

Title page:

Diagnostic performance of LIVERFASt as a non-invasive liver fibrosis test: Data from three cohorts in patients with metabolic dysfunction-associated steatotic liver disease.

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ABSTRACT

Background & Aims

Effective non-invasive tests are essential for the detection and treatment of metabolic dysfunction-associated steatotic liver disease (MASLD). This study evaluates the diagnostic performance of the novel blood-based device, LIVERFAS_t to detect fibrosis stages in patients with MASLD, including those with type 2 diabetes, compared to FIB-4 in a subgroup analysis.

Methods

LIVERFAS_t is computed with 10 blood biomarkers and four anthropometric measures and provides a quantitative score (0.00-1.00) to stage cirrhosis (F4), advanced fibrosis (\geq F3), and clinically significant fibrosis (\geq F2). Three cohorts of patients (two retrospective and one prospective) from tertiary centers in Europe and the U.S. with histological-proven biopsy were used to assess LIVERFAS_t and FIB-4 diagnostic performance using the area under the receiver operating curve, sensitivity, specificity, and predictive values for varying fibrosis prevalence levels.

Results

In total, 497 MASLD adult patients were included in the pooled analysis of the three cohorts (median age 56 years, 56.7% female, 50.3% type 2 diabetes, 44.1% advanced fibrosis, and 20.1% cirrhosis). In the pooled analysis, the AUROCs for fibrosis stages F4, \geq F3, and \geq F2 were: 0.868, 0.846, 0.748, as well as for the T2DM subgroup (n=250): 0.846, 0.798, 0.736, respectively. For 35% advanced fibrosis prevalence, the positive/negative predictive values were 77.2%/81.3% for the overall cohort and 65.52%/79.81% for the subgroup with type 2 diabetes, respectively. At high (90%) to low (1%) advanced fibrosis prevalences, the positive and negative predictive values ranged from 93% to 4.28% and from 43.06% to 99.73%, respectively. For F4 and \geq F3 fibrosis stages, LIVERFAS_t outperformed FIB-4: AUROC 0.870 vs 0.851 and 0.874 vs 0.821 ($P < 0.01$), with sensitivity 74.07 vs 48.15 and 65.54 vs 37.29, respectively.

Conclusions

LIVERFAS_t is a highly sensitive and clinically useful diagnostic test for staging fibrosis in MASLD patients, including those with type 2 diabetes and has a higher sensitivity for

detecting advanced fibrosis when compared with FIB-4. More studies are warranted to compare LIVERFASt with other recommended NITs.

Keywords: Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD), Metabolic-Associated Steatohepatitis (MASH), NAFLD Activity Score (NAS), fibrosis stage, cirrhosis, steatohepatitis, fibrosis, biomarkers, diagnostic panel, LIVERFAST, validation

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BACKGROUND & AIMS

Metabolic dysfunction-associated steatotic liver disease (MASLD), with its more severe form, metabolic dysfunction-associated steatohepatitis (MASH), are conditions that lead to liver fibrosis. The stage of fibrosis is an important predictor of liver-related outcomes, including progression to cirrhosis, hepatocellular carcinoma and mortality¹⁻⁴.

The global prevalence of MASLD is estimated to be approximately 38%, and this is expected to dramatically increase in the next 15 years due to the increasing rates of obesity and type 2 diabetes mellitus (T2DM)^{3,5-8}. T2DM is a major risk factor for the development of MASLD and is associated with fibrosis progression and primary liver cancer^{6,7,9,10}. A recent meta-analysis indicated that approximately 65% of patients with T2DM may have MASLD (prevalence rates estimated during the 1990-2021 period), with rates as high as 81% in Eastern Europe, 71% in the Middle East, and 68% in North America, Western Europe, and Australia^{5,10}. Effective screening strategies for early MASLD diagnosis can help prevent disease progression and improve survival through specific measures or therapies, although the optimal diagnostic approaches remain undefined^{11,12}. The World Health Organization's screening policy criteria highlight the need for established treatments in patients with recognized diseases, justifying population-based screening initiatives by health authorities¹¹.

The recently updated American Association for the Study of Liver Diseases (AASLD) guidelines recommend a two-step diagnostic pathway to detect liver fibrosis, first using a blood biomarker score such as Fibrosis-4 index (FIB-4), followed by either enhanced liver fibrosis (ELF) score or non-invasive imaging techniques (such as vibration-controlled transient elastography [VCTE], or magnetic resonance elastography), taking the reliance away from using invasive liver biopsies¹³. However, these two-step current combinations of NITs are not as accurate as expected and can have non-negligible variability¹⁴ as well as cost and accessibility issues¹⁵, especially in non-tertiary centers. In addition, the accuracy of these NITs is further reduced when patients also exhibit T2DM or obesity¹⁶. With the growing prevalence of MASLD, T2DM, and obesity, and the approval of the new drug resmetirom for MASH patients in the U.S¹⁷⁻¹⁹, there is an urgent need for more accurate and reliable NITs in clinical settings to detect stages of fibrosis and initiate drug therapy.

LIVERFAST test was previously shown as a novel prognostic enrichment biomarker alongside FIB-4 and liver stiffness measurements that could predict global and liver-related mortality and morbidity in MAFLD patients²⁰, but its performance as a diagnostic tool for staging fibrosis in

MASLD patients has yet to be validated. LIVERFAS_t uses a diagnostic algorithm to generate a normalized score for assessing liver fibrosis, specifically for diagnosing cirrhosis (stage 4 or F4), advanced fibrosis (\geq stage 3 or F3), and clinically significant fibrosis (\geq stage 2 or F2). It has two additional features that determine MASH activity and steatosis. The algorithm is based on a standard set of ten blood measurements, along with four anthropometric and demographic attributes.

This study evaluated the diagnostic performance of the LIVERFAS_t Fibrosis Test (without assessing MASH activity and steatosis. Herein, described as LIVERFAS_t test) compared to liver biopsy-confirmed data and FIB-4 in three international cohorts. Data from the three cohorts were pooled to assess the overall MASLD population, as well as a subset of patients with T2DM.

METHODS

The original terms of non-alcoholic fatty liver disease ('NAFLD') and non-alcoholic steatohepatitis ('NASH') used during the initial data collection were replaced with 'MASLD' and 'MASH,' respectively, reflecting updated nomenclature and the assumption that are substitutable^{1,4}. This study assessed the overall performance of LIVERFAS_t test in three cohort groups: one retrospective European database (DB1), one prospective U.S. database (DB2), and one retrospective U.S. database (DB3).

Study Population

DB1 Cohort

DB1 consisted of a retrospective dataset from a non-interventional registry, collected between 2003 and 2020, which included real-world data of adult French steatotic liver disease (SLD) patients. Data were obtained from the Hepatology Unit at the Hospital Haut Lévêque, Bordeaux University Hospital, France. The registry was established to collect clinical information, imaging, and outcomes for longitudinal analysis of non-invasive liver tests and was registered at ClinicalTrials.gov (identifier NCT01241227).

Data were obtained from patients with histopathological evidence of liver disease, who had previously provided informed consent. MASLD was diagnosed retrospectively based on histological criteria and the presence of metabolic dysfunction. The LIVERFAS_t test was performed on anonymized historical data collected concurrently with the liver biopsies. During original collection of samples, a multitude of biomarker tests were ran on the fresh samples, and data stored in the register. For this study, we calculated the LIVERFAS_t score using this

pre-analyzed data. No new serum analysis was rerun. Patients were excluded if the biopsy samples were inadequate, if essential data were missing, or if the biopsy length was less than 20 mm in the absence of advanced fibrosis, or cirrhosis, or if alcohol consumption was not compatible with the MASLD diagnosis. Patients were included if the time between blood test and liver biopsy was <1 year from each other.

DB2 Cohort

DB2 included MASLD (formerly ‘NAFLD’) patients aged 18–80 years, enrolled prospectively between January 2021 and December 2021 from 26 secondary and tertiary hepatology centers across the U.S. (ClinicalTrials.gov identifier NCT04579874). The full list of site participants can be found in the supplementary material Table S1. Eligible patients had a confirmed histological MASLD diagnosis, with a liver biopsy performed within six months of blood collection for the LIVERFASt test. Patients with a history of liver transplantation, advanced cirrhosis (Child-Pugh-Turcotte score ≥ 10), or conditions that could interfere with blood biomarkers (e.g., drug-induced liver injury, acute inflammatory syndrome, and sepsis) were excluded. All patients had blood samples and liver biopsy performed within <6 months.

DB3 Cohort

DB3 was a retrospective collection of MASLD patients from the NASH-Clinical Research Network (CRN) registry maintained by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) in the U.S. The NASH-CRN enrolled over 3,500 adults to analyze the associations between demographic, clinical, and laboratory variables and the severity of ‘NAFLD/NASH’. The data used here for DB3 were obtained from the Non-alcoholic Fatty Liver Disease (NAFLD) Adult Database [(V4)/<https://doi.org/10.58020/53bk-jk73>] supported by NIDDK and made available through the NIDDK central repository (NIDDK-CR) for request at Resources for Research (R4R), <https://repository.niddk.nih.gov/> (request ID number 22849, study number FIBRO-NIDDK-ADU-004-0600).

The DB3 study was approved as an ancillary study of NASH-CRN, with samples randomly assigned by the NIDDK from its biorepository. The DB3 cohort excluded active patient participation and relied solely on previously collected liver histopathology data and eligible samples that met the quality criteria, including biopsy length (≥ 20 mm) and availability of stored serum samples at -80°C to ensure stability when running LIVERFASt analysis. Blood serum samples and biopsy were taken within <1 year of each other. Samples from this biorepository have been used and analyzed in previous publications, and we followed similar

protocols²¹. The original studies that generated DB3 data were approved by the individual institutional review board, and informed consent was obtained from all participants.

The full inclusion/exclusion criteria for all cohorts can be found in the supplementary material Table S2.

Histopathological Reference

Histological assessments varied across cohorts. In DB1, liver biopsies were performed as part of clinical care, with local pathologists providing initial readings. In DB2, histological assessment occurred within six months of blood sampling and was centralized using high-definition, digitized slides (Pacific Rim Pathology Labs Services) evaluated by two experienced central pathologists (PS and MY) blinded to LIVERFAST test results. Slide preparations included hematoxylin-eosin, trichrome staining, and additional stains such as PAS-H, PAS-D, and Prussian Blue. Central pathologists reached a consensus on fibrosis staging, biopsy adequacy, and MASLD classification. In DB3, the NASH-CRN pathology committee performed histological assessments following a standardized protocol, blinded to clinical and laboratory data²².

Fibrosis was assessed in all three cohorts using the NASH-CRN system: stage 0 (F0, none), stage 1 (F1, 1a or 1b perisinusoidal zone 3 or 1c portal fibrosis), stage 2 (F2, clinically significant fibrosis, perisinusoidal and periportal fibrosis without bridging), stage 3 (F3, advanced fibrosis or bridging fibrosis), and stage 4 (F4, cirrhosis)^{22,23}.

Stages 1a, 1b, and 1c were assigned for statistical analysis as fibrosis stage 1, without any distinction between the categories.

Patients with cirrhosis without features of MASLD (steatosis <5%, grade S0) were still included in the study as they may be in a stage where the active elements of steatohepatitis may not be retained in cirrhosis.

LIVERFAST Device and Biomarkers Description

The LIVERFAST device, developed by Fibronostics U.S, Inc. (Florida, U.S), is a software-based system that uses a proprietary algorithm internally derived with liver biopsy and validated against mortality²⁰ to identify serum patterns associated with liver fibrosis, MASH activity, and steatosis. The generated normalized scores ranging from 0.00 to 1.00 are assigned by mapping to semi-quantitative stage ratings similar to the liver biopsy classifications for MASLD²²⁻²⁴. The LIVERFAST cut-offs were pre-specified (\geq F1 0.07, \geq F2 0.21, \geq F3 0.52, F4

0.75) and have been determined from non published internal data in accordance with the regulatory guidance.

LIVERFAST's algorithm utilizes the most discriminating variables based on a standard set of inputs, 10 blood measurements (alpha-2 macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, gamma-glutamyl transferase, alanine and aspartate aminotransferases, fasting glucose, total cholesterol, and triglycerides), along with four anthropometric and demographic attributes (age, height, weight, and sex). Variables are embedded in a neural network to produce a final LIVERFAST score (as above)²²⁻²⁴.

This study aimed to assess only the clinical performance of the LIVERFAST test for assessing fibrosis stage (cirrhosis stage 4, advanced fibrosis stage ≥ 3 , and clinically significant fibrosis stage ≥ 2).

Blood samples were anonymized for analysis and drawn from registry data (DB1), fresh samples (DB2), or stored biorepository specimens (DB3). Fresh specimens were shipped to a central laboratory in the U.S. within 24 h under controlled temperature conditions, where testing complied with the College of American Pathologists (CAP), Clinical Laboratory Improvement Act (CLIA), with methods aligned with FDA and ISO standards. Retrospective specimens were also centralized in a certified laboratory, fulfilling the regulatory requirements in accordance with the international standards for clinical biology analysis (ISO 15189). Consistent procedures were followed for blood sample handling, with either fresh or biorepository specimens processed according to standard protocols. This harmonization ensured the quality of pre-analytical and analytical procedures, contributing to the reliability of LIVERFAST inputs across the study.

LIVERFAST test was performed using deidentified inputs on the secured Fibronostics portal, which generated a normalized score for fibrosis.

FIB-4

For those patients in all cohorts who had platelets count, we independently calculated FIB-4 score as follows:

$(\text{age [years]} \times \text{aspartate aminotransferase [U/L]}) / (\text{platelets [giga/L]} \times \text{SQRT (alanine aminotransferase [U/L])})$. Thresholds were 1.30 to rule out and 2.68 to rule in advanced fibrosis and for cirrhosis (F4) 3.48.

Statistical Analysis

This study described only the performance of the LIVERFASt test generated from each database. Due to the nature of the retrospective collections, overall sample size calculations were not performed but were run in the prospective cohort.

The main diagnostic endpoints included the area under receiver operating characteristics (AUROC) and its 95% confidence interval (95% CI) for fibrosis stages \geq F2, \geq F3, and F4 in each cohort and in the overall pooled MASLD cohort, with secondary analysis stratified by T2DM status. According to FDA guidance and previous independent NITs validations, non-invasive devices require an AUROC of at least 0.70 with a p-value <0.05 ²⁵. Sensitivity and specificity were calculated at fixed points (90% for each). Positive predictive values (PPVs) and negative predictive values (NPVs) were estimated across different modelled fibrosis prevalence rates (1%, 5%, 10%, 20%, 35%, 50%, 75%, and 90%), given that the study cohorts were drawn primarily from tertiary centers, which could skew the prevalence. Due to the heterogenous patient pool of DB1 and the historical and prospective datasets, we decided to run separate sensitivity analysis without DB1 and only on DB2 and DB3 to check for consistency and bias from heterogenous cohorts. Diagnostic accuracy has been compared between groups in those patients with a time lapse of more than 6 months or less than 6 months from biopsy to blood draw for biomarkers.

Statistical software NCSS-2023 and SAS (versions t.14 and t.16) were used for analysis^{26,27}.

RESULTS

A total of 1,162 patients were initially considered from the three cohorts, of which 633 were excluded due to various factors, such as missing data, failure to meet the inclusion criteria, inadequate histopathology, or LIVERFASt input data. The final sample consisted of 497 MASLD patients: 191 from DB1, 201 from DB2, and 105 from DB3. For the pooled data analysis, 497 patients with MASLD were included (Figure 1).

The mean interval between blood sampling and biopsy was less than six months for 80.7% (DB1 70.2%, DB2 100%, DB3 62.9%) of the patients, and over six months and up to a year for 19.1%. A total of 80.5% of the biopsies were performed on specimens measuring at least 20 mm (combined fragments for DB2), and the remainder were <20 mm.

The median age of the pooled MASLD cohort was 56 years (range: 18-83), 56.7% were female and 50.3% had T2DM (n=250). In terms of histological fibrosis staging confirmed by biopsy, 343 (69.0%) patients had clinically significant fibrosis (\geq stage F2), 219 (44.1%) had advanced fibrosis (\geq stage F3), and 100 (20.1%) had cirrhosis (stage F4). Patients in the DB2 and DB3 cohorts tended to be younger, with a higher proportion of females, elevated body mass index (BMI), lower liver enzyme levels, and less frequent cirrhosis than those in the DB1 cohort (all $P<0.05$) (Table I).

Diagnosing Cirrhosis (Stage 4) with LIVERFASt Test

The prevalence of cirrhosis was 30.9% for DB1, 13.9% for DB2, 12.4% for DB3, and 20.1% for the pooled MASLD cohort and 27.2% in the T2DM subgroup. LIVERFASt test AUROC values for diagnosing cirrhosis were 0.869 for DB1, 0.855 for DB2, 0.823 for DB3, and 0.868 for the overall MASLD pooled population, all of which were statistically significant ($P<0.001$, compared to hazard) (Table II). In the pooled MASLD cohort, the AUROC values were 0.887 for patients without T2DM and 0.846 for those with T2DM, with no statistically significant difference between the groups (Table II, supplementary Figure S1). The sensitivities for detecting cirrhosis were 68.0%, 75.0%, and 64.7% for the pooled population, non-T2DM, and T2DM subgroups, respectively, whereas the specificities were 90.2%, 91.6%, and 88.5%, respectively (Table IIIa). When sensitivity was fixed at 90%, the corresponding specificities were 63%, 65.6%, and 56.7% for the overall MASLD pooled population, non-T2DM, and T2DM subgroups, respectively, and the corresponding NPVs were 96.2%, 97.9%, and 94.5%, respectively. When specificity was fixed at 90%, the sensitivities were 68%, 75%, and 60.3%, and the PPV were 63.6%, 54.5%, and 70.7%, respectively (Table IIIb). To remove any potential bias from the heterogenous DB1 cohort, a sensitivity analysis was run to exclude DB1 and pool

only DB2 and DB3. The AUROC values for diagnosing cirrhosis in the pooled DB2 and DB3 (N=306) population were 0.843, 0.848 in T2DM subgroup and 0.826 in the subgroup without T2DM, all non-significant from DB1 (P=NS). (see supplementary Table S4.) Pooled DB2 and DB3 had lower sensitivities and higher specificities, while NPV and PPV were similar to DB1 cohort alone. (see supplementary Table S5) There was no significant difference in LIVERFAST performance (AUROC) when comparing those patients with < 6 months versus those with ≥ 6 month interval between biopsy and blood draw (all p=NS). (see supplementary table S6)

Diagnosing Advanced Fibrosis (Stage ≥ 3) with LIVERFAST Test

The prevalence of advanced fibrosis was 53.4% for DB1, 39.3% for DB2, 36.2% for DB3, and 44.1% for the pooled MASLD cohort and 54.4% in the T2DM subgroup. For diagnosing advanced fibrosis (\geq stage 3), all AUROCs were significant (P<0.001, compared to hazard), with values of 0.888 for DB1, 0.773 for DB2, 0.872 for DB3, and 0.846 for the overall MASLD pooled population (Table II and supplementary Figure S1). The performance was comparable across subgroups, with AUROCs of 0.884 in non-T2DM patients and 0.798 in T2DM patients, showing no significant difference (Table II). Sensitivities were 62.1%, 61.5%, and 62.5%, and specificities were 86.0%, 90.2%, and 79.8% for the pooled population, non-T2DM group, and T2DM group, respectively (Table IIIa and supplementary Table S3). With sensitivity fixed at 90%, the specificities were 59.7%, 72.6%, and 42.1% for the overall MASLD pooled cohort, non-T2DM, and T2DM groups, respectively, and the NPVs were 88.8%, 93.7%, and 78.7%, respectively. At 90% fixed specificity, the sensitivities were 56.2%, 61.5%, and 56.6%, and the PPVs were 82%, 76.1% and 87.5%, respectively (Table IIIb). When excluding DB1, the sensitivity analysis (AUROC) for diagnosing stages \geq F3 in the pooled DB2 and DB3 population was 0.803, significantly different from DB1 (0.888), mainly due to the T2DM subgroup with an AUROC of 0.730, both p<0.05. In the subgroup without T2DM, the AUROC was 0.867 not different from DB1 (P=NS). (see supplementary Table S4.) In the pooled DB2 and DB3 cohort, LIVERFAST had lower sensitivities and higher specificities, while NPV and higher PPV were similar to those observed in the DB1 cohort alone. (see supplementary Table S5) For \geq F3 stages, the AUROCs were higher in the subgroup without T2DM and < 6 months delay between liver biopsy and blood draw, while the contrary was observed in the subgroup with T2DM, all p<0.01. (see supplementary Table S6)

Diagnosing Clinically Significant Fibrosis (Stage ≥ 2) with LIVERFAS_t Test

The prevalence of clinically significant fibrosis was 75.4% for DB1, 70.7% for DB2, 54.3% for DB3, and 69.0% for the pooled MASLD cohort and 81.2% in the T2DM subgroup. All AUROCs were significant ($p < 0.001$, compared to hazard) for identifying clinically significant fibrosis (\geq stage 2) using the LIVERFAS_t Test. The AUROCs were 0.779 for DB1, 0.705 for DB2, 0.783 for DB3, and 0.748 for the pooled populations, respectively (Table II and supplementary Figure S1). In the overall MASLD pooled cohort, LIVERFAS_t Fibrosis Test showed similar performance in detecting fibrosis regardless of T2DM status, with AUROCs of 0.717 in non-T2DM and 0.736 in T2DM patients (Table II). Sensitivities for diagnosing \geq stage 2 fibrosis were 70.9%, 62.9%, and 76.4%, while specificities were 64.9%, 72.0%, and 48.9% for the overall cohort, non-T2DM, and T2DM subgroups, respectively (Table IIIa). For a fixed sensitivity of 90%, specificities were 25.3%, 16.8%, and 31.9%, and NPVs were 53.4%, 56.3%, and 42.9%, respectively, while for fixed specificity at 90%, sensitivities were 46.7%, 40.7%, and 50.3%, and PPVs were 91.2%, 85.1%, and 96.2% for the overall pooled MASLD cohort, non-T2DM, and T2DM subgroups, respectively (Table IIIb). When excluding DB1 cohort in the sensitivity analysis, the AUROC for diagnosing \geq F2 stages in the pooled DB2 and DB3 population were 0.721, 0.683 in the T2DM subgroup, and 0.722 in the subgroup without T2DM, all non-significant from DB1 ($P=NS$). (see supplementary Table S4) In the pooled DB2 and DB3 cohort, LIVERFAS_t had lower sensitivities and higher specificities, while NPV and PPV were similar to those observed in DB1 cohort alone. (see supplementary Table S5) There was no significant difference in LIVERFAS_t performance (AUROC) for staging \geq F2 according to the interval between biopsy and blood draw in the subgroup without T2DM. A difference was observed in the subgroup with T2DM with ≥ 6 months delay; a result that should be interpreted with caution due to the low number ($N=5$) of patients without \geq F2 fibrosis. (see supplementary Table S6)

Sensitivities, Specificities and Predictive Values Generated by LIVERFAS_t Test

Sensitivity and specificity for each fibrosis endpoint at varying cut-off points were plotted across the dynamic range of LIVERFAS_t fibrosis scores (Figures 2a, 2b, and 2c). The predictive values of the LIVERFAS_t test were examined across various prevalence levels of fibrosis. The PPVs increased with the prevalence of fibrosis, with PPV/NPV values in the overall pooled MASLD cohort ranging from 18.9%/97.7% for a low prevalence of advanced

fibrosis setting (5%) to 81.8%/40.8% at a higher prevalence (75%). In scenarios where the prevalence of advanced fibrosis was 35%, as often seen in tertiary centers screening for T2DM patients, the PPV/NPV was 70.5%/80.8% for the overall cohort and 62.5%/79.8% for the T2DM subgroup (Table IV).

Comparison of LIVERFASt sensitivity, specificity, PPV and NPV with FIB-4 score

In a separate analysis, we isolated patients who also had platelet biomarkers to calculate FIB-4 score. Out of the 497 patients included in the study, 394 had data to calculate FIB-4. Using a LIVERFASt cut-off of 0.75 and FIB-4 cut-off of 3.48 for staging cirrhosis (F4), the LIVERFASt AUROC was 0.870, 0.879 and 0.854 for pooled MASLD, non-T2DM and with T2DM compared to 0.851, 0.870 and 0.828 for FIB-4, respectively (all were non-significant. Table V). Furthermore, the sensitivity, specificity, PPV and NPV were 74.07, 88.18, 61.86 and 92.93, respectively for LIVERFASt, and 48.15, 96.17, 76.47 and 87.76 for FIB-4. Similar trends were seen for non-T2DM patients and T2DM patients (Table VI).

For advanced fibrosis (\geq F3), LIVERFASt had a cut-off of 0.52 and FIB-4 a cut-off of 2.68. The LIVERFASt AUROC was 0.874, 0.898 and 0.842 for the pooled MASLD, non-T2DM and T2DM, respectively and for FIB-4 0.821, 0.828 and 0.799, respectively. LIVERFASt was significant for the pooled MASLD and the non-T2DM ($P < 0.01$ and $P < 0.05$, respectively. Table V). The sensitivity, specificity, PPV and NPV values were 65.54, 85.25, 78.38 and 75.20 for LIVERFASt, meanwhile for FIB-4 they were 37.29, 94.93, 85.71 and 64.98, respectively (Table VI). As there is no recommended cut-off for FIB-4 F2, we did not make a comparison with sensitivity, specificity, NPV and PPV.

DISCUSSION

This study shows, for the first time, the performance of the LIVERFAST test as a diagnostic enrichment device for assessing clinically significant fibrosis, advanced fibrosis, and cirrhosis in patients with MASLD, with a strong performance in patients with T2DM and MASLD. We show the independent performance of LIVERFAST as an accurate diagnostic test with an acceptable AUROC (>0.7) for all stages of fibrosis in the overall pooled population and in the T2DM subgroup. In addition, we offer a comparison of its performance with FIB-4, the most widely accepted NIT for staging fibrosis in MASLD patients. For both cirrhosis and advanced fibrosis, LIVERFAST had a markedly higher sensitivity and NPV score when compared to FIB-4. Previously, a LIVERFAST and FIB-4 combination was shown to have a good prognostic performance of predicting overall and excellent performance for predicting liver-related outcomes in MAFLD patients, with patients having a higher initial LIVERFAST score having the highest risk of liver-related mortality versus FIB-4 (hazard ratios 3.04 [2.13-4.33] versus 1.30 [1.18-1.32], respectively) per additional point of fibrosis²⁰. Thus, LIVERFAST, with additional investigation, could be considered a reliable prognostic and diagnostic enrichment biomarker for patients with MASLD. As mentioned, recently updated guidelines recommend a two-step diagnostic pathway for staging fibrosis using blood biomarker scores and imaging techniques¹³. However, there are several pitfalls associated with the current NITs: 1. The FIB-4 score varies with age and its accuracy is lower in MASLD populations with T2DM²⁸. Using an age-adapted FIB-4 cut-off could help minimize false-negative rates when screening general populations²⁹; 2. As a general rule, biomarkers such as FIB-4 and ELF that utilize two cut-offs (one to rule in and one to rule out advanced fibrosis) result in a high number of indeterminate outcomes (between the cut-offs), necessitating further evaluations; 3. When VCTE is used as a second step, its PPV is low, often leading to the need for liver biopsy; 4. The main limitation of VCTE is related to unreliable results in individuals with high BMI, with up to 20% of examinations fielding unreliable results, particularly in obese patients with MASLD^{30,31}; and 5. Furthermore, VCTE results can fluctuate, presenting a high rate of false positives with inflammatory activity and intrinsic variability in patients with MASLD without significant activity fluctuations¹⁴. Thus, with all these limitations, there are several areas for improvement and a combination method of NITs for diagnosing fibrosis in patients with MASLD, obesity, and T2DM is warranted. This study provides strong evidence that LIVERFAST test, with its high accuracy, ability to detect \geq F2, and high NPV in low prevalence populations, could be

used as an upstream diagnostic test for MASLD and could potentially fit into tier 1 of the two-step diagnostic approach recommended.

The Non-Invasive Biomarkers for Metabolic Liver Disease (NIMBLE) consortium has initiated a regulatory roadmap for qualifying biomarkers for diagnostic enrichment and has led efforts to validate several NITs for liver fibrosis staging and for detecting ‘at-risk MASH’ defined as having fibrosis \geq stage 2 with a NAS ≥ 4 ²⁵. These include NIS4 (Genfit, Lille, France), OWLiver (One Way Lipidomics, Bilbao, Spain), PROC3 (Nordic Bioscience, Copenhagen, Denmark), ELF test (Siemens Healthineers, New Jersey, USA), and FibroMeter VCTE (Echosens, Paris, France). These tests were validated using a cohort of 1,078 patients with MASLD from the NASH-CRN, which had a balanced distribution of fibrosis severity. Our study included a smaller subset from the NASH-CRN cohort, with approximately two-thirds of the participants having undergone LIVERFAST testing within 180 days of liver biopsy. Although in the current study, we did not conduct a direct comparison of the LIVERFAST test with VCTE nor evaluate at-risk MASH patients, when we compare the data obtained from the NIMBLE consortium and our initial data with high PPV and NPV across a range of fibrosis prevalence rates, from 1% to 90% (Table IV), we could speculate that LIVERFAST could achieve comparable or superior diagnostic accuracy in detecting fibrosis. The data provided by comparing LIVERFAST performance with FIB-4 performance in a subgroup of patients, already indicates that LIVERFAST has the potential to be highly sensitive with a good NPV. . In addition, LIVERFAST has several other advantages, such as although LIVERFAST is not a free resource, unlike FIB-4, it has minimal safety concerns, as it operates under rigorous quality control required by medical device regulations. Furthermore, in some geographical locations e.g. USA, payment models for LIVERFAST are already established, where the test is reimbursed, and costs are widely covered by major national commercial insurers and government programs such as Medicare and Medicaid. Moreover, it has the advantage of a high sensitivity and NPV where it can identify individuals that usually fall within the FIB-4 grey zone with a better efficacy of identifying cases that eventually require a secondary assessment (e.g. VCTE). According to guidelines, in GI/hepatology care, when using imaging-based NITs, LIVERFAST could strengthen the stratification of patients at risk for advanced fibrosis and cirrhosis.

The high diagnostic performance of LIVERFAST test suggests that it can meet regulatory standards for fibrosis assessment in MASLD patients with or without the presence of T2DM. Based on LIVERFAST parameters, it has the potential to be run on routine collection of biochemistry samples (no need for a hematology sample as in the case of FIB-4); thus, it can

be readily integrated into clinical care pathways, and is potentially suitable for primary care settings, where it can help rule out advanced fibrosis in lower prevalence populations due to its high NPVs, and identify and manage patients with significant fibrosis, advanced fibrosis, or cirrhosis in specialized hepatology settings. LIVERFAST's inputs include common laboratory parameters that are inexpensive and widely accessible. Some of these fibrosis biomarkers overlap with those used in other validated NITs for fibrosis assessment and prognosis in MASLD³²⁻³⁵, meaning that it can be used to complement and enhance the staging and prognostic assessment in the two-step approach. However, this finding needs to be validated in future studies as well as LIVERFAST's potential role in identifying patients for specific therapy, such as resmetirom, a recently approved THR- β agonist for non-cirrhotic MASH with fibrosis^{17,18,19}.

Most proprietary NITs have a lower accuracy when applied to T2DM cohorts¹⁶. In our study, the LIVERFAST test performance for cirrhosis as for clinically significant fibrosis was very high and not impacted by T2DM, potentially recommending it as a forefront test in such populations. In the pooled MASLD population, despite the higher performance (AUROC 0.88) observed for advanced fibrosis in patients without T2DM, LIVERFAST test performed well in patients with T2DM (AUROC 0.80). In addition, when compared to FIB-4 performance in T2DM patients, LIVERFAST has a higher sensitivity and NPV in both \geq F3 and F4 fibrosis. This study highlights that LIVERFAST is more sensitive and offers superiority over FIB-4 to detect advanced fibrosis. This is worth further investigation with more diverse populations and future studies are needed to fully determine the clinical application of LIVERFAST in combination with existing NITs, specifically FIB-4 and VCTE.

Limitations of the Study

Despite its strengths, this study had several limitations that warrant consideration. First, all three cohorts were based at tertiary care centers, where patients typically have more advanced fibrosis than those seen in primary care centers. This selection bias may limit the generalizability of the results to the broader MASLD population, where the distribution of fibrosis stages would likely differ, as stated in Harrison et al. 2021, who found upon imaging and reference liver biopsy an overall prevalence of 38% MASLD, 14% MASH and significant liver fibrosis in 6% of asymptomatic middle-aged U.S. cohort⁷.

Second, the pooled analysis of patients with MASLD was based on three heterogeneous datasets. While this approach increases the sample size, it also introduces heterogeneity that could affect the findings. Notably, patients in DB1 were older and had more severe fibrosis than those in DB2 and DB3, possibly reflecting a longer disease duration. In addition, DB2 cohort was a combination of multiple sites and liver biopsy quality was slightly variable and in some instances of lower quality sample size. Ethnicity data were not collected for DB1, but were likely predominantly Caucasian, as in the other cohorts.

Furthermore, a small subset of patients had a longer duration between blood sample and biopsy (between 6 months and 1 year). However, to address this we ran a sensitivity analysis to compare the time interval of less than 6 months and more than 6 months between biopsy and blood draw in all subgroups. It revealed that the time interval between the biopsy and the blood sampling did not change the performance (AUROC) in the overall population and in the subgroup without T2DM for cirrhosis, advanced fibrosis and for $\geq F2$. However, as expected, a significant improvement in the performance up to 0.97 was seen in the subgroup without T2DM and time interval less than 6 months. Paradoxically high AUROC were noticed in the subgroups with T2DM and time interval more than 6 months for both $\geq F3$ and $\geq F2$, but this is likely due to the low number of subjects without $\geq F3$ (N=12) and $\geq F2$ (N=5), respectively.

Finally, another limitation is the absence of direct comparisons between LIVERFAST test and other NITs such as VCTE. VCTE was not collected in the prospective dataset, some of the data is historical in which VCTE was not a requirement at the time or it was used to calibrate the quality of the biopsy and therefore, the comparison was not possible. In addition, having a FIB-4 measurement was not an inclusion criterion; however, we did manage to calculate FIB-4 in almost 80% of patients which allowed us to run analysis to compare the two NITs in this diverse cohort population.

Conclusion

This is the first validation study on the performance of LIVERFAST test as a clinical NIT against biopsy and offers promising accuracy in assessing liver fibrosis across various stages in MASLD patients and, encouragingly, in T2DM patients. This sets the stage for further investigation as a tier 1 NIT that could work well in combination with tier 2 utilities, such as VCTE. Additional studies should focus on larger, more diverse populations to confirm these

results and address the limitations related to cohort diversity, selection bias and comparative NITs.

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Table I. Demographic, clinical and laboratory data from the study cohorts. DB, database; MASLD, metabolic dysfunction-associated liver disease; BMI, body mass index; HDL, high-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase.

| | DB1 | DB2 | DB3 | Overall MASLD pooled cohort |
|---|-------------------|------------------|-------------------|------------------------------------|
| <i>Demographics</i> | | | | |
| N | 191 | 201 | 105 | 497 |
| Age (years) | 60 (18-83) | 56 (18-76) | 49 (18-75) | 56 (18-83) |
| Female | 83 (43.5%) | 137 (68%) | 62 (59%) | 282 (56.7%) |
| BMI (kg/m ²) | 31.5 (20.8-48.7) | 35.4 (22-76) | 33.7 (24.0-54.8) | 33.5 (20.8-75.7) |
| <i>Cardiometabolic criteria</i> | | | | |
| Type 2 Diabetes | 97 (50.8%) | 109 (54.2%) | 44 (41.9%) | 250 (50.3%) |
| Fasting glucose \geq 5.6 mmol/L (100 mg/dL) | 110 (57.6%) | 114 (56.7%) | 56 (53.9%) | 281 (56.5%) |
| BMI \geq 25 kg/m ² | 168 (87.9%) | 192 (95.5%) | 100 (96.2%) | 461 (92.8%) |
| Triglycerides \geq 1.70 mmol/L (150 mg/dL) | 90 (48.7%) | 95 (47.3%) | 60 (57.7%) | 248 (49.9%) |
| HDL-Cholesterol \leq 1.0 mmol/L (40 mg/dL) (M) and \leq 1.3 mmol/L (50 mg/dL) (F) | 95 (50.5%), n=188 | 33 (68.8%), n=48 | 72 (69.2%), n=104 | 200 (58.8%), n=340 |
| Hypertension | 121 (63.4%) | 78 (38.8%) | 59 (56.2%) | 258 (51.9%) |
| <i>Blood (Biochemistry and blood count)</i> | | | | |
| AST (IU/L) | 46 (15-318) | 31 (6-154) | 38 (16-460) | 38 (6-318) |
| ALT (IU/L) | 58 (12-442) | 38 (10-257) | 47 (10-310) | 48 (10-442) |
| GGT (IU/L) | 99 (17-1540) | 40 (12-1100) | 54 (10-820) | 56 (10-1540) |

| | | | | |
|---|----------------------|---------------------|---------------------|------------------------|
| Total bilirubin (micromol/L) | 11 (3-126) | 8.55 (1.71-30.79) | 7.0 (2.9-30.0) | 10.0 (1.7-126.0) |
| Fasting glucose (mmol/L) | 5.9 (2.2-18.9) | 5.83 (3.89-15.10) | 5.77 (3.88-19.09) | 5.88 (2.20-19.09) |
| Triglyceride (mmol/L) | 1.68 (0.60-7.12) | 1.67 (0.42-4.62) | 1.93 (0.47-9.03) | 1.69 (0.42-9.03) |
| Total cholesterol (mmol/L) | 5.12 (2.34-10.07) | 5.01 (2.23-8.41) | 4.94 (2.30-7.63) | 4.82 (2.02-10.07) |
| Alpha-2 macroglobulin (g/l) | 2.40 (1.00-6.00) | 2.17 (0.74-4.32) | 1.81 (0.87-4.39) | 2.15 (0.74-6.00) |
| Apolipoprotein A1 (g/L) | 1.35 (0.66-2.31) | 1.44 (0.93-2.54) | 1.36 (0.88-1.96) | 1.39 (0.66-2.54) |
| Haptoglobin (g/L) | 1.20 (0.08-3.14) | 1.48 (0.08-2.53) | 1.26 (0.09-3.28) | 1.34 (0.08-3.53) |
| Albumin (g/L) | 4.2 (1.3-5.1) | 4.3 (3.3-6.7), n=89 | 4.20 (1.40-5.20) | 4.3 (1.30-6.70), n=385 |
| HbA1c (%) | 6.4 (4.8-10.6), n=34 | NA | 5.9 (4.1-12), n=101 | 6.1 (4.1-11.0), n=135 |
| LIVERFASt | | | | |
| LIVERFASt Fibrosis score (0.00-1.00) | 0.60 (0.01-1.00) | 0.19 (0.00-0.96) | 0.28 (0.001-0.99) | 0.29 (0.01-1.00) |
| Liver histology | | | | |
| Length of liver biopsy specimen (mm) *Sum of fragments sizes in DB2 | 28 (12-95) | 22 (5-84)* | 27 (21-62) | 26 (5-95)* |
| Number of liver biopsy specimen with length (mm) \geq 20 mm. *Sum of fragments sizes in DB2 | 175 (91.6%) | 120 (59.7%)* | 105 (100%) | 400 (80.5%)* |
| Fibrosis stage (NASH-CRN) | | | | |
| <i>0 – none</i> | 16 (8.4%) | 23 (11.4%) | 21 (20.0%) | 60 (12.1%) |

| | | | | |
|---|-------------|------------|------------|-------------|
| <i>1 - perisinusoidal or portal</i> | 31 (16.2%) | 36 (17.9%) | 27 (25.7%) | 94 (18.9%) |
| <i>2 - perisinusoidal and periportal</i> | 42 (22.0%) | 63 (31.3%) | 19 (18.1%) | 124 (25.0%) |
| <i>3 – bridging</i> | 43 (22.5%) | 51 (25.4%) | 25 (28.8%) | 119 (23.9%) |
| <i>4 – cirrhosis</i> | 59 (30.9%) | 28 (13.9%) | 13 (12.4%) | 100 (20.1%) |
| <i>Time lapse between blood analyses and liver biopsy</i> | | | | |
| <i>≤ 6 months</i> | 134 (70.2%) | 201 (100%) | 66 (62.9%) | 401 (80.7%) |
| <i>>6 months</i> | 57 (29.8%) | 0 | 39 (37.1%) | 96 (19.1%) |
| Data are n, median (range), n (%), or mean (SD), unless otherwise specified. | | | | |
| *NA Not available. Data are missing because the investigator has not agreed to share those data or the data has not been collected. | | | | |

Table II. Standard-area under the receiver-operating curves according to the histological feature's endpoint (fibrosis) across all cohorts and pooled population. MASLD, metabolic dysfunction-associated liver disease; T2DM, type 2 diabetes mellitus; AUROC, area under the receiver-operating characteristics curve; DB, database; CI, confidence interval.

| Group | Cohorts | | | | | | | | | | | |
|--|-----------------------------|-------------------------|----------------|---------------------------|-------------------------|----------------|-----------------------------|-------------------------|----------------|-------------------|-------------------------|---------------------|
| | DB1 MASLD, retrospective | | | DB2 MASLD, prospective | | | DB3 MASLD, retrospective | | | Pooled population | | |
| | Prevalence | AUROC P-value | 95% CI | Prevalence | AUROC P-value | 95% CI | Prevalence | AUROC P-value | 95% CI | Prevalence | AUROC P-value | 95% CI |
| Cirrhosis (Fibrosis stage 4) | | | | | | | | | | | | |
| <i>All MASLD</i> | 59 / 191 | 0.868 <0.001 | 0.799 0.914 | 28 / 201 | 0.855 <0.001 | 0.751 0.917 | 13 / 105 | 0.823 <0.001 | 0.620 0.923 | 100 / 497 | 0.868 <0.001 | 0.820 - 0.903 |
| <i>Without T2DM</i> | 20 / 94 | 0.914 <0.001 | 0.819 0.960 | 8 / 92 | 0.891 <0.001 | 0.734 0.958 | 4 / 61 | 0.737 NS | 0.013 0.954 | 32 / 247 | 0.887 <0.001 | 0.799 - 0.938 |
| <i>With T2DM</i> | 39 / 97 | 0.817 <0.001* | 0.704 0.889 | 20 / 109 | 0.829 <0.001* | 0.676 0.914 | 9 / 44 | 0.876 <0.001* | 0.710 0.950 | 68 / 250 | 0.846 <0.001* | 0.779 - 0.894 |
| Advanced fibrosis (fibrosis stages ≥ 3) | | | | | | | | | | | | |

| | | | | | | | | | | | | |
|---|-----------|-------------------------|----------------|-----------|-------------------------|----------------|----------|-------------------------|----------------|-----------|--------------------------|---------------------|
| <i>All MASLD</i> | 102 / 191 | 0.888 <0.001 | 0.834 0.926 | 79 / 201 | 0.773 <0.001 | 0.699 0.830 | 38 / 105 | 0.872 <0.001 | 0.770 0.930 | 219 / 497 | 0.846 <0.001 | 0.808 - 0.877 |
| <i>Without T2DM</i> | 36 / 94 | 0.903 <0.001 | 0.824 0.947 | 25 / 92 | 0.836 <0.001 | 0.715 0.908 | 22 / 61 | 0.896 <0.001 | 0.757 0.958 | 83 / 247 | 0.884 <0.001 | 0.831 - 0.920 |
| <i>With T2DM</i> | 66 / 97 | 0.864 <0.001* | 0.772 0.921 | 54 / 109 | 0.706 <0.001* | 0.595 0.790 | 16 / 44 | 0.859 <0.001* | 0.664 0.945 | 136 / 250 | 0.798 <0.001** | 0.738 - 0.846 |
| Fibrosis (Fibrosis stages ≥ 2) | | | | | | | | | | | | |
| <i>All MASLD</i> | 144 / 191 | 0.779 <0.001 | 0.701 0.839 | 142 / 201 | 0.705 <0.001 | 0.622 0.773 | 57 / 105 | 0.783 <0.001 | 0.678 0.857 | 343 / 497 | 0.748 <0.001 | 0.702 - 0.788 |
| <i>Without T2DM</i> | 58 / 94 | 0.711 <0.001 | 0.592 0.800 | 50 / 92 | 0.659 <0.001 | 0.532 0.757 | 32 / 61 | 0.815 <0.001 | 0.676 0.897 | 140 / 247 | 0.717 <0.001 | 0.647 - 0.774 |
| <i>With T2DM</i> | 86 / 97 | 0.765 <0.001* | 0.598 0.869 | 92 / 109 | 0.712 <0.001* | 0.563 0.817 | 25 / 44 | 0.773 <0.001* | 0.582 0.883 | 203 / 250 | 0.736 <0.001* | 0.659 - 0.797 |
| *P-value = ns versus the group without T2DM | | | | | | | | | | | | |

Table IIIa. Sensitivity and specificity of LIVERFAST test for its intended uses in the overall MASLD pooled population and according to the presence of type 2 diabetes. MASLD, metabolic dysfunction-associated liver disease; T2DM, type 2 diabetes mellitus; AUROC, area under the receiver-operating characteristics curve.

| Group | Prevalence | Sensitivity (%) | Specificity (%) | AUROC | 95% confidence interval | Significance versus hazard |
|--|-------------------|------------------------|------------------------|--------------|--------------------------------|-----------------------------------|
| Cirrhosis (Fibrosis stage 4) | | | | | | |
| Overall MASLD | 100 / 497 | 68.0 | 90.2 | 0.868 | 0.820 0.903 | < 0.001 |
| <i>Without T2DM</i> | 32 / 247 | 75.0 | 91.6 | 0.887 | 0.799 0.938 | < 0.001 |
| <i>With T2DM</i> | 68 / 250 | 64.7 | 88.5 | 0.846 | 0.779 0.894 | < 0.001 |
| Advanced fibrosis (fibrosis stages ≥ 3) | | | | | | |
| Overall MASLD | 219 / 497 | 62.1 | 86.0 | 0.846 | 0.808 0.877 | < 0.001 |
| <i>Without T2DM</i> | 83 / 247 | 61.5 | 90.2 | 0.884 | 0.831 0.920 | < 0.001 |

| | | | | | | |
|--|-----------|------|------|-------|-------------|---------|
| <i>With T2DM</i> | 136 / 250 | 62.5 | 79.8 | 0.798 | 0.738 0.846 | < 0.001 |
| Clinically significant fibrosis (Fibrosis stages ≥ 2) | | | | | | |
| Overall MASLD | 343 / 497 | 70.9 | 64.9 | 0.748 | 0.702 0.788 | < 0.001 |
| <i>Without T2DM</i> | 140 / 247 | 62.9 | 72.0 | 0.717 | 0.647 0.774 | < 0.001 |
| <i>With T2DM</i> | 203 / 250 | 76.4 | 48.9 | 0.736 | 0.659 0.797 | < 0.001 |

Table IIIb. Performance of LIVERFASt test at high sensitivity and specificity in the overall MASLD pooled population and according to the presence of type 2 diabetes. MASLD, metabolic dysfunction-associated liver disease; NPV, negative predictive value; PPV, positive predictive value; T2DM, type 2 diabetes mellitus.

| | When constraining sensitivity to be at least 90% | | | When constraining specificity to be at least 90% | | |
|---------------------|--|---------|---------|--|---------|---------|
| Group | Specificity (%) | NPV (%) | PPV (%) | Sensitivity (%) | NPV (%) | PPV (%) |
| | Cirrhosis (Fibrosis stage 4) | | | | | |
| Overall MASLD | 63.0 | 96.2 | 38.0 | 68.0 | 91.8 | 63.6 |
| <i>Without T2DM</i> | 65.6 | 97.9 | 28.2 | 75.0 | 96.1 | 54.5 |
| <i>With T2DM</i> | 56.7 | 94.5 | 44.0 | 60.3 | 85.9 | 70.7 |
| | Advanced fibrosis (Fibrosis stages ≥ 3) | | | | | |
| Overall MASLD | 59.7 | 88.8 | 63.9 | 56.2 | 72.3 | 82.0 |
| <i>Without T2DM</i> | 72.6 | 93.7 | 62.2 | 61.5 | 82.2 | 76.1 |
| <i>With T2DM</i> | 42.1 | 78.7 | 65.1 | 56.6 | 63.6 | 87.5 |
| | Clinically significant fibrosis (Fibrosis stages ≥ 2) | | | | | |
| Overall MASLD | 25.3 | 53.4 | 72.9 | 46.7 | 43.2 | 91.2 |

| | | | | | | |
|-------------------------|------|------|------|------|------|------|
| <i>Without T2DM</i> | 16.8 | 56.3 | 58.6 | 40.7 | 53.9 | 85.1 |
| <i>With T2DM</i> | 31.9 | 42.9 | 85.1 | 50.3 | 29.9 | 96.2 |

Table IV. Predictive values of LIVERFASt test for their intended use in the overall MASLD pooled population with varying prevalence of disease phenotype. MASLD, metabolic dysfunction-associated liver disease; NPV, negative predictive value; PPV, positive predictive value; T2DM, type 2 diabetes mellitus.

| Group | Prevalence of disease phenotype | | | | | | | |
|--------------------------|--|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| | 1% | 5% | 10% | 20% | 35% | 50% | 75% | 90% |
| | PPV | PPV | PPV | PPV | PPV | PPV | PPV | PPV |
| | NPV | NPV | NPV | NPV | NPV | NPV | NPV | NPV |
| | Cirrhosis (stage F4) | | | | | | | |
| Overall MASLD population | 6.54% / 99.64% | 26.70% / 98.17% | 43.48% / 96.21% | 63.38% / 91.85% | 78.85% / 83.96% | 87.38% / 73.81% | 95.41% / 48.44% | 98.42% / 23.85% |
| <i>Without T2DM</i> | 8.30% / 99.73% | 38.42% / 97.00% | 49.88% / 97.06% | 69.13% / 93.61% | 82.83% / 87.19% | 89.96% / 78.56% | 96.41% / 54.99% | 98.78% / 28.94% |
| <i>With T2DM</i> | 5.36% / 99.60% | 22.79% / 97.94% | 38.39% / 95.76% | 58.42% / 90.30% | 75.12% / 82.32% | 84.87% / 71.48% | 94.39% / 45.52% | 98.06% / 21.78% |
| | Advanced fibrosis (≥ stage F3 fibrosis) | | | | | | | |
| Overall MASLD population | 4.28% / 99.56% | 18.90% / 97.73% | 32.97% / 95.33% | 52.53% / 90.07% | 70.45% / 80.82% | 81.57% / 69.40% | 93.00% / 43.06% | 97.55% / 20.13% |
| <i>Without T2DM</i> | 5.98% / 99.57% | 17.39% / 98.59% | 41.17% / 95.47% | 61.16% / 90.35% | 77.23% / 81.30% | 86.30% / 70.07% | 94.97% / 43.83% | 98.27% / 20.64% |

| | | | | | | | | |
|--------------------------|--|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| <i>With T2DM</i> | 3.03% / 99.53% | 14.02% / 97.59% | 25.61% / 95.04% | 43.65% / 89.49% | 62.52% / 79.81% | 75.60% / 68.04% | 90.29% / 41.51% | 96.54% / 19.13% |
| | Clinically significant fibrosis (\geq stage F2 fibrosis) | | | | | | | |
| Overall MASLD population | 2.00% / 99.55% | 9.61% / 97.69% | 18.33% / 95.25% | 33.56% / 89.91% | 52.11% / 80.53% | 66.89% / 69.01% | 85.84% / 42.61% | 94.79% / 19.84% |
| <i>Without T2DM</i> | 2.21% / 99.48% | 10.55% / 97.36% | 19.94% / 94.58% | 35.92% / 88.57% | 54.69% / 78.25% | 69.15% / 65.96% | 87.06% / 39.24% | 95.28% / 17.71% |
| <i>With T2DM</i> | 1.49% / 99.51% | 7.30% / 97.52% | 14.95% / 94.91% | 27.21% / 89.22% | 44.60% / 79.35% | 58.92% / 67.42% | 81.77% / 40.82% | 93.08% / 18.70% |

Table V. Standard-area under the receiver operating curves for LIVERFAST versus FIB-4 according to the histological feature's endpoint (fibrosis) in the overall MASLD population.

| N=394 | Prevalence | LIVERFAST | | FIB-4 | | P value vs. LIVERFAST |
|--|------------|--------------------------|-------------|-------------------------|----------------|-----------------------|
| | | AUC P value | 95% CI | AUC P-value | 95% CI | |
| Cirrhosis (fibrosis stage 4) | | | | | | |
| Overall MASLD | 81 / 394 | 0.870 < 0.001 | 0.816 0.909 | 0.851 < 0.001 | 0.794 0.892 | NS |
| <i>Without T2DM</i> | 27 / 201 | 0.879 < 0.001 | 0.773 0.937 | 0.870 < 0.001 | 0.764 0.931 | NS |
| <i>With T2DM</i> | 54 / 193 | 0.854 < 0.001* | 0.777 0.905 | 0.828 < 0.001 | 0.777 0.905 | NS |
| Advanced fibrosis (fibrosis stages ≥ 3) | | | | | | |
| Overall MASLD | 177 / 394 | 0.874 < 0.001 | 0.836 0.904 | 0.821 < 0.001 | 0.775 0.858 | <0.01 |
| <i>Without T2DM</i> | 72 / 201 | 0.898 < 0.001* | 0.845 0.933 | 0.828 < 0.001 | 0.672 0.829 | <0.05 |
| <i>With T2DM</i> | 105 / 193 | 0.842 < 0.001* | 0.779 0.888 | 0.799 < 0.001 | 0.729 0.853 | NS ^s |

Fibrosis (Fibrosis stages ≥ 2)

| | | | | | | |
|---------------------|-----------|--------------------------|-------------|-------------------------|----------------|----|
| Overall MASLD | 270 / 394 | 0.766 < 0.001 | 0.715 0.809 | 0.753 < 0.001 | 0.700 0.798 | NS |
| <i>Without T2DM</i> | 58 / 94 | 0.738 < 0.001 | 0.662 0.799 | 0.712 < 0.001 | 0.634 0.775 | NS |
| <i>With T2DM</i> | 155 / 193 | 0.750 < 0.001* | 0.664 0.816 | 0.749 < 0.001 | 0.645 0.779 | NS |

AUC Area under receiver operator curve; CI, confidence interval; T2DM, Type 2 diabetes mellitus.

*P value = ns for LIVERFAST versus the group without T2-diabetes

\$ P value between binormal AUROCs <0.01 (AUROCs LIVERFAST 0.852 vs. FIB-4 0.769)

Table VI. Sensitivity, specificity, PPV and NPV for LIVERFASt and FIB-4.

PPV, positive predictive value; NPV, negative predictive value; MASLD, metabolic dysfunction-associated steatotic liver disease; T2DM, type 2 diabetes mellitus.

| | Sensitivity | Specificity | PPV | NPV |
|--|--------------------|--------------------|------------|------------|
| Cirrhosis (fibrosis stage 4) | | | | |
| LIVERFASt | | | | |
| Overall MASLD | 74.07 | 88.18 | 61.86 | 92.93 |
| <i>Non-T2DM</i> | 74.07 | 90.23 | 54.05 | 95.73 |
| <i>T2DM</i> | 74.07 | 86.33 | 67.80 | 89.55 |
| FIB-4 | | | | |
| Overall MASLD | 48.15 | 96.17 | 76.47 | 87.76 |
| <i>Non-T2DM</i> | 55.56 | 95.98 | 68.18 | 92.30 |
| <i>T2DM</i> | 44.44 | 96.40 | 82.76 | 81.71 |
| Advanced fibrosis (fibrosis stages ≥ 3) | | | | |
| LIVERFASt | | | | |
| Overall MASLD | 65.54 | 85.25 | 78.38 | 75.20 |
| <i>Non-T2DM</i> | 63.89 | 89.15 | 76.67 | 81.56 |
| <i>T2DM</i> | 68.57 | 79.55 | 80.00 | 67.96 |
| FIB-4 | | | | |
| Overall MASLD | 37.29 | 94.93 | 85.71 | 64.98 |

| | | | | |
|-----------------|-------|-------|-------|-------|
| <i>Non-T2DM</i> | 38.89 | 94.57 | 80.00 | 73.49 |
| <i>T2DM</i> | 36.19 | 96.59 | 92.68 | 55.92 |

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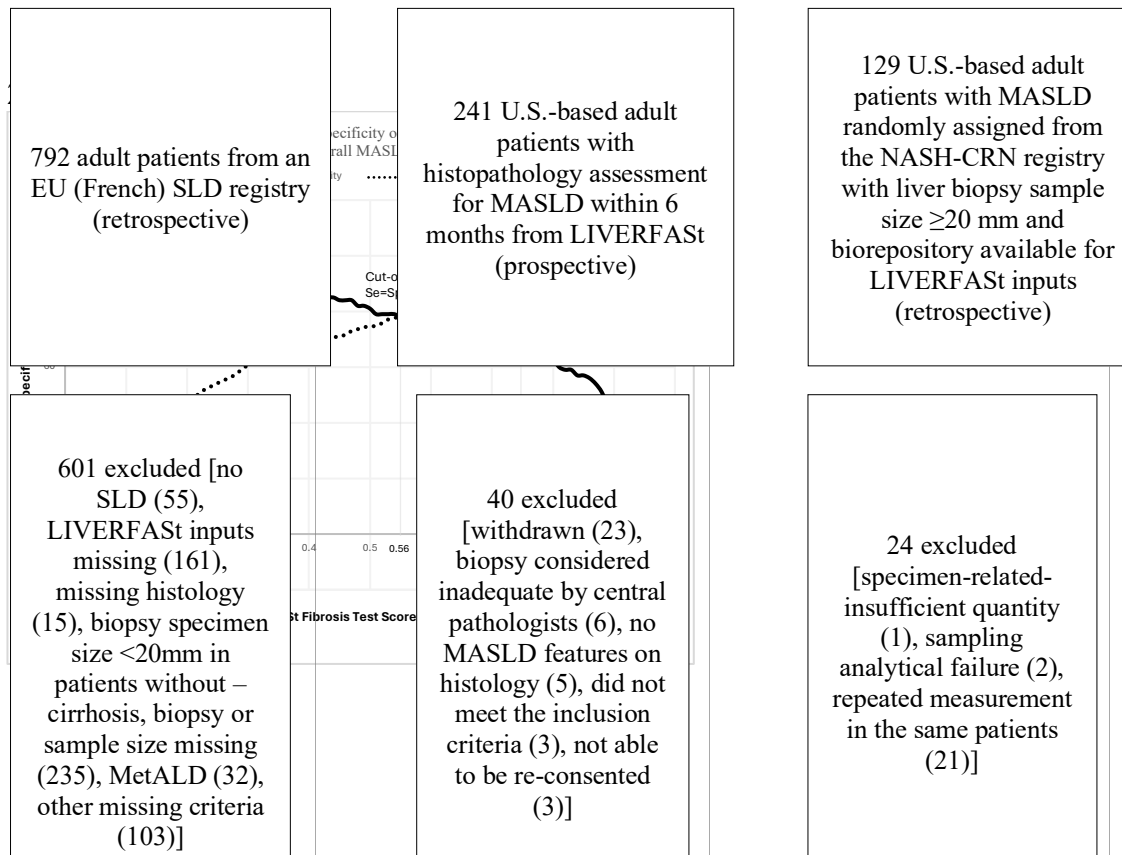
FIGURES

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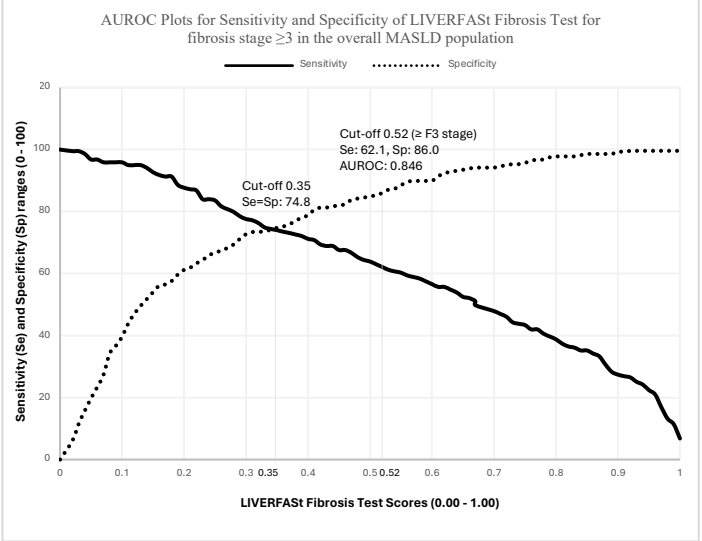
Figure 1. Study population. EU, Europe; SLD, steatotic liver disease; MASLD, metabolic dysfunction-associated steatotic liver disease; T2DM, type 2 diabetes mellitus; DB, database.

Figure 2. Performance of LIVERFASt Fibrosis Test for fibrosis in the overall MASLD pooled population. Sensitivity and specificity of LIVERFASt Fibrosis Test for the intended uses are shown as a function of the cut-off scores. The panels demonstrate changes in sensitivity and specificity at varying LIVERFASt Fibrosis Test cut-off scores for the diagnosis of cirrhosis (fibrosis stage F4) (**2a**), for the diagnosis of advanced fibrosis (stage ≥ 3) (**2b**) and for the diagnosis of clinically significant fibrosis (stage ≥ 2) (**2c**).

1.



2b.



2c.

