AGILE 3+ >0.67 for detection of occurrence of LRE, CVE and EHC was assessed using time-dependent receiver operating characteristic curves described as area under the receiver operating characteristic (AUROC) with 95% CI. Comparison among AUROCs was done by using DeLong test. Bonferroni test adjustment was used for multiple comparisons. Kaplan Meier curves were also used to depict the time-dependent risk of developing events. All data was analysed using R studio. In addition to the base packages in R, tidy verse, survival, survminer, boot, reshape2, and readxl packages were used.

RESULTS

Baseline features of patients

Baseline characteristics of the histological cohort of 1938 patients, overall and stratified for F0-F1 or F2-F4 fibrosis and for presence or absence of MASH, are summarized in **Table 1**. As expected, the median age was significant higher in patients with fibrosis F2-F4 than those with fibrosis F0-F1, regardless of the presence of MASH. Metabolic comorbidities (obesity, diabetes, and arterial hypertension) increased according to the fibrosis severity, especially in those with F2-F4 and in the presence of steatohepatitis. Supplemental Table 1, http://links.lww.com/HEP/I19 reports the comparison of patients with F2-F4 fibrosis stratified according to presence/absence of MASH.

Occurrence of liver-related events and extra-hepatic events in the entire cohort of patients with histological diagnosis of MASLD

The 664 patients with F0-F1 fibrosis without MASH had a median follow-up of 7.9 years and experienced 5 LRE (4 LD and 1 HCC), 16 CVE and 21 EHC; those with F0-F1 and MASH (n=208) had a median follow-up of 8 years and experienced 3 LRE (2 LD and 1 HCC), 14 CVE and 13 EHC. The 507 patients with F2-F4 without MASH, in a median follow-up of 7 years, experienced 28 LRE (19 LD and 9 HCC), 16 CVE and 13 EHC; finally, those with F2-F4 and presence of MASH (n=559) during a median follow-up of 5.7 years, experienced 57 LRE (36 LD and 21 HCC), 33 CVE and 35 EHC.

The Kaplan-Meier curve in **Figure 1A** reports the probability of LRE occurrence over time according to histological status; it showed that the risk progressively increased from patients with F0-F1 fibrosis with/without MASH to those with F2-F4 fibrosis without and with MASH. The observed 5-year actuarial rate of LRE was 0.4%, 0.2%, 5.1% and 6.6% in patients with F0-F1 fibrosis without MASH, F0-F1 fibrosis with MASH, F2-F4 fibrosis without MASH, and high-risk MASH, respectively. At multivariate Cox regression analysis, male gender (HR 1.96, 95% C.I. 1.19 - 3.23, p=0.008), age ≥50 years (HR 2.95, 95% C.I. 1.65 - 5.28, p<0.001), and both F2-F4 fibrosis without MASH (HR 9.96, 95% C.I. 3.35 - 29.62, p<0.001) and high-risk MASH (HR 10.14, 95% C.I. 3.51 - 29.25, p<0.001), using F0-F1 fibrosis without MASH as reference, were associated with LRE occurrence (**Table 2**).

When considering separately HCC occurrence and development of LD, the risk of events increased according to the severity of fibrosis (**Figure 1B and 1C**) and we confirmed F2-F4 fibrosis without MASH (HR 9.68, 95% C.I. 3.22 - 29.1, p<0.001 for LD; HR 14.8, 95% C.I. 1.83 – 119.84, p=0.01 for HCC) and high-risk MASH (HR 7.67, 95% C.I. 2.6 - 22.57, p<0.001 for LD; HR 13.36, 95% C.I. 1.7 – 104.8, p=0.01 for HCC) as independent risk factors (**Table 2**).

The Kaplan-Meier curve in **Figure 1** reports the probability of CVE (D) and EHC (E) occurrence over time according to histological status, and showed that the risk progressively increased from patients with F0-F1 fibrosis with/without MASH to those with F2-F4 fibrosis without and with MASH. At multivariate Cox regression analysis, only F2-F4 fibrosis without

MASH (aHR 2.2, 95% C.I. 1.08 - 4.52, p=0.03) and marginally high-risk MASH (aHR 1.75, 95% C.I. 0.91 - 3.35, p=0.09) resulted associated with CVE occurrence, while no independent association was observed for development of EHC (**Table 2**).

Occurrence of liver-related events and extra-hepatic events in patients without cirrhosis and with histological diagnosis of MASLD

The characteristics of the population of MASLD patients without cirrhosis overall and stratified for F2-F3 fibrosis and MASH are reported in Supplemental Table 2, http://links.lww.com/HEP/I19. As expected, the total number of LRE, CVE and EHC was lower. Patients with F2-F3 fibrosis without MASH (N=160), in a median follow-up of 6.8 years, experienced 7 LRE (4 LD and 3 HCC), 12 CVE and 9 EHC; while those with F2-F3 fibrosis and MASH (N=490) during a median follow-up of 5.9 years, experienced 18 LRE (10 LD and 8 HCC), 27 CVE and 28 EHC. The Kaplan-Meier curves in Supplemental Figure 1, http://links.lww.com/HEP/I20 report the probability of LRE, CVE and EHC occurrence over time according to histological status, showing that the risk increased according to the severity of liver fibrosis. The observed 5-year actuarial rate of LRE was 0.4%, 0.2%, 1.3% and 2.7% in patients with F0-F1 fibrosis without MASH, F0-F1 fibrosis with MASH, F2-F3 fibrosis without MASH, and F2-F3 fibrosis with MASH, respectively. At multivariate Cox regression analysis, F2-F3 fibrosis with MASH resulted independently associated with LRE (aHR 4.6, 95% C.I. 1.42 - 14.95, p=0.01), CVE (aHR 2.02, 95% C.I. 1.04 - 3.93, p=0.03), and EHC (aHR 1.82, 95% C.I. 0.99 - 3.35, p=0.05) occurrence, while F2-F3 fibrosis without MASH only with CVE development (aHR 2.27, 95% C.I. 1.04 - 4.93, p=0.03) (Supplemental Table 3, http://links.lww.com/HEP/I19). The analyses for patients with F2-F3 fibrosis without MASH were however limited by the small sample size.

Occurrence of liver-related events and extra-hepatic events in patients with histological diagnosis of MASLD and with availability of liver stiffness assessment

The characteristics of the 1074 patients with histological diagnosis of MASLD and with availability of LSM are reported in Supplemental Table 4, http://links.lww.com/HEP/I19. In this sub-group, 24% of patients had F0-F1 fibrosis without MASH, 25.8% F0-F1 fibrosis with MASH, 8.5% F2-43 fibrosis without MASH, and 41.7% high-risk MASH.

The 503 patients with LSM<8 KPa experienced 5 LRE (2 LD and 3 HCC), 9 CVE and 14 EHC; those with LSM ≥8 KPa (n=571) experienced 37 LRE (23 LD and 14 HCC), 25 CVE and 25 EHC. The 407 patients with LSM<10 KPa experienced 6 LRE (2 LD and 4 HCC), 19 CVE and 24 EHC; those with LSM ≥10 KPa (n=667) experienced 36 LRE (23 LD and 13 HCC), 15 CVE and 15 EHC. The 775 patients with LSM<12 KPa experienced 7 LRE (2 LD and 5 HCC), 23 CVE and 30 EHC; finally, those with LSM ≥12 KPa (n=299) experienced 35 LRE (23 LD and 12 HCC), 11 CVE and 9 EHC. The 569 patients with AGILE 3+ ≤0.67 experienced 2 LRE (2 HCC), 15 CVE and 25 EHC; finally, those with AGILE 3+ >0.67 (n=276) experienced 33 LRE (20 LD and 13 HCC), 12 CVE and 10 EHC.

The Kaplan-Meier curves in Supplemental Figure 2, http://links.lww.com/HEP/I21 report the probability of LRE, CVE and EHC occurrence over time according to LSM ≥8 KPa -noninvasive expression of intermediate/high risk of advanced fibrosis-. Kaplan-Meier curves according to F2-F4 fibrosis and according to high-risk MASH were also reported for indirect comparison (Supplemental Figure 2, http://links.lww.com/HEP/I21). Notably, the 5-year risk of LRE in patients with LSM ≥8 KPa was 4.7%, similar to the 6.4% and 6% observed -in the same subgroup of 1074 patients- in patients with high-risk MASH and with F2-F4 fibrosis, respectively. At multivariate Cox regression analysis, LSM ≥8 KPa independently predicted the development of LRE (aHR 3.84, 95% C.I. 1.4 - 10.54, p=0.009) and of CVE (aHR 2.60, 95% C.I. 1.11 - 6.09, p=0.02), but not of EHC (aHR 1.46, 95% C.I. 0.69 - 3.06, p=0.31) (**Table 3**). Supplemental figure 3, http://links.lww.com/HEP/I22 shows the time-dependent AUROCs (tAUROCs) for LSM ≥8 KPa versus F2-F4 fibrosis and high-risk MASH in predicting LRE, CVE and EHC. Notably, the

tAUROC of LSM ≥8 KPa for LRE, CVE and EHC was similar to that of high-risk MASH, but significantly lower with respect to F2-F4 fibrosis for LRE at 36, 60 and 120 months (5-year AUROC 0.665, 0.739, and 0.780, respectively) (Supplemental Table 5, http://links.lww.com/HEP/I19).

The Kaplan-Meier curves in **Figure 2** depicts the probability of LRE, CVE and EHC occurrence over time according to LSM ≥10 KPa -noninvasive expression of cACLD- and to AGILE 3+ >0.67 -noninvasive expression of advanced fibrosis-. Kaplan-Meier curves according to F2-F4 fibrosis and according to high-risk MASH were also reported for indirect comparison (**Figure 2**). Notably, the 5-year risk of LRE in patients with LSM ≥10 KPa was 6.4% similar to the 6.4% and 6% observed -in the same subgroup of 1074 patients- in patients with high-risk MASH and with F2-F4 fibrosis, respectively, while the 5-year risk of LRE in patients with AGILE 3+ >0.67 was slightly higher (9.5%). At multivariate Cox regression analysis, LSM ≥10 KPa independently predicted the development of LRE (aHR 6.31, 95% C.I. 2.46 - 16.2, p<0.001), not of CVE (aHR 1.05, 95% C.I. 0.49 - 2.28, p=0.89) or of EHC (aHR 0.89, 95% C.I. 0.43 - 1.85, p=0.75) (**Table 3**). Similarly, AGILE 3+ score > 0.67 independently predicted the development of LRE (aHR 27.45, 95% C.I. 5.72 – 131.74, p<0.001), not of CVE (aHR 0.95, 95% C.I. 0.36 - 2.49, p=0.91) or of EHC (aHR 0.43, 95% C.I. 0.18 - 1.05, p=0.06) (**Table 3**). **Figure 3** shows the tAUROCs for LSM ≥10 KPa and AGILE 3+ >0.67 versus F2-F4 fibrosis and high-risk MASH in predicting LRE, CVE and EHC. Notably, the tAUROC of LSM ≥10 KPa for LRE, CVE and EHC was similar to that of high-risk MASH and F2-F4 fibrosis (5-year AUROC for LRE 0.772, 0.739, and 0.780, respectively), and slightly lower than that of AGILE 3+ >0.67 for LRE (5-year AUROC 0.818) (Supplemental Table 6, http://links.lww.com/HEP/I19).

Finally, as a sensitivity analysis, the Kaplan-Meier curves in Supplemental Figure 4, http://links.lww.com/HEP/I23 report the probability of LRE, CVE and EHC occurrence over time according to LSM ≥12 KPa -recently proposed for the noninvasive diagnosis of cACLD-. Kaplan-Meier curves according to F2-F4 fibrosis and according to high-risk MASH were also reported for

indirect comparison (Supplemental Figure 4, http://links.lww.com/HEP/I23). Notably, the 5-year risk of LRE in patients with LSM ≥12 KPa was 8.7%, similar to the 6.4% and 6% observed -in the same subgroup of 1074 patients- in patients with high-risk MASH and with F2-F4 fibrosis, respectively. At multivariate Cox regression analysis, LSM ≥12 KPa independently predicted the development of LRE (aHR 9.81, 95% C.I. 4.0 - 24.08, p<0.001) but not of CVE (aHR 1.03, 95% C.I. 0.46 - 2.33, p=0.94) and of EHC (aHR 0.61, 95% C.I. 0.26 - 1.39, p=0.23) (**Table 3**). Supplemental figure 5, http://links.lww.com/HEP/I24 shows the time-dependent AUROCs (tAUROCs) for LSM ≥12 KPa versus F2-F4 fibrosis and high-risk MASH in predicting LRE, CVE and EHC. Notably, the tAUROC of LSM ≥12 KPa for LRE, CVE and EHC was similar to that of high-risk MASH and of F2-F4 fibrosis (5-year AUROC 0.766, 0.739, and 0.780, respectively) (Supplemental Table 7, http://links.lww.com/HEP/I19).

Discussion

In this study on a large cohort of individuals with a histological diagnosis of MASLD, we found that patients with high-risk MASH have a similar risk of developing LRE with respect to those with F2-F4 fibrosis but without MASH. We also showed that noninvasive stratification of LRE risk by LSM or AGILE 3+, compared to histological defined high-risk MASH and significant fibrosis, adequately identified at risk MASLD patients worthy of enrollment in pharmacological trials.

The FDA and EMA recommend testing for drug therapy patients with compensated MASLD-related cirrhosis or in those with high-risk MASH because they are at risk of developing LRE [9-11], while patients with significant liver fibrosis but without MASH are excluded from clinical trials. Consequently, these rules are applied in phase III clinical trials looking at the impact of new MASH-targeted drugs on the 5-year risk of developing LRE.

We observed that the risk of developing LRE significantly increased according to the presence of F2-F4 fibrosis regardless of the presence/absence of histological steatohepatitis, with a 5-year actuarial rate of 5.1% and 6.6% in patients with F2-F4 fibrosis without and with MASH, respectively. Notably, after adjusting for demographic and metabolic risk factors, patients with high-risk MASH had a roughly 10-times higher probability of LRE occurrence compared to patients with F0-F1 fibrosis and without MASH, and a similar increased independent risk was also observed in patients with F2-F4 fibrosis but without MASH. These results were also observed when considering separately HCC and LD. When looking at extrahepatic events, we also confirmed a higher risk of CVE in patients with F2-F4 fibrosis with and without MASH. This data is overall in line with the study by Angulo and colleagues, on a multicenter cohort of 619 patients with biopsy-proven MASLD. In that study, even though data on high-risk MASH were not available, patients with any stage fibrosis, regardless of MASH, had shorter survival times than patients without fibrosis [14]. Considering the possibility that MASLD patients with cirrhosis, even if at high risk of LRE and EHC, can lose some histological features of MASH - burnt-out MASH [30, 31]-, and consequently could be responsible of an ascertainment bias leading to the observed high rate of LRE in F2-F4 patients without MASH, we performed a separate sub-group analysis in MAFLD patients without cirrhosis. In this clinical scenario also, we showed a progressive increase in LRE in patients with F2-F3 fibrosis regardless of the presence of steatohepatitis. However, the risk of LRE was more pronounced in those with MASH -5-year LRE actuarial rate of 1.3% and 3.7% in patients without and with MASH, respectively-. Furthermore, after adjusting for demographic and metabolic confounders only presence of F2-F3 fibrosis with MASH remained independently associated with a 4.6-time higher risk of LRE occurrence. The association with F2-F3 fibrosis without MASH was lost, but this is worthy of further investigation because of the small sample size of the studied sub-group (N=160).

In sum, our data might suggest that until there are data on the role of histological steatohepatitis in predicting treatment response, we should recognize that patients with significant

fibrosis, with or without steatohepatitis, are at risk of adverse outcomes and should not be ignored but considered in future phase 2b and phase 3 trials of new agents. This consideration is further supported by 1) the low intra and inter pathologist agreement for the diagnosis of steatohepatitis leading to discordant results and to a very high rate (>50%) of screening failure in clinical trials, 2) the possibility that the histological features of steatohepatitis -as well as documented for fibrosis- may not be homogeneously distributed across the liver, thus leading to suboptimal estimation of steatohepatitis diagnosis, and 3) the knowledge that histological features of MASH -especially ballooning and lobular inflammation- can dynamically change over time according to evolving risk factors -weight, glycated hemoglobin, etc- and consequently making a one-time histological evaluation not representative of the mid-term risk of outcomes (some patients can have MASH at some point but not at the moment of the histological assessment).

International guidelines suggest that, in MASLD patients, a liver stiffness threshold lower than 8 KPa can be used to exclude those at risk of advanced liver fibrosis [23], and a cut-off ≥10 KPa [24], or -as recently proposed- of 12 KPa [25], can identify patients with cACLD. These LSM values have been also validated as able to stratify the risk of developing LRE [27]. Moreover, recent studies suggest the use of AGILE 3+ as a LSM-based noninvasive tool for identifying patients at high risk of advanced fibrosis and of LRE, because leading a slight improvement respect to LSM [26,27,32].

We confirmed that the presence of LSM values ≥8 KPa or ≥10 KPa or ≥12 KPa, compared to their counterparts, independently predicted a 4-, 6-time and 9-time higher risk of developing LRE, respectively, LSM ≥8 KPa also predicting a higher risk of CVE occurrence. Moreover, we showed that the 5-year LRE actuarial rate for LSM ≥8 KPa, for LSM ≥10 KPa and for LSM ≥12 KPa was 4.7%, 6.4% and 8.7%, respectively, similar to the 6.4% and the 6% observed in the same cohort of patients with high-risk MASH or in those with significant liver fibrosis, respectively. Consequently, when comparing the accuracy of LSM in predicting LRE with respect to the presence of high-risk MASH or of significant liver fibrosis, we show that the 5-year accuracy of

LSM ≥8 KPa was poor and significantly lower than that of histological assessment. In contrast, the diagnostic performance of LSM ≥10 KPa or LSM ≥12 KPa was acceptable and similar to that of both significant liver fibrosis and high-risk MASH. The choose of the cut-off of 10 KPa or 12 KPa should be based on further studies also assessing the risk of cirrhosis development in patients with a LSM between 10 and 12 KPa, even if a strategy based on LSM ≥10 could be more conservative and probably preferrable. When looking at AGILE 3+ at the cut-off of 0.67, our study confirmed its good accuracy for predicting LRE, also showing a nonsignificant trend -worthy to be externally validated- for a better accuracy respect to LSM >10 KPa, F2-F4 fibrosis and high-risk MASH.

Overall, our data might support the use of LSM ≥10 KPa or LSM ≥12 KPa or AGILE 3+ >0.67 in place of histologically defined high-risk MASH as an easy tool to identify patients at risk of developing LRE and so worthy of being considered for inclusion in clinical trials of new pharmacological agents for metabolic-related liver disease. Such a change of paradigm, worthy to be validated against histology in prospective cohorts, could greatly increase enrollment in clinical trials and enable the testing of new drugs in a setting nearer to clinical practice. This is a particular consideration currently where more than half of screened patients are excluded for discrepant histology, particularly for diagnosis of steatohepatitis. Moreover, the use of LSM or AGILE 3+ to identify MASLD patients for pharmacological treatment could also allow to evaluate whether the same test can be used for monitoring treatment efficacy. Consistent with this notion, cohort studies have demonstrated that changes over time in LSM accurately predict the risk of developing LRE [33].

The main limitation of the present study lies in its validity for the different populations and settings where patients are seen. Our study only included patients referred to tertiary referral centers for suspected liver damage and thus it is possible that the obtained results might not be replicated in populations differing for age, ethnicity, biological sex, biochemical parameters, metabolic comorbidities and seen in different setting such as primary care. The present study is also affected by selection bias related to the inclusion of only patients who underwent liver biopsy, and to the

availability of LSM only in a subgroup of patients. Lower length of follow-up in patients with high-risk MASH compared to those with F2-F4 fibrosis without MASH could further affect the final interpretation of the results. The lack of data about the FAST score -based on the combination of AST, LSM and controlled attenuation parameter [34]- is another limitation. MRI-based evaluation of liver stiffness or the application of the MAST score [35], might better identify patients for clinical trials, but high cost and low availability limit their testing, validation and large-scale implementation. The retrospective nature of the study, its relatively small sample size for some sub-group analyses, the lack of an external validation cohort and of central biopsy reading, on site variability -even if all models were adjusted for enrolling center-, and potentially hidden alcohol abuse in some patients could further limit the interpretation of our results.

In conclusion, we found that the 5-year risk of developing LRE is similar in patients with high-risk MASH and those with F2-F4 fibrosis without MASH. Further, the use of LSM ≥10 KPa or LSM ≥12 KPa or AGILE 3+ >0.67 appears to be an accurate predictor to identify MASLD patients at risk for LRE and worthy to be included in clinical trials testing new pharmacological agents.

Figure 1. Kaplan Meier curves depicting the probability of LRE (A), LD (B), HCC (C), CVE (D) and EHC (E) in patients split for fibrosis stage (F0-F1 vs F2-F4) and for presence or absence of MASH.

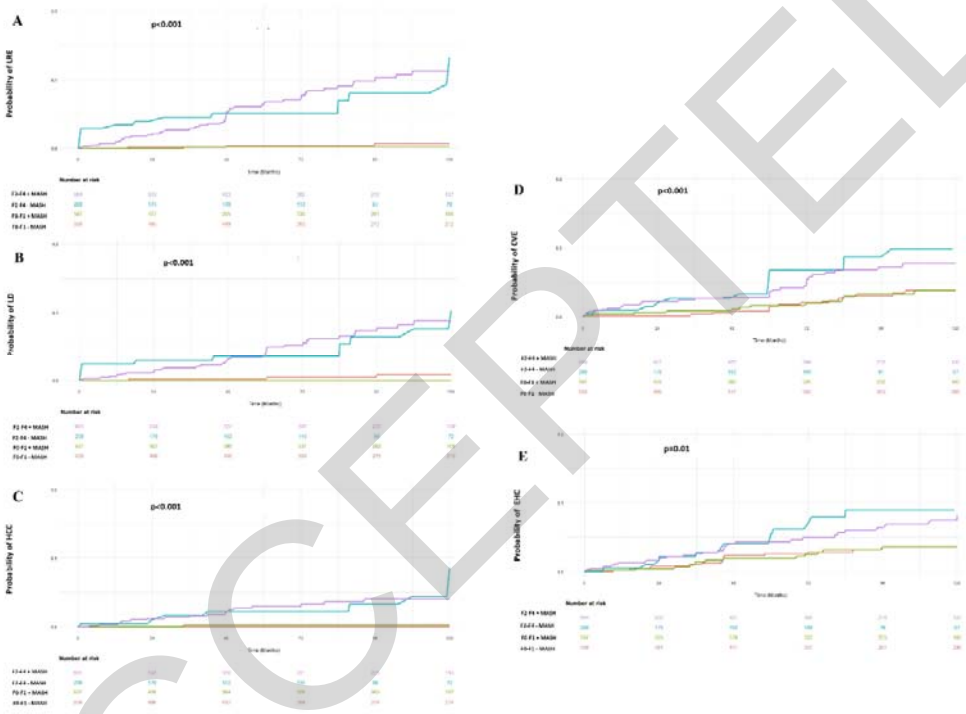
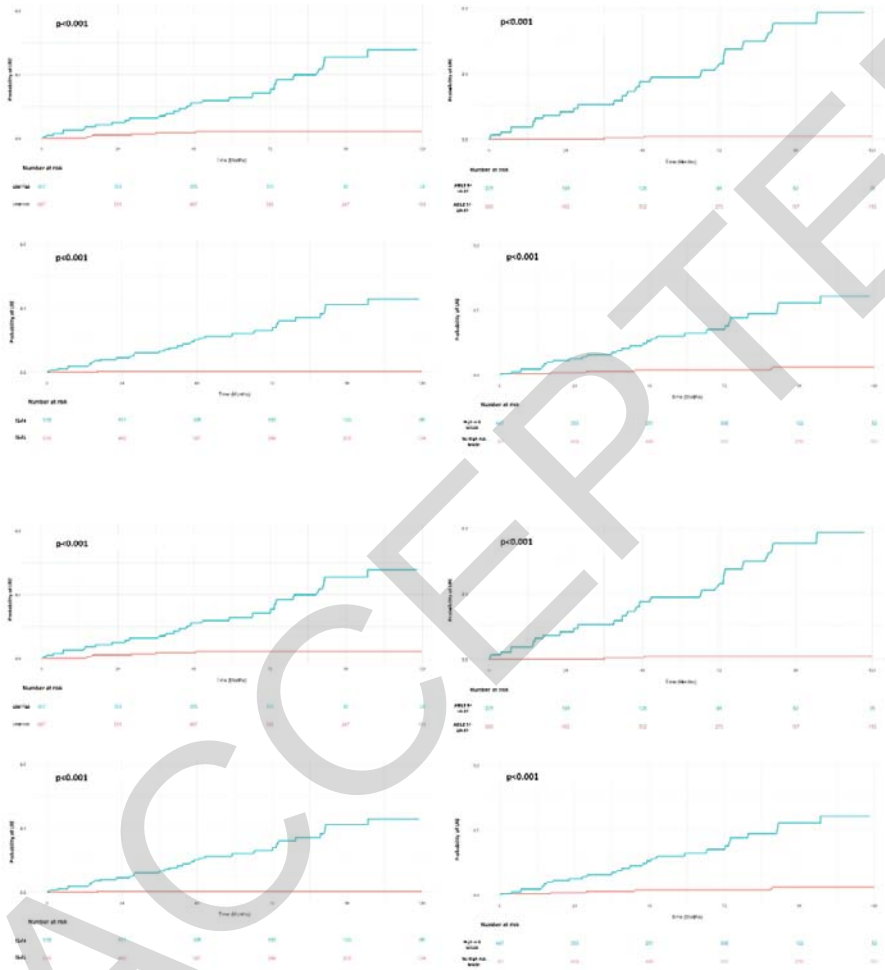


Figure 2. Kaplan Meier curves depicting the probability of LRE (A), CVE (B) and EHC (C) in patients splitted according to LSM ≥10 KPa, AGILE 3+ >0.67, F2-F4 fibrosis and High-risk MASH.



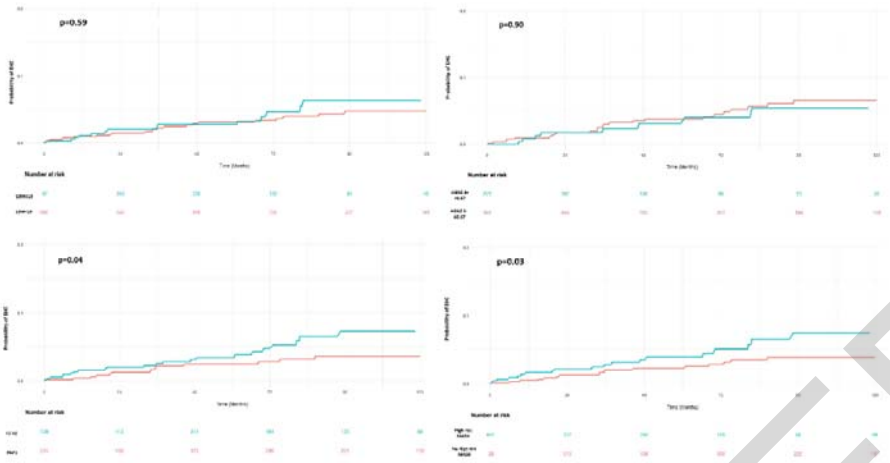
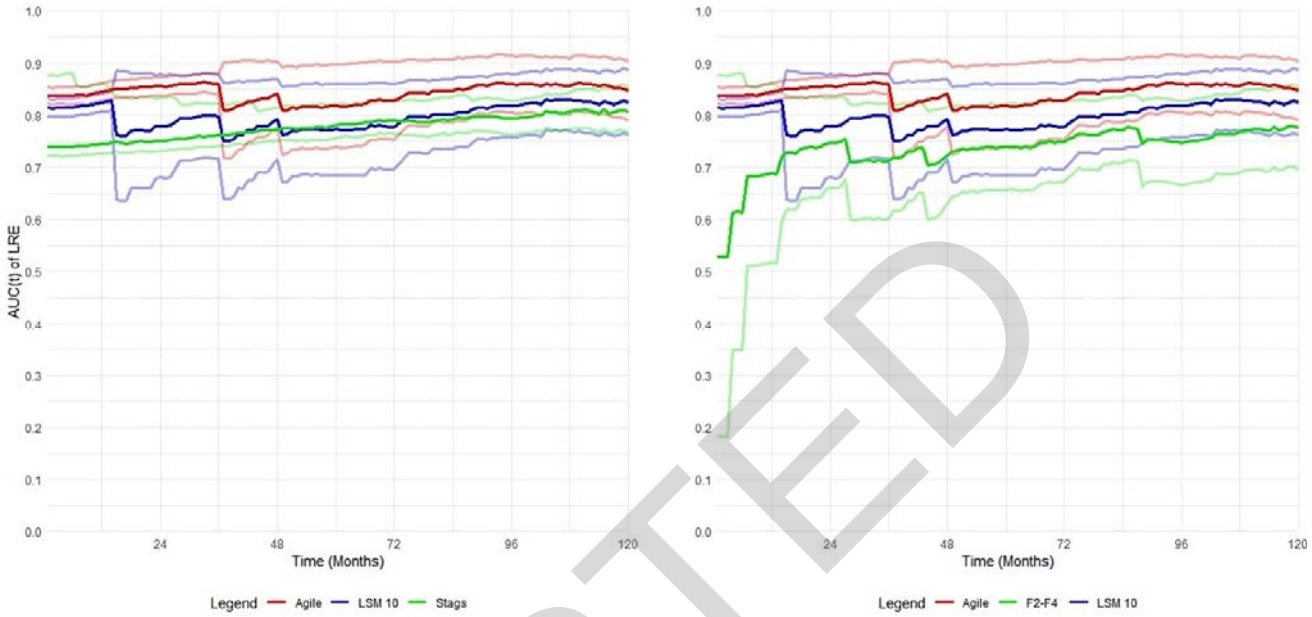
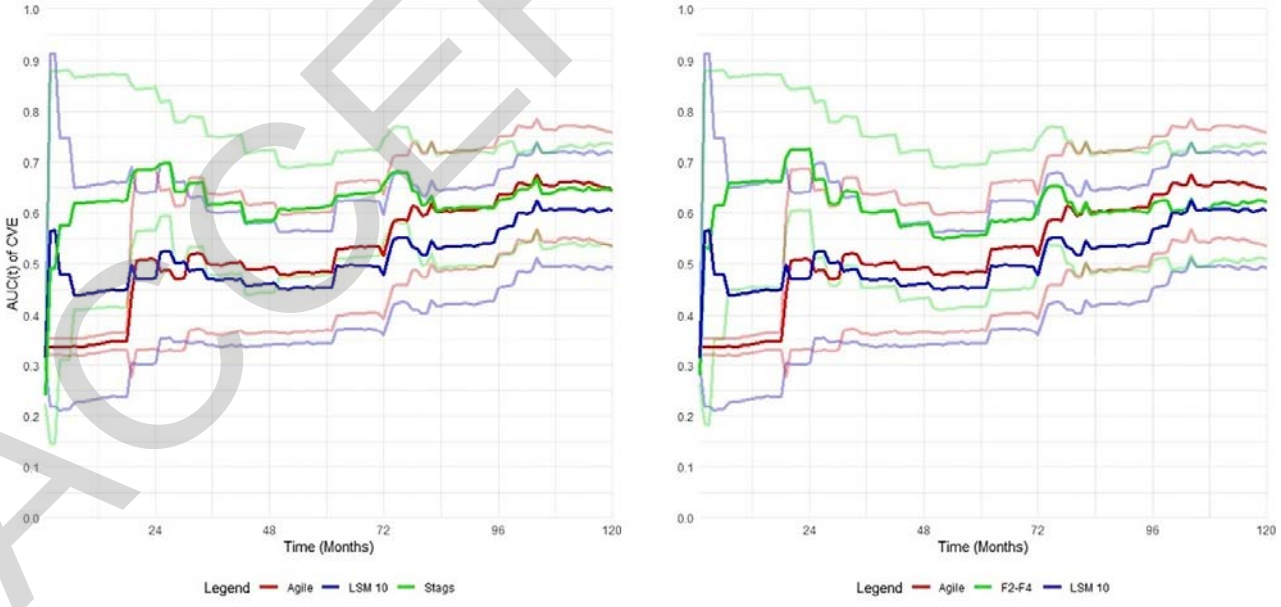
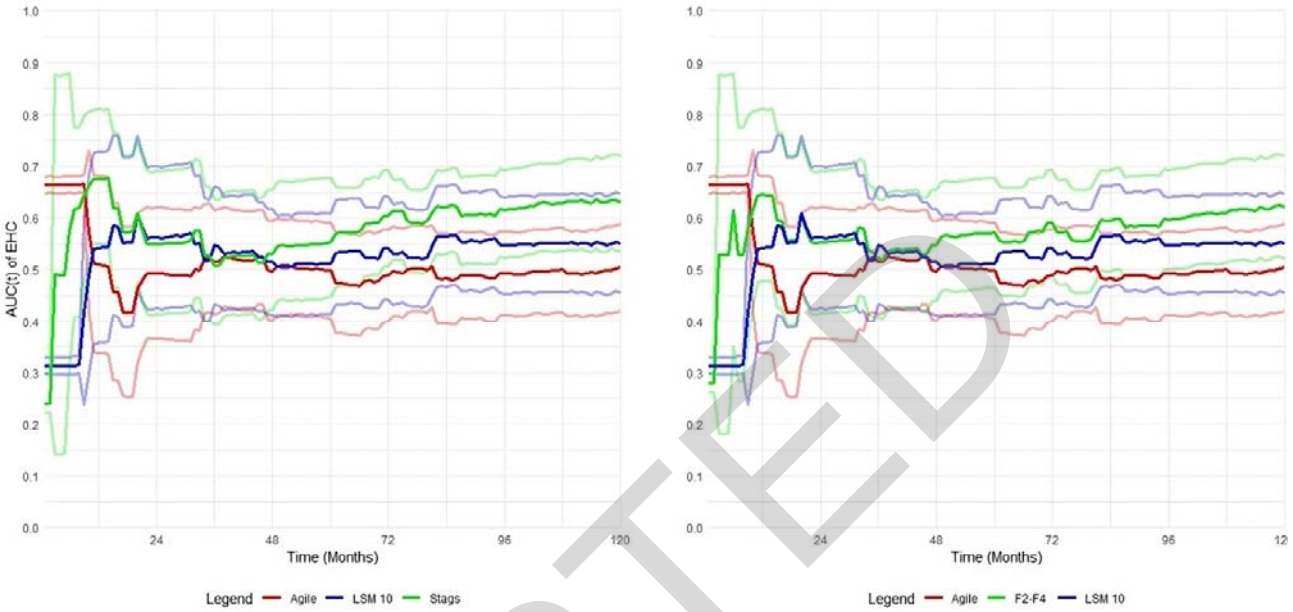


Figure 3. Time-dependent areas under the receiver operating characteristic (tAUROCs) for LSM ≥10 KPa and AGILE 3+ >0.67 versus F2-F4 fibrosis (left) and high-risk MASH (right) in predicting LRE (A), CVE (B) and EHC (C).







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	Entire Cohort N=1938	F0-F1-NoMASH N=664	F0-F1-MASH N=208	F2-F4-NoMASH N=507	F2-F4-MASH N=559	P value Comparison between F0-F1-NoMASH vs all conditions	P value Comparison between F0-F1-MASH vs all conditions	P value Comparison between F2-F4-NoMASH vs all conditions	P value Comparison between F2-F4-MASH vs all conditions

Table 1. Baseline features of the entire histological cohort of 1938 patients and stratified for fibrosis F0-F1 or F2-F4 and for presence or absence of MASH.

Age #	50.5 (39.02 - 58)	46 (37.67 - 55)	46 (36 - 55)	54 (45.27 - 60)	55 (47 - 62)	<0.001	<0.001	<0.001	<0.001
Male *	61.66	70.84	63.51	66.35	51.05	<0.001	<0.001	<0.001	<0.001
Diabetes *	34.54	17.09	25.69	46.63	52.11	<0.001	<0.001	<0.001	<0.001
BMI ≥30 Kg/m2 *	46.06	32.97	43.18	46.67	59.37	<0.001	<0.001	0.918	<0.001
Arterial hypertension *	43.23	29.96	34.26	50.48	55.98	<0.001	<0.001	0.013	<0.001
Smoking *	16.65	15.62	13.07	14.17	20.73	0.778	<0.001	<0.001	<0.001
PLT – mmc #	230 (187 - 275)	240 (200 - 286)	250 (212 - 293)	216 (166 - 249.5)	211 (171 - 256.5)	<0.001	<0.001	<0.001	<0.001
Albumin - mg/dl #	4.5 (4.2 - 4.7)	4.5 (4.4 - 4.8)	4.5 (4.26 - 4.8)	4.4 (4.2 - 4.6)	4.4 (4.1 - 4.62)	<0.001	<0.001	0.019	<0.001
AST - U/L #	41 (28.4 - 62)	33 (26 - 52)	40 (28 - 56.75)	46 (32 - 67)	48 (34 - 70)	<0.001	0.029	0.029	<0.001
ALT U/L #	52 (34 - 81)	47 (30 - 67)	53 (35 - 86)	47 (29 - 75)	58 (38 - 88.25)	<0.001	0.058	0.011	<0.001
Total cholesterol - mg/dL #	192 (165.51 - 223.04)	201 (173 - 227.8)	197.2 (170 - 224.21)	184 (150.86 - 216.46)	184.2 (158 - 219)	<0.001	0.043	0.012	<0.001
Tryglicerides - mg/dL #	128 (92 - 183)	124 (89 - 179.5)	124 (88.78 - 177.57)	119.6 (88.57 - 175.54)	134 (97.43 - 189.99)	0.225	0.507	0.507	0.001
INR #	1 (0.94 - 1.03)	1 (0.93 - 1.02)	1 (0.92 - 1)	1 (0.96 - 1.07)	1 (0.96 - 1.06)	<0.001	<0.001	<0.001	<0.001
LSM – Kpa #	8.3 (5.9 - 12.35)	6.2 (4.82 - 8.17)	6.6 (5.3 - 8.8)	10.3 (6.9 - 13.45)	11.8 (8.1 - 18.4)	<0.001	<0.001	0.004	<0.001

Steatosis grade 1/2/3 *	42.78/32.2/25.03	59.21/27.19/13.6	39.05/35.7/25.25	47.12/25.96/26.92	30.42/35.69/33.89	<0.001	0.176	<0.001	<0.001
Lobular Inflammation 1/2/3/4 *	19.5/56.4/22.14/1.96	52.77/42.04/5.01/0.18	0/80.28/19.53/0.2	39.9/37.98/19.23/2.88	0/56.02/39.46/4.52	<0.001	<0.001	<0.001	<0.001
Ballooning 0/1/2 *	28.17/50.15/21.67	69.95/26.83/3.22	0/75.94/24.06	74.52/19.23/6.25	0/59.79/40.21	<0.001	<0.001	<0.001	<0.001
Time of follow-up – months #	7 (3.7 – 10.93)	7.9 / 4.85 - 12	8 / 4.25 - 10.87	7 / 3.92 - 12.05	5.7 / 2.76 - 9.47	<0.001	0.242	0.326	<0.001

Abbreviations: MASH, metabolic dysfunction-associated steatohepatitis; BMI, body mass index; PLT, platelets; AST, aspartate transaminase; ALT, alanine transaminase; INR, international normalized ratio; LSM, liver stiffness measurement.

Data are given as: (#) median (1st quartile-3rd quartile) or (*) percentage of cases (%).

Table 2. Multivariate Cox regression analysis* of factors associated with LRE (cumulative and split in LD and HCC), CVE and EHC occurrences, in patients with F0-F1 and F2-F4 fibrosis, with and without MASH.

Event		HR	95% - CI	P Value
LRE	**Male**	1.96	(1.19 - 3.23)	0.008
	Diabetes	1.5	(0.93 - 2.41)	0.092
	F0-F1-MASH"	0.96	(0.21 - 4.33)	0.960
	F2-F4-No MASH"	9.96	(3.35 - 29.62)	<0.001
	F2-F4-MASH"	10.14	(3.51 - 29.25)	<0.001
	BMI ≥30 Kg/m2	1.16	(0.73 - 1.85)	0.519
	Age ≥50 years	2.95	(1.65 - 5.28)	<0.001
LD	**Male**	1.57	(0.9 - 2.76)	0.112
	Diabetes	1.38	(0.81 - 2.35)	0.232
	F0-F1-MASH"	0.64	(0.12 - 3.49)	0.603
	F2-F4-No MASH"	9.68	(3.22 - 29.1)	<0.001
	F2-F4-MASH"	7.67	(2.6 - 22.57)	<0.001
	BMI ≥30 Kg/m2	1.22	(0.72 - 2.06)	0.459
	Age ≥50 years	2.52	(1.34 - 4.73)	0.004
HCC	**Male**	2.69	(1.18 - 6.1)	0.018
	Diabetes	1.62	(0.77 -	0.202

			3.43)	
	F0-F1-MASH”	1.19	(0.07 - 19.11)	0.011
	F2-F4-No MASH”	14.8	(1.83 – 119.84)	0.011
	F2-F4-MASH”	13.36	(1.7 - 104.8)	0.013
	BMI ≥30 Kg/m2	1.63	(0.77 - 3.44)	0.200
	Age ≥50 years	2.44	(1.01 - 5.9)	0.040
CVE	**Male**	1.58	(0.96 - 2.58)	0.069
	Diabetes	1.07	(0.66 - 1.73)	0.782
	F0-F1-MASH”	1.07	(0.52 - 2.21)	0.851
	F2-F4-No MASH”	2.2	(1.08 - 4.52)	0.030
	F2-F4-MASH”	1.75	(0.91 - 3.35)	0.092
	BMI ≥30 Kg/m2	1.12	(0.71 - 1.78)	0.627
	Age ≥50 years	2.55	(1.5 - 4.33)	<0.001
EHC	**Male**	1.12	(0.7 - 1.79)	0.633
	Diabetes	1.04	(0.65 - 1.67)	0.867
	F0-F1-MASH”	0.81	(0.4 - 1.62)	0.545
	F2-F4-No MASH”	1.45	(0.71 - 2.97)	0.304
	F2-F4-MASH”	1.59	(0.88 - 2.87)	0.123
	BMI ≥30 Kg/m2	0.92	(0.58 - 1.44)	0.704
	Age ≥50 years	3.1	(1.8 - 5.32)	<0.001

*All analysis were corrected for center.

“F0-F1-No MASH as reference

Abbreviations: MASH, metabolic dysfunction-associated steatohepatitis; LRE, liver-related events; LD, liver decompensation; HCC, hepatocellular carcinoma; CVE, cardiovascular events; EHC, extra-hepatic cancer; BMI, body mass index.

Table 3. Multivariate Cox regression analysis* of factors associated with LRE, CVE and EHC occurrences, in the subgroup of 1075 patients with histological diagnosis of MASLD and with availability of liver stiffness.

Event		HR	95% - CI	P Value	HR	95 % - CI	P Value	HR	95% - CI	P Value	HR	95 % - CI	P Value
LRE	**Male**	2.11	(1.04 - 4.3)	0.03	2.21	(1.08 - 4.51)	0.02	1.59	(0.74 - 3.42)	0.23	2.12	1.04- 4.32	0.03
	Diabetes	1.75	(0.83 - 3.71)	0.14	1.68	(0.79 - 3.58)	0.17	0.8	(0.36 - 1.76)	0.57	1.44	0.67- 3.08	0.35
	LSM ≥8 KPa	3.84	(1.4 - 10.54)	0.009	-			-			-		

	LSM ≥10 KPa	-			6.31	(2.46 - 16.2)	<0.001	-			-		
	LSM ≥12 KPa	-			-			-			9.81	4.0 - 24.08	<0.001
	AGILE 3+ >0.67	-			-			27.45	(5.72 – 131.74)	<0.001	-		
	BMI ≥30 Kg/m2	1.35	(0.66 - 2.75)	0.41	1.2	(0.59 - 2.46)	0.61	1.35	(0.6 - 3.03)	0.47	1.15	0.56- 2.37	0.70
	Age ≥50 years	4.02	(1.5 - 10.79)	0.005	3.29	(1.21 - 8.93)	0.01	2.2	(0.71 – 6.79)	0.17	3.15	1.16- 8.57	0.02
CVE	**Male**	2.99	(1.32 - 6.74)	0.008	2.98	(1.32 - 6.74)	0.008	2.73	(1.1 – 6.73)	0.02	2.89	1.28- 6.54	0.01
	Diabetes	0.98	(0.47 - 2.05)	0.95	1.17	(0.56 - 2.45)	0.68	1.19	(0.49 – 2.89)	0.70	1.44	0.67- 3.08	0.35
	LSM ≥8 KPa	2.6	(1.11 - 6.09)	0.02	-			-			-		
	LSM ≥10 KPa	-			1.05	(0.49 - 2.28)	0.89	-			-		
	LSM ≥12 KPa	-			-			-			1.03	0.46- 2.33	0.94
	AGILE 3+ >0.67	-			-			0.95	(0.36 – 2.49)	0.91	-		
	BMI ≥30 Kg/m2	1.17	(0.57 - 2.42)	0.66	1.48	(0.71 - 3.08)	0.29	1.51	(0.66 – 3.44)	0.33	1.56	0.76- 3.20	0.22
	Age ≥50 years	2.6	(1.14 - 5.94)	0.02	2.97	(1.28 - 6.87)	0.01	3.55	(1.33 – 9.52)	0.01	3.09	1.34- 7.11	0.007
EH	**Male**	1.	(0.86 -	0.12	1.	(0.8	0.12	1.8	(0.9	0.09	1.	0.8	0.12

C		69	3.35)		72	7 - 3.4)		9	– 3.97)		69	6- 3.3 5	
	Diabetes	1	(0.5 - 1.99)	0.99	1. 11	(0.5 6 - 2.2 1)	0.75	1.5 3	(0.7 2 – 3.28)	0.27	1. 19	0.6 0- 2.3 6	0.61
	LSM ≥8 KPa	1. 46	(0.69 - 3.06)	0.31	-			-			-		
	LSM ≥10 KPa	-			0. 89	(0.4 3 - 1.8 5)	0.75	-			-		
	LSM ≥12 KPa	-			-			-			0. 61	0.2 6- 1.3 9	0.23
	AGILE 3+ >0.67	-			-			0.4 3	(0.1 8 – 1.05)	0.06	-		
	BMI ≥30 Kg/m2	0. 74	(0.37 - 1.47)	0.38	0. 83	(0.4 1 - 1.6 5)	0.59	0.8 5	(0.4 1 – 1.75)	0.65	0. 90	0.4 6- 1.7 8	0.76
	Age ≥50 years	4. 84	(1.91 - 12.27)	<0.0 01	5. 26	(2.0 7 - 13. 34)	<0.0 01	6.6 9	(2.5 5 – 17.6 1)	<0.0 01	5. 56	2.2 - 14. 05	<0.0 01

*All analysis were corrected for center.

Abbreviations: MASH, metabolic dysfunction-associated steatohepatitis; LRE, liver-related events; LD, liver decompensation; HCC, hepatocellular carcinoma; CVE, cardiovascular events; EHC, extra-hepatic cancer; BMI, body mass index.