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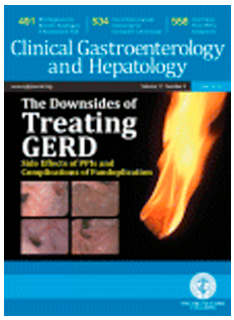

Validation of the blood test MACK-3 for the noninvasive diagnosis of fibrotic NASH: an international study with 1,924 patients

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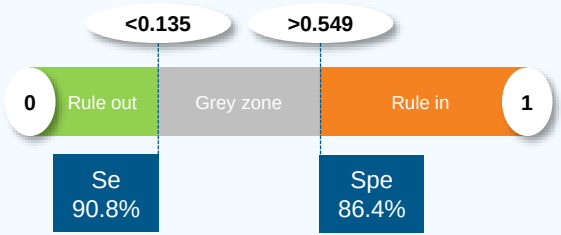
Non-invasive diagnosis of FIBROTIC NASH (NASH + NAS ≥4 + F ≥2)

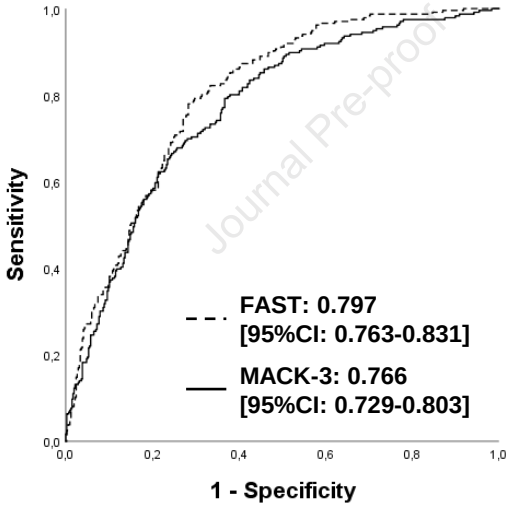
All study population (n=1,924)

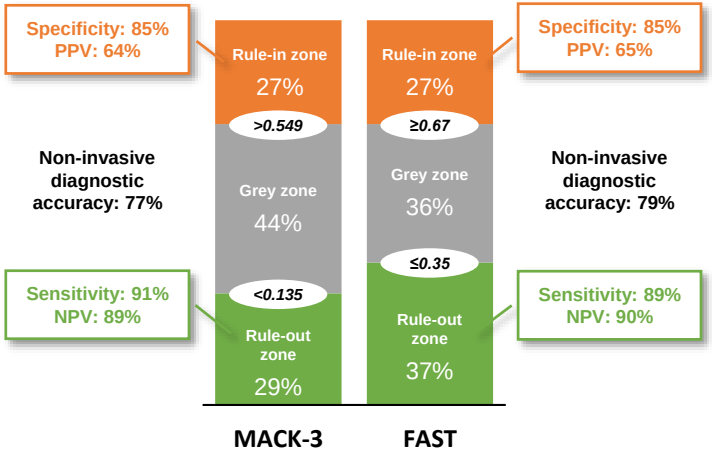
MACK-3: AST, HOMA, CK18

AUROC : 0.791
[95%CI: 0.768-0.814]



MACK-3 versus FAST (n=655)





Clinical Gastroenterology and Hepatology

Validation of the blood test MACK-3 for the noninvasive diagnosis of fibrotic NASH: an international study with 1,924 patients

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K.H. Chuah		X		
C. Costentin		X		X
J. George		X		X
A. Armandi		X		
L. Adams		X		X
N.F. Lange		X		X
O. Blanchet		X		X
V. Moal		X		X
R. Younes		X		
M. Roux	X		X	X
W.K. Chan		X		X
N. Sturm		X		
M. Eslam		X		
E. Bugianesi		X		X
J. Wang		X		
J-F. Dufour		X		X
S. Francque		X		X
H. Yki-Järvinen		X		
K. I. Zheng		X		
J. Boursier	X	X	X	X

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ABBREVIATIONS

AST: aspartate aminotransferase

AUROC: area under the receiver operating characteristic

CK18: cytokeratin 18

FAST: FibroScan-AST score

HOMA: Homeostasis Model Assessment

NAFLD: non-alcoholic fatty liver disease

NAS: NAFLD Activity Score

NASH: non-alcoholic steatohepatitis

NIDA: noninvasive diagnostic accuracy

Rs: Spearman correlation coefficient

ABSTRACT

Background and Aims: Drug development in NASH is hampered by a high screening failure rate that reaches 60-80% in therapeutic trials, mainly because of the absence of fibrotic NASH on baseline liver histology. MACK-3, a blood test including three biomarkers (aspartate aminotransferase, homeostasis model assessment and cytokeratin 18), was recently developed for the noninvasive diagnosis of fibrotic NASH. We aimed to validate the diagnostic accuracy of this noninvasive test in an international multicenter study.

Methods: 1,924 patients with biopsy-proven NAFLD from 10 centers in Asia, Australia, and Europe were included. The blood test MACK-3 was calculated for all patients. FAST, an elastography-based test for fibrotic NASH, was also available in a subset of 655 patients. Fibrotic NASH was defined as the presence of NASH on liver biopsy with NAFLD Activity Score ≥4 and fibrosis stage F≥2 according to the NASH CRN scoring system.

Results: AUROC of MACK-3 for fibrotic NASH was 0.791 [0.768-0.814]. Sensitivity at the previously published MACK-3 threshold of <0.135 was 91% and specificity at its >0.549 threshold was 85%. The MACK-3 AUROC was not affected by age, sex, diabetes, or BMI. MACK-3 and FAST results were well correlated (Rs=0.781, $P < .001$). Except an 8% higher rate of patients included in the grey zone, MACK-3 provided similar accuracy to that of FAST. Both tests included 27% of patients in their rule-in zone, with 85% specificity and 35% false positives (screen failure rate).

Conclusion: The blood test MACK-3 is an accurate tool to improve patient selection in NASH therapeutic trials.

Key words: NAFLD; NASH; fibrotic NASH; blood test; FAST

INTRODUCTION

In parallel with the global obesity pandemic, non-alcoholic fatty liver disease (NAFLD) has become the main cause of chronic liver disease worldwide[1]. Non-alcoholic steatohepatitis (NASH) is the aggressive form of NAFLD, with liver inflammation that promotes the progressive accumulation of fibrosis in the liver parenchyma. NASH has risen to become the leading cause of cirrhosis and hepatocellular carcinoma in some regions[2,3], and the second for liver transplantation in Europe and the United State[4,5]. There is no approved pharmacological treatment for NASH, but extensive research is underway to find new therapeutic agents able to halt progression to cirrhosis and hepatocellular carcinoma and enable the ultimate goal of reducing the worldwide burden of NASH[6]. Experience from phase 2b and 3 therapeutic trials shows a dramatically high rate of screening failure, reaching 60-80%, resulting from biopsy results not meeting the in- and exclusion histological criteria. In this context, noninvasive liver tests represent a very attractive option to better select patients, limit screening failures, and therefore reduce unnecessary, invasive and costly procedures. Therapeutic trials include patients in need of an intervention namely those with "fibrotic NASH," a composite histological criterion combining NASH + NAFLD Activity Score (NAS) ≥4 and fibrosis stage F ≥2. Most of the noninvasive tests currently available in clinical practice are mainly intended for advanced liver fibrosis F≥3 and show limited accuracy for earlier stages of the disease, such as fibrotic NASH[7,8]. In addition to considerably improving patient selection for therapeutic trials, any new test that can better target fibrotic NASH would be a very useful tool for identifying patients who would benefit from NASH therapeutics once they become available in clinical practice.

MACK-3 is such a blood-based test specifically developed for the noninvasive diagnosis of fibrotic NASH[8]. This new test combines three biomarkers, respectively associated with liver inflammation (AST: aspartate aminotransferase), insulin resistance (HOMA: Homeostasis Model Assessment), and apoptosis (CK18: cytokeratin 18). In its development study, MACK-3 showed very good diagnostic accuracy for fibrotic NASH with an AUROC at 0.85[8]. The aim of the study we present here was to validate the MACK-3 in a large international multicenter cohort of biopsy-proven NAFLD patients.

PATIENTS AND METHODS

Patients

The study population was obtained by pooling the data from ten cohorts. The patients from the Angers (France) and Antwerp (Belgium) cohorts for the present work were enrolled after the end of recruitment for the MACK-3 development study. Thus, these two cohorts are independent of the originally published study[8]. The cohorts from Helsinki (Finland), Kuala Lumpur (Malaysia), Wenzhou (China), Turin (Italy), and Sydney (Australia) came from previously published works[9–12]. The last three were local cohorts of patients with biobanked samples from centers in Bern (Switzerland), Grenoble (France), and Perth (Australia). Each cohort received approval from its local ethics committee, and all patients gave written informed consent before their inclusion.

All ten cohorts included adult patients who underwent a liver biopsy for suspected NAFLD, after exclusion of concomitant steatosis-inducing drugs (such as corticosteroids, tamoxifen, amiodarone, or methotrexate), excessive alcohol consumption (>30 g/day in men or >20 g/day in women), chronic hepatitis B or C infection, and the other causes of chronic liver disease. Patients were not included if they had a history of liver-related complications (ascites, variceal bleeding, jaundice, encephalopathy, hepatocellular carcinoma) or biopsy length less than 10 mm. More information on the cohorts of the ten investigating centers is presented in **Supplementary Table s1**.

Histology

Pathological examination of liver biopsies was performed in each center by a senior expert pathologist specialized in hepatology and blinded for patient data. Steatosis, lobular inflammation, ballooning, and fibrosis were semi-quantitatively evaluated using the NASH-CRN scoring system in all centers[13], except in Bern (65 patients) where the SAF score was used[14]. NASH was defined as a grade ≥1 in each component of steatosis, lobular inflammation, and hepatocellular ballooning. The NAS (ranging from 0 to 8) corresponded to the sum of the steatosis, lobular inflammation, and ballooning grades. “Fibrotic NASH,” the primary diagnostic target of the study, was defined as the presence of NASH with NAS ≥4 and F≥2.

Blood tests

Blood markers were measured in the local labs of the investigating centers. The serum levels of CK18 were measured on frozen (-80°C) samples using the M30-Apoptosense enzyme-linked immunosorbent assay kit from PEVIVA (Bromma, Sweden), except in the Wenzhou center where the kit from Suzhou Herui Biomed Technology Co. Ltd (China) was used. HOMA was calculated as follows: fasting glucose

(mmol/L) * fasting insulin (µU/mL) / 22.5. The blood fibrosis test MACK-3 (AST, HOMA, CK18) was calculated as previously published[8]. The thresholds for ruling out and ruling in fibrotic NASH were, respectively, <0.135 and >0.549[8].

Liver elastography

When available, liver elastography (FibroScan™; Echosens, Paris, France) data were collected to calculate the FibroScan-AST score (FAST), an elastography-based score recently developed for the noninvasive diagnosis of fibrotic NASH[15]. FAST is a combination of serum AST with the two FibroScan results: liver stiffness as measured by vibration controlled transient elastography and the controlled attenuation parameter, this latter being a surrogate of liver steatosis. In each center, FibroScan examinations were performed in fasting conditions, by experienced operators blinded for patient data, and according to the manufacturer's recommendations. FAST was calculated as per the previously published formula. The thresholds for ruling out and ruling in fibrotic NASH were ≤0.35 and ≥0.67[15].

Statistical analysis

Continuous variables were expressed as means ± standard deviation and compared using the Mann-Whitney or the Kruskal-Wallis tests. Categorical variables were expressed as percentages and compared using the Chi-squared or Fisher tests. Correlations between quantitative variables were determined using the Spearman correlation coefficient (Rs). The diagnostic accuracies of noninvasive tests were evaluated using the area under the receiver operating characteristic (AUROC), presented with their 95% confidence interval and compared with the Delong test[16]. We also calculated the noninvasive diagnostic accuracy (NIDA), which corresponds to the diagnostic accuracy of a noninvasive test when used with two thresholds (rule-out, rule-in). In this situation, the diagnosis relies on the noninvasive test only when its result is in the rule-out or the rule-in zone. Indeed, when the test result is in the grey zone between the two thresholds, the diagnosis remains undetermined with requirement for further second-line testing. NIDA thus corresponds to the rate of correctly classified patients by the noninvasive test in the pooled rule-out and rule-in zones. NIDA was calculated as follow: ([true negatives in the rule-out zone] + [true positives in the rule-in zone]) / (all patients included in both rule-out and rule-in zones). Statistical analyses were performed using SPSS version 25.0 software (IBM, Armonk, NY, USA).

RESULTS

Patients

The characteristics of the 1,924 patients included in the present study are summarized in **Table 1**. Mean age was 49.2±13.0 years, 55.4% of the patients were male, mean BMI was 32.4±7.5 kg/m^2, and 36.5% had type 2 diabetes mellitus. The mean biopsy length was 21±10 mm and 84.3% of the biopsies were at least 15 mm in length. The prevalence of NASH was 53.8%, significant fibrosis 33.4%, and fibrotic NASH 23.1%. As expected, patients with fibrotic NASH had greater age, more diabetes and more elevated liver tests. Patients with fibrotic NASH had significantly higher levels of AST, HOMA and CK18, thus confirming the relevance of their combination in MACK-3. The characteristics of the patients included in each of the ten investigating centers are summarized in **Supplementary Table s2**.

Accuracy of MACK-3

MACK-3 showed significant correlation with all individual liver lesions (**Supplementary Table s3**) and its results progressively increased as a function of steatosis, lobular inflammation, and ballooning grades, as well as across NAS and fibrosis stages (**Supplementary Figures s1 to s5**). The AUROC of MACK-3 for the diagnosis of fibrotic NASH was 0.791 [0.768-0.814] (**Figure 1**), and similar across the 10 investigating centers (**Table 2**). The <0.135 rule-out threshold of MACK-3 provided an excellent 90.8% sensitivity for fibrotic NASH, and the specificity was 86.4% at the >0.549 rule-in threshold. As expected, the negative and positive predictive values of MACK-3 were influenced by the prevalence of the diagnostic target: the increasing prevalence of fibrotic NASH observed across the 10 investigating centers was associated with a linear increase in the positive predictive value, while the negative predictive value decreased only slightly (**Supplementary Figure s6**). Importantly, 86% of the misclassified patients from the rule-out zone showed only borderline misclassification (i.e., only one NAS point or only one fibrosis stage discrepancy, **Supplementary Table s4**). In the rule-in zone, misclassification was borderline in 67% of the cases.

Sensitivity analysis

Sensitivity analysis showed that MACK-3 AUROC was not influenced by age, sex, diabetes, or the biopsy length (**Table 3**). Accuracy was higher in patients with BMI ≥35 kg/m^2, but half of these patients (48.6%) came from the bariatric surgery setting, which corresponds to a particular population with only 3.6% prevalence of fibrotic NASH despite the highly elevated BMI risk factor. The AUROC of MACK-3 was 0.937 [0.884-0.990] in patients with BMI ≥35 kg/m^2 coming from bariatric surgery, whereas it was 0.793 [0.744-0.842] in patients with BMI ≥35 kg/m^2 outside bariatric surgery. Thus, the results in this

latter group of patients were similar to those of patients with BMI <30 kg/m^2 or those with BMI 30-35 kg/m^2 (**Table 3**). Accuracy of MACK-3 in the non-bariatric group (n=1,589 patients) is presented in **Table 2** (see last line of the Table).

Comparison between MACK-3 and FAST

FAST score was available in 655 patients whose characteristics are summarized in **Supplementary Table s5**. MACK-3 and FAST were well correlated, with a Spearman correlation coefficient at 0.781 (*P* < .001, **Figure 2**). The AUROC of MACK3 (0.766 [0.729-0.803]) and FAST (0.797 [0.763-0.831]) were close, but significantly different (*P* = .033; **Figure 3**). This translated in less patients included in the grey zone with FAST as compared to MACK-3 (36% vs 44%, *P* < .001; **Figure 4**). Within the rule-out zone, accuracy of MACK-3 and FAST were similar with sensitivity and negative predictive value around 90%. In terms of patient selection for clinical trials, MACK-3 and FAST both included 27% of the patients in their rule-in zone. Within the rule-in zone, the two tests showed the same accuracy with 85% specificity and 65% positive predictive value (35% screen failure rate). Finally, NIDA was similar between MACK-3 (77%) and FAST (79%). **Supplementary Figure s7** shows there were very few discrepant results between MACK-3 and FAST, i.e., patients with FAST ≤0.35 and MACK-3 >0.549 or patients with FAST ≥0.67 and MACK-3 <0.135. Interestingly, the positive predictive value increased to 73% when both FAST and MACK-3 agreed for fibrotic NASH.

DISCUSSION

Therapeutics in the setting of NASH is a highly active field of research with numerous ongoing clinical trials. All these efforts are however hampered by a dramatically high screening failure rate, reaching 60-80% in some instances. Therefore, there is an urgent need to develop new tools able to accurately target fibrotic NASH, the histological criterion that usually defines the indication for patient inclusion in NASH therapeutic trials. Because they have the potential to be accessible to all physicians, blood tests appear as an attractive option to facilitate the identification and referral to liver clinics of candidates for therapeutic trials. In this context, our study validates the good accuracy of the blood test MACK-3 for the noninvasive diagnosis of fibrotic NASH, and the relevance of its rule-out and rule-in thresholds (<0.135 and >0.549) as determined in the development study[8].

A range of currently available noninvasive tests can accurately diagnose advanced liver fibrosis, but they perform less well for earlier stages of the disease. Therefore, several novel noninvasive tests have been recently developed specifically to diagnose fibrotic NASH. These include blood tests like MACK-3 and NIS4, and elastography-based tests such as FAST and MAST. FAST and MAST have shown promising diagnostic accuracy[15,17], but it remains that the need for specific elastography devices limits their availability, which is especially tangible with MAST that requires magnetic resonance elastography technology. On the other hand, blood-based tests offer an opportunity for more generalized use, which is a crucial advantage given the very large number of at-risk patients to evaluate. NIS4 (combination of HbA1c, alpha-2 macroglobulin, YKL-40, and the micro-RNA miR-34a-5p) shows good accuracy for fibrotic NASH with an AUROC of 0.80 [0.77-0.84][18]. However, the deployment of this test is limited by the micro-RNA assay that requires sophisticated and expensive technology with currently limited availability. In this context, MACK-3 is a very attractive option for the first-line evaluation of fibrotic NASH in at-risk patients with metabolic factors. Indeed, the accuracy of MACK-3 is comparable to that of NIS4, with an AUROC of 0.791 [0.768-0.814] in our validation study. Moreover, MACK-3 combines simple biomarkers that are already available (AST and HOMA index) or easily implementable in biological platforms (CK18). These advantages should enable the widespread use of MACK-3 in clinical practice.

Fibrotic NASH is difficult to diagnose because it results from the combination of four different liver lesions (steatosis, lobular inflammation, ballooning, and fibrosis). We therefore evaluated how MACK-3 behaved according to each liver lesion. Our results show that MACK-3 is a sensitive test: it increased significantly from the first grade of steatosis, lobular inflammation, and ballooning, and from the first

stage of liver fibrosis. Except an 8% higher rate of patients included in the grey zone, MACK-3 provided similar accuracy to that of FAST (**Figure 4**). Importantly, when considering patient selection for therapeutic trials, it is noteworthy that MACK-3 included the same rate of patients as FAST in its rule-in zone (27%), and that the accuracy within this zone was similar between the two tests with only 35% false positives. FibroScan device and FAST score are now widely available in liver clinics, and MACK-3 has the potential to be implemented in biological platforms. Therefore, these tests provide physicians with two different solutions they can use according to their local resources with comparable accuracy for fibrotic NASH.

A limitation of the study was the lack of a central reading of liver biopsies, with histological diagnosis of fibrotic NASH relying on the local expert reading at each of the 10 investigating centers. It should however emphasized that previous works have shown that the reliability of histological diagnosis remains suboptimal even with a central reading[19]. Knowing these limitations, our study is nevertheless of interest in demonstrating that MACK-3 and FAST target groups of patients enriched in fibrotic NASH, which is very relevant in the current context of high screening failure rates observed in NASH therapeutic trials. We also acknowledge that CK18 is currently not widely available, but such biomarker can be implemented in biology platforms to overcome this limitation and allow wide dissemination of MACK-3 in clinical practice. Finally, as our study was designed to validate the MACK-3 in the same context it was initially developed, further studies are now required to validate its accuracy in less specialized settings where it is assumed to be used in clinical practice.

In conclusion, MACK-3 is an accurate blood test for the noninvasive diagnosis of fibrotic NASH. Currently, it represents an attractive option to improve patient selection in ongoing NASH therapeutic trials. In the near future, when drugs for NASH have been approved for clinical practice, MACK-3 could help physicians identify patients in need of pharmacological therapy for their liver disease.

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Table 1: Patient characteristics

	All (n=1924)	No fibrotic NASH (n=1480)	Fibrotic NASH (n=444)	*P*
Age (years)	49.2 ± 13.0	48.0 ± 12.6	53.3 ± 13.5	< .001
Male sex (%)	1065 (55.4)	831 (56.1)	234 (52.7)	.211
BMI (kg/m^2)	32.4 ± 7.5	32.4 ± 7.8	32.4 ± 6.2	.095
Diabetes (%)	703 (36.5)	465 (31.4)	238 (53.6)	< .001
Bariatric patients (%)	335 (17.4)	323 (21.8)	12 (2.7)	< .001
Steatosis grade (%):				
- 0	234 (12.2)	234 (15.8)	0 (0.0)	< .001
- 1	717 (37.3)	629 (42.5)	88 (19.8)	
- 2	603 (31.1)	412 (27.8)	191 (43.0)	
- 3	370 (19.2)	205 (13.9)	165 (37.2)	
Ballooning (%):				< .001
- 0	661 (35.6)	661 (45.4)	0 (0.0)	
- 1	788 (42.4)	625 (43.0)	163 (40.3)	
- 2	410 (22.1)	169 (11.6)	241 (59.7)	
Lobular inflammation (%)				< .001
- 0	602 (32.4)	602 (41.4)	0 (0.0)	
- 1	958 (51.5)	712 (48.9)	246 (60.9)	
- 2	288 (15.5)	138 (9.5)	150 (37.1)	
- 3	11 (0.6)	3 (0.2)	8 (2.0)	
NAFLD Activity Score (NAS)	3.3 ± 1.9	2.7 ± 1.7	5.2 ± 1.0	< .001
NASH (%)	1001 (53.8)	597 (41.0)	404 (100.0)	< .001
Fibrosis stage (%):				< .001
- 0	678 (35.2)	678 (45.8)	0 (0.0)	
- 1	604 (31.4)	604 (40.8)	0 (0.0)	
- 2	287 (14.9)	90 (6.1)	197 (44.4)	
- 3	241 (12.5)	59 (4.0)	182 (41.0)	
- 4	114 (5.9)	49 (3.3)	65 (14.6)	
AST (IU/L)	44 ± 35	39 ± 31	61 ± 42	< .001
ALT (IU/L)	66 ± 58	60 ± 54	87 ± 67	< .001
Gamma-GT (IU/L)	93 ± 130	79 ± 117	142 ± 158	< .001
Alkaline phosphatase (IU/L)	84 ± 35	82 ± 35	93 ± 35	< .001
Albumin (g/L)	43.2 ± 4.7	43.2 ± 4.8	43.2 ± 4.5	.950
Platelets (G/L)	244 ± 67	248 ± 67	229 ± 66	< .001
HOMA	6.7 ± 13.2	5.7 ± 12.5	10.1 ± 15.0	< .001
CK18 (IU/L)	346 ± 449	274 ± 319	587 ± 678	< .001
FIB4	1.28 ± 1.03	1.14 ± 0.92	1.75 ± 1.21	< .001

Table 2: Diagnostic accuracy of MACK-3 across the ten investigating centers

Center	Patients	Fibrotic	AUROC	Rule-out zone (MACK-3 <0.135)					Rule-in zone (MACK-3 >0.549)					Grey zone	NIDA
	(n)	NASH (%)	(95% CI)	Patients (%) [a]	Se (%)	Spe (%)	-LR	+LR	Patients (%) [a]	Se (%)	Spe (%)	-LR	+LR	Patients (%) [a]	(%)
HELSINKI	356	5.9	0.926 [0.887-0.966]	61.0	100.0	64.8	0.00	2.84	7.3	47.6	95.2	0.55	9.97	31.7	93.4
WENZHOU	440	13.9	0.721 [0.653-0.790]	49.3	77.0	53.6	0.43	1.66	20.0	42.6	83.6	0.69	2.61	30.7	75.1
SYDNEY	128	16.4	0.826 [0.749-0.902]	31.3	100.0	37.4	0.00	1.60	24.2	57.1	82.2	0.52	3.22	44.5	73.2
KUALA LUMPUR	193	21.8	0.801 [0.734-0.868]	16.6	100.0	21.2	0.00	1.27	37.3	73.8	72.8	0.36	2.72	46.1	60.6
ANTWERP	211	23.2	0.836 [0.777-0.895]	35.1	93.9	43.8	0.14	1.67	19.9	49.0	88.9	0.57	4.41	45.0	81.9
TORINO	101	25.7	0.741 [0.631-0.851]	48.5	80.8	58.7	0.33	1.95	15.8	26.9	88.0	0.83	2.24	35.6	78.5
PERTH	65	27.7	0.801 [0.690-0.913]	29.2	100.0	40.4	0.00	1.68	33.8	55.6	74.5	0.60	2.18	36.9	70.7
ANGERS	236	42.4	0.798 [0.742-0.855]	23.3	95.0	36.8	0.14	1.50	29.2	50.0	86.0	0.58	3.58	47.5	80.6
GRENOBLE	129	51.2	0.763 [0.682-0.845]	38.0	80.3	57.1	0.34	1.87	16.3	30.3	98.4	0.71	19.09	45.7	80.0
BERN	65	61.5	0.781 [0.665-0.897]	9.2	97.5	20.0	0.13	1.22	41.5	57.5	84.0	0.51	3.59	49.2	84.8
ALL	1,924	23.1	0.791 [0.768-0.814]	39.4	90.8	48.4	0.19	1.76	21.5	48.0	86.4	0.60	3.53	39.1	79.4
ALL (no bariatric) [b]	1,589	27.2	0.761 [0.735-0.786]	33.5	90.5	42.4	0.22	1.57	25.2	47.9	83.3	0.63	2.87	41.3	74.9

NIDA: noninvasive diagnostic effectiveness (rate of correct classification among the patients noninvasively diagnosed, i.e., those included in pooled rule-out and rule-in zones);
Se: sensitivity (%); Spe: specificity (%); -LR: negative likelihood ratio; +LR: positive likelihood ratio

[a] Rate of patients (%) included in the test interval

[b] After exclusion of the 335 patients coming from bariatric surgery

Table 3: Accuracy of MACK-3 for fibrotic NASH, sensitivity analysis

		Fibrotic NASH (%)	AUROC (95% CI)	Sensitivity (%) [a]	Specificity (%) [b]
Sex	Female	24.4	0.810 [0.778-0.843]	90.0	88.1
	Male	22.0	0.777 [0.745-0.809]	91.5	85.1
Age (years)	<40	17.1	0.815 [0.768-0.863]	94.9	80.4
	40–59	19.5	0.787 [0.752-0.823]	85.9	89.1
	≥60	37.5	0.788 [0.746-0.830]	94.6	86.7
Bariatric	No	27.2	0.761 [0.735-0.786]	90.5	83.3
	Yes	3.6	0.938 [0.887-0.989]	100.0	97.5
BMI (kg/m^2)	<30	18.8	0.758 [0.718-0.798]	86.4	86.9
	30–35	34.7	0.716 [0.666-0.765]	89.9	77.9
	>35	20.8	0.880 [0.850-0.910]	98.4	90.4
Diabetes	No	16.9	0.775 [0.742-0.809]	86.4	88.4
	Yes	33.9	0.781 [0.746-0.816]	94.5	82.2
Biopsy length (mm)	<20	18.7	0.783 [0.744-0.822]	91.3	81.9
	≥20	38.6	0.755 [0.717-0.793]	89.8	85.2

[a] Sensitivity at the <0.135 threshold of MACK-3
[b] Specificity at the >0.549 threshold of MACK-3

FIGURE LEGENDS

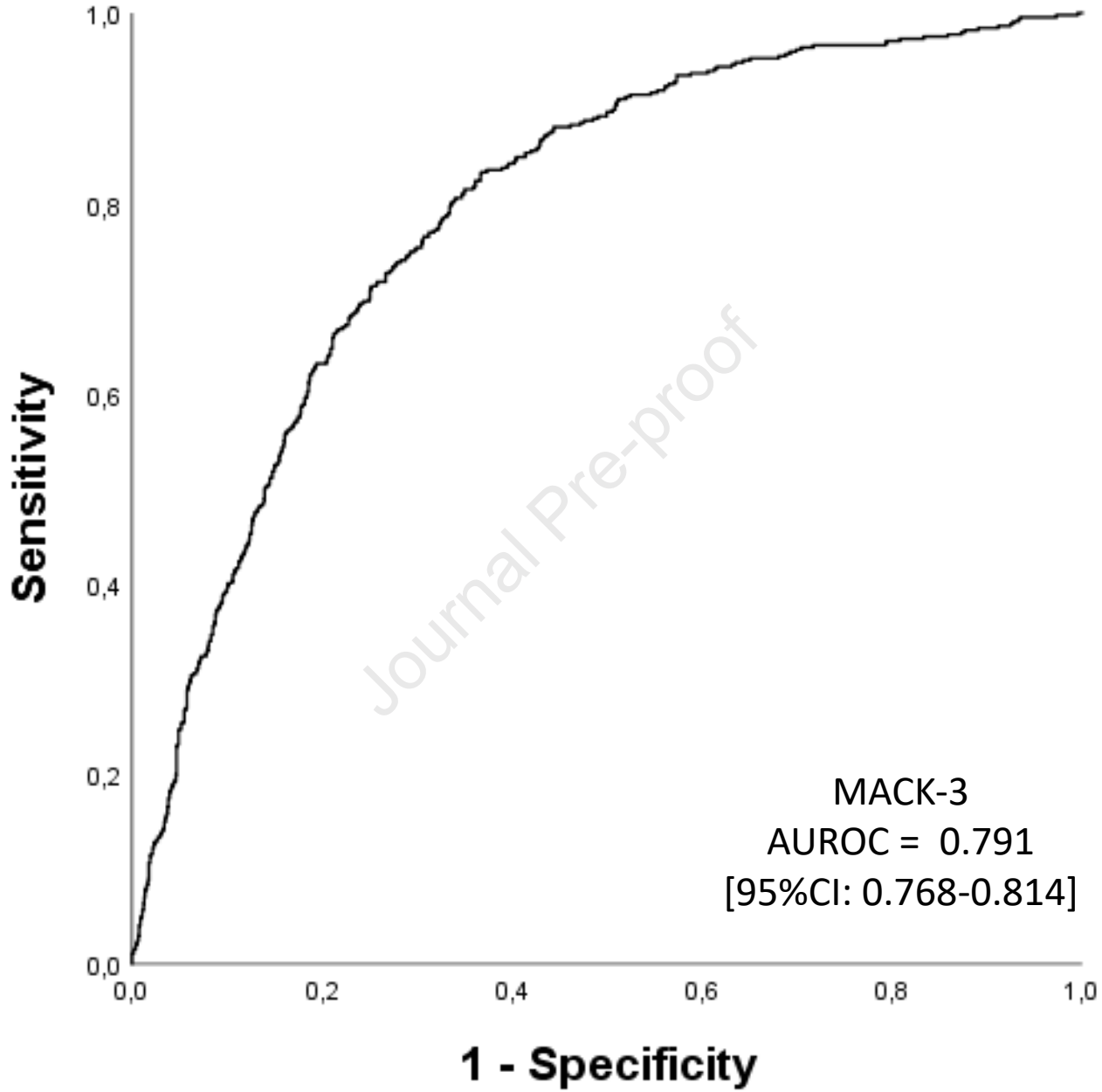
Figure 1: AUROCs of MACK-3 for the diagnosis of fibrotic NASH in the whole study set

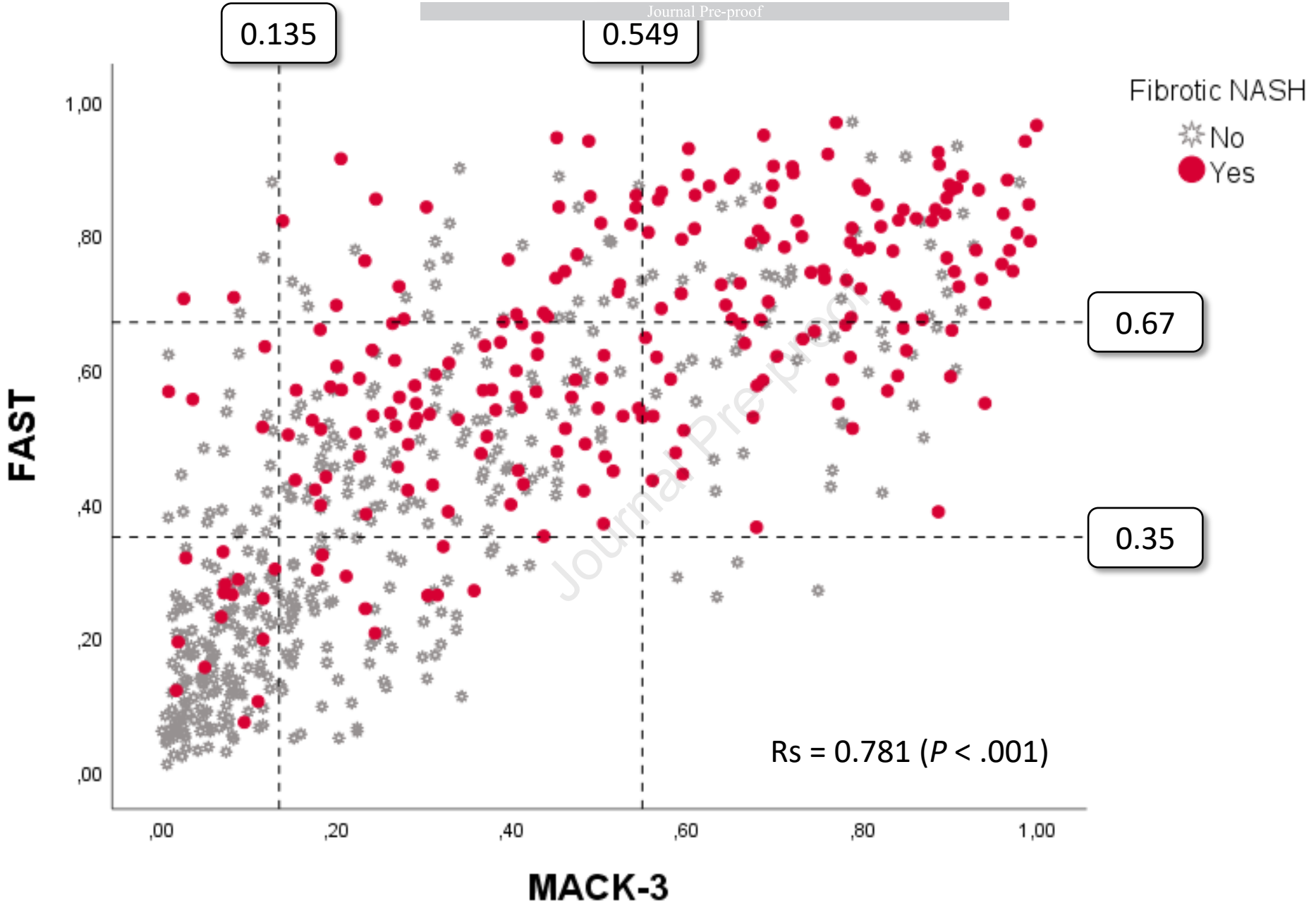
Figure 2: Correlation between MACK-3 and FAST in the 655 patients having both tests available.

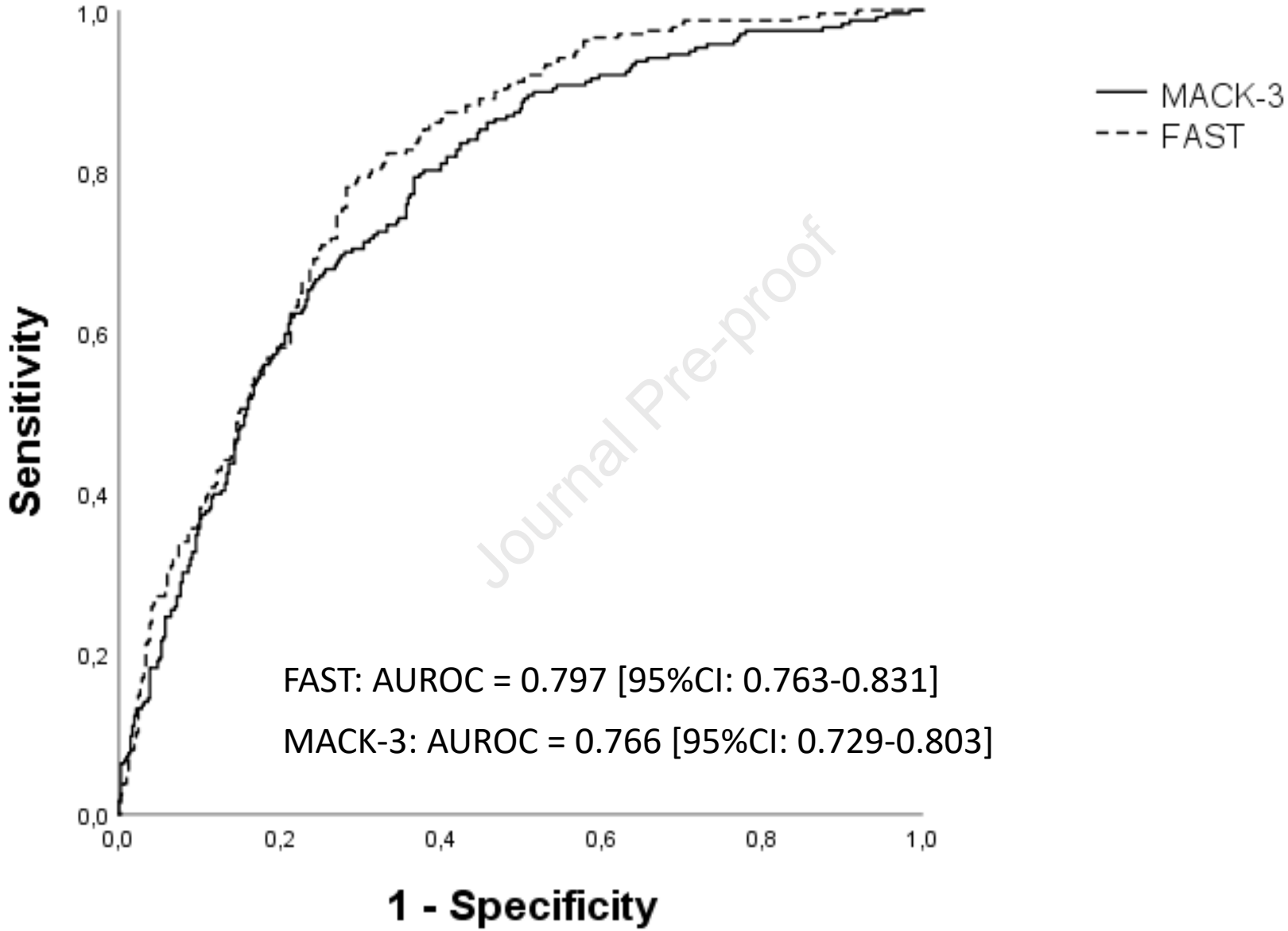
Figure 3: AUROC of MACK-3 and FAST for the diagnosis of fibrotic NASH in the 655 patients having both tests available.

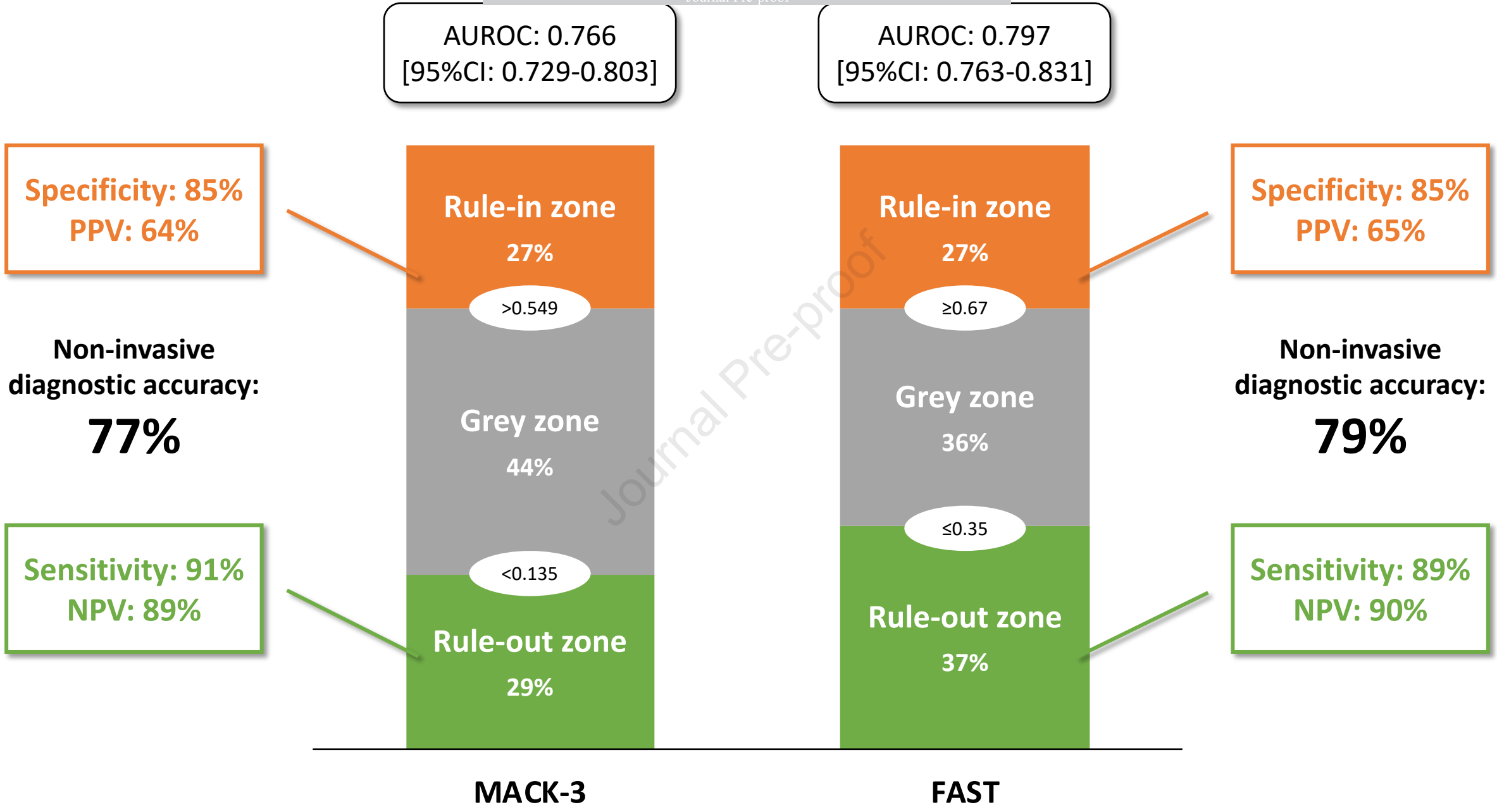
Figure 4: Accuracy of MACK-3 and FAST for the noninvasive diagnosis of fibrotic NASH in the 655 patients having both tests available.

PPV: positive predictive value; NPV: negative predictive value









Validation of the blood test MACK-3 for the noninvasive diagnosis of fibrotic NASH: an international study with 1,924 patients

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Supplementary Material

Supplementary Table s1: Study cohorts

	Angers	Antwerp	Bern	Helsinki	Grenoble	Kuala Lumpur	Perth	Sydney	Torino	Wenzhou
Enrolment dates (first and last inclusion)	From May 2016 to July 2020	From September 2012 to December 2020	From September 2017 to September 2019	From April 2007 to March 2020	From November 2014 to February 2019	From November 2012 to May 2015	From February 2010 to January 2020	From January 1999 to March 2011	From September 2008 to October 2018	From December 2016 to December 2018
Study design	Prospective cross-sectional single-center	Prospective cross-sectional single-center	Retrospective cross-sectional single-center	Prospective cross-sectional single-center	Prospective cross-sectional single-center	Prospective cross-sectional single-center	Prospective cross-sectional single-center	Prospective cross-sectional single-center	Prospective cross-sectional single-center	Prospective cross-sectional single-center
Center description	Hepatology tertiary care	Hepatology tertiary care	Hepatology tertiary care	Obesity clinic tertiary care	Hepatology tertiary care	Hepatology tertiary care	Hepatology tertiary care	Hepatology tertiary care	Hepatology tertiary care	Hepatology tertiary care
Eligibility criteria	Liver biopsy scheduled for the evaluation of NAFLD	Liver biopsy scheduled for the evaluation of NAFLD	Liver biopsy scheduled for the evaluation of NAFLD	Patients with suspected NAFLD undergoing weight-loss surgery	Liver biopsy scheduled for the evaluation of NAFLD	NAFLD patients screened and enrolled for a clinical trial	Liver biopsy scheduled for the evaluation of NAFLD	Liver biopsy scheduled for the evaluation of NAFLD	Liver biopsy scheduled for the evaluation of NAFLD	Liver biopsy scheduled for the evaluation of NAFLD
Reason to send a patient for a liver biopsy	Abnormal liver function tests, hyperferritinemia, metabolic syndrome, abnormal non-invasive tests of liver fibrosis	Abnormal liver tests, hyperferritinemia, metabolic syndrome, abnormal non-invasive tests of liver fibrosis	Abnormal liver function tests, hyperferritinemia, metabolic syndrome, abnormal non-invasive tests of liver fibrosis	Routine liver biopsy during weight-loss surgery to exclude advanced liver disease in patients with obesity and metabolic risk factors	Abnormal liver function tests, hyperferritinemia, metabolic syndrome, abnormal non-invasive tests of liver fibrosis	Abnormal liver function tests, metabolic syndrome, abnormal non-invasive tests of liver fibrosis	Abnormal liver function tests, hyperferritinemia, abnormal non-invasive tests of liver fibrosis	Abnormal liver function tests, hyperferritinemia, abnormal non-invasive tests of liver fibrosis	Abnormal liver function tests, hyperferritinemia, metabolic syndrome, abnormal non-invasive tests of liver fibrosis	CAP, US, CT or MRI imaging showing fatty liver disease; and/or abnormal ALT but below 5 ULN; no alcohol drinking history or daily alcohol intake < 20 g for male and 10 g for female
Liver biopsy reading	Reading by expert pathologist	Reading by expert pathologist	Reading by expert pathologist	Reading by expert pathologist	Reading by expert pathologist	Reading by expert pathologist	Reading by expert pathologist	Reading by expert pathologist	Reading by expert pathologist	Reading by expert pathologist

Supplementary Table s2: Patient characteristics in each of the ten investigating centers

	Angers	Antwerp	Bern	Helsinki	Grenoble	Kuala Lumpur	Perth	Sydney	Torino	Wenzhou
Patients (n)	236	211	65	356	129	193	65	128	101	440
Age (years)	57.5±11.9	49.6±14.3	55.4±14.3	50.7±9.5	54.5±11.3	49.9±11.3	54.0±13.1	48.4±12.2	44.4±12.9	41.3±11.8
Male sex (%)	146 (61.9)	97 (46.0)	43 (66.2)	100 (28.1)	77 (59.7)	96 (49.7)	26 (40.0)	79 (61.7)	73 (72.3)	328 (74.5)
BMI (kg/m^2)	33.9±6.8	34.9±7.4	31.3±5.0	40.4±7.2	31.1±5.2	29.9±4.5	35.8±6.8	30.7±5.1	28.1±4.0	26.6±3.3
Diabetes (%)	111 (47.0)	58 (27.5)	27 (41.5)	155 (43.5)	57 (44.2)	90 (46.6)	35 (53.8)	31 (24.2)	17 (16.8)	122 (27.7)
Bariatric patients (%)	6 (2.5)	25 (11.8)	0 (0.0)	304 (85.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Steatosis grade (%):										
- 0	17 (7.2)	26 (12.3)	2 (3.1)	135 (37.9)	2 (1.6)	3 (1.6)	1 (1.5)	3 (2.3)	1 (1.0)	44 (10.0)
- 1	106 (44.9)	86 (40.8)	19 (29.2)	139 (39.0)	29 (22.5)	46 (23.8)	18 (27.7)	65 (50.8)	50 (49.5)	159 (36.1)
- 2	61 (25.8)	71 (33.6)	19 (29.2)	52 (14.6)	41 (31.8)	100 (51.8)	30 (46.2)	43 (33.6)	31 (30.7)	155 (35.2)
- 3	52 (22.0)	28 (13.3)	25 (38.5)	30 (8.4)	57 (44.2)	44 (22.8)	16 (24.6)	17 (13.3)	19 (18.8)	82 (18.6)
Ballooning (%):										
- 0	63 (26.7)	18 (8.5)	-	305 (85.7)	19 (14.7)	59 (30.6)	20 (30.8)	70 (54.7)	20 (19.8)	87 (19.8)
- 1	98 (41.5)	127 (60.2)	-	35 (9.8)	64 (49.6)	88 (45.6)	31 (47.7)	39 (30.5)	44 (43.6)	262 (59.5)
- 2	75 (31.8)	66 (31.3)	-	16 (4.5)	46 (35.7)	46 (23.8)	14 (21.5)	19 (14.8)	37 (36.6)	91 (20.7)
Lobular inflammation (%)										
- 0	48 (20.3)	67 (31.8)	-	299 (84.0)	40 (31.0)	3 (1.6)	18 (27.7)	45 (35.2)	24 (23.8)	58 (13.2)
- 1	161 (68.2)	126 (59.7)	-	47 (13.2)	70 (54.3)	105 (54.4)	37 (56.9)	70 (54.7)	68 (67.3)	274 (62.3)
- 2	27 (11.4)	18 (8.5)	-	10 (2.8)	18 (14.0)	82 (42.5)	10 (15.4)	13 (10.2)	9 (8.9)	101 (23.0)
- 3	0 (0.0)	0 (0.0)	-	0 (0.0)	1 (0.8)	3 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	7 (1.6)
NAFLD Activity Score (NAS)	3.6±1.7	3.5±1.6	2.0±1.0 [a]	1.3±1.6	4.2±1.8	4.3±1.4	3.7±1.5	2.9±1.6	3.7±1.3	3.8±1.6
NASH (%)	155 (65.7)	135 (64.0)	54 (83.1)	46 (12.9)	85 (65.9)	132 (68.4)	37 (56.9)	52 (40.6)	61 (60.4)	298 (67.7)
Fibrosis stage (%):										
- 0	39 (16.5)	75 (35.5)	5 (7.7)	199 (55.9)	17 (13.2)	66 (34.2)	16 (24.6)	37 (28.9)	34 (33.7)	190 (43.2)
- 1	47 (19.9)	61 (28.9)	15 (23.1)	116 (32.6)	23 (17.8)	81 (42.0)	20 (30.8)	52 (40.6)	17 (16.8)	172 (39.1)
- 2	68 (28.8)	25 (11.8)	19 (29.2)	18 (5.1)	43 (33.3)	15 (7.8)	4 (6.2)	17 (13.3)	18 (17.8)	60 (13.6)
- 3	62 (26.3)	28 (13.3)	23 (35.4)	14 (3.9)	23 (17.8)	25 (13.0)	14 (21.5)	12 (9.4)	25 (24.8)	15 (3.4)
- 4	20 (8.5)	22 (10.4)	3 (4.6)	9 (2.5)	23 (17.8)	6 (3.1)	11 (16.9)	10 (7.8)	7 (6.9)	3 (0.7)
Fibrotic NASH (%)	100 (42.4)	49 (23.2)	40 (61.5)	21 (5.9)	66 (51.2)	42 (21.8)	18 (27.7)	21 (16.4)	26 (25.7)	61 (13.9)
AST (IU/L)	45±39	38±29	63±42	33±14	42±22	48±26	54±37	49±24	44±32	50±50
ALT (IU/L)	62±46	58±48	82±84	39±25	71±35	79±45	80±76	74±41	72±44	79±84
Gamma-GT (IU/L)	118±175	80±117	162±197	54±100	151±190	96±80	134±184	110±102	104±117	77±98
Alkaline phosphatase (IU/L)	82±29	89±36	86±28	70±31	90±35	85±29	93±36	100±36	83±38	88±38
Albumin (g/L)	43.3±3.5	43.0±5.5	38.5±3.8	38.6±3.7	45.2±4.1	43.1±3.4	41.8±3.5	44.6±3.4	45.2±4.1	46.2±3.6

Platelets (G/L)	222±65	245±76	224±62	252±62	223±64	275±67	222±66	253±68	231±74	246±61
HOMA	9.8±19.7	10.6±23.2	8.8±16.5	3.9±3.2	5.6±10.1	7.8±7.2	11.8±26.5	5.5±5.7	3.5±2.7	5.4±7.8
CK18 (IU/L)	386±472	410±447	364±277	218±162	424±466	516±461	503±985	313±348	345±484	285±461
FIB4	1.67±1.04	1.28±1.34	2.11±1.75	1.23±0.82	1.40±0.94	1.07±0.62	1.89±1.74	1.28±0.85	1.15±0.96	1.02±0.79

[a] According to the SAF scoring system

Supplementary Table s3: Correlation of MACK-3 results with individual liver lesions

	MACK-3	
	Rs	***P***
Steatosis grade	0.469	< .001
Lobular inflammation grade	0.434	< .001
Ballooning grade	0.417	< .001
NAFLD Activity Score (NAS)	0.566	< .001
Fibrosis stage	0.479	< .001

Rs: Spearman correlation coefficient

Supplementary Table s4: Contingency tables for patients ruled out (<0.135), ruled in (>0.549), or in the grey zone (0.135 – 0.549) with MACK-3

Green: well classified for fibrotic NASH

Yellow and red: misclassified for fibrotic NASH

Among the misclassified, yellow represents borderline misclassification with a discrepancy of only one NAS point or one fibrosis stage

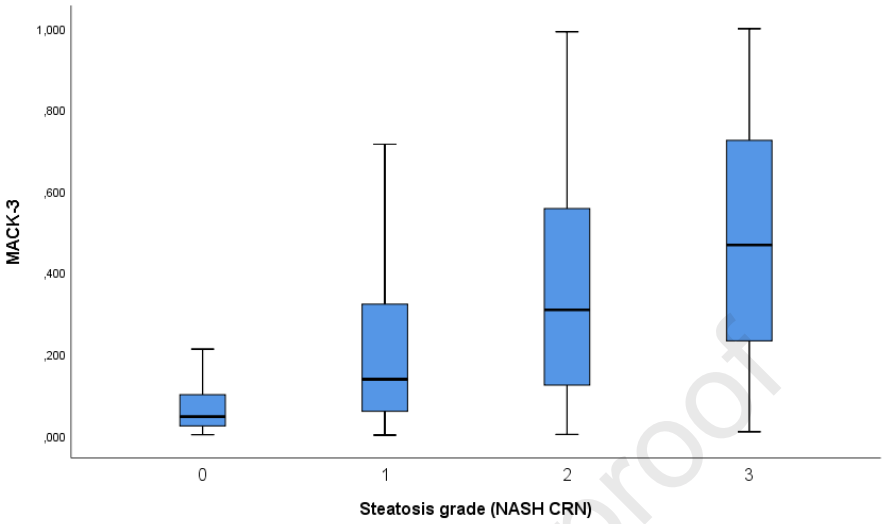
MACK-3	Fibrosis stage	NAS								
		0	1	2	3	4	5	6	7	8
<0.135	0	13.7% 103	14.0% 105	9.0% 68	12.9% 97	4.7% 35	2.1% 16	0.3% 2	0.1% 1	0.0% 0
	1	4.5% 34	5.2% 39	4.7% 35	7.3% 55	5.9% 44	2.3% 17	0.7% 5	0.0% 0	0.0% 0
	2	0.3% 2	0.3% 2	1.5% 11	1.5% 11	2.5% 19	1.5% 11	0.1% 1	0.3% 2	0.1% 1
	3	0.1% 1	0.3% 2	0.4% 3	0.8% 6	0.9% 7	0.5% 4	0.3% 2	0.0% 0	0.0% 0
	4	0.1% 1	0.5% 4	0.3% 2	0.3% 2	0.1% 1	0.1% 1	0.0% 0	0.0% 0	0.0% 0
0.135–0.549	0	2.1% 15	5.0% 36	4.9% 35	6.8% 49	6.0% 43	3.3% 24	0.8% 6	0.1% 1	0.0% 0
	1	0.4% 3	2.6% 19	3.9% 28	7.1% 51	9.6% 69	6.9% 50	2.6% 19	0.4% 3	0.0% 0
	2	0.0% 0	1.1% 8	1.1% 8	3.2% 23	4.3% 31	5.0% 36	2.6% 19	0.8% 6	0.0% 0
	3	0.3% 2	0.3% 2	1.3% 9	2.1% 15	3.8% 27	2.8% 20	1.4% 10	0.4% 3	0.0% 0
	4	0.4% 3	1.1% 8	0.3% 2	1.4% 10	1.1% 8	1.5% 11	1.0% 7	0.1% 1	0.0% 0
>0.549	0	0.0% 0	1.0% 4	0.0% 0	1.8% 7	3.4% 13	2.1% 8	1.0% 4	0.3% 1	0.0% 0
	1	0.0% 0	1.0% 4	2.3% 9	3.1% 12	5.9% 23	8.8% 34	7.0% 27	2.1% 8	0.3% 1
	2	0.0% 0	0.0% 0	0.5% 2	1.6% 6	2.3% 9	7.8% 30	5.9% 23	1.6% 6	0.3% 1
	3	0.0% 0	0.3% 1	0.3% 1	2.6% 10	4.9% 19	6.5% 25	8.8% 34	3.6% 14	0.3% 1
	4	0.0% 0	0.8% 3	1.3% 5	1.0% 4	3.9% 15	1.3% 5	4.4% 17	0.3% 1	0.0% 0

Supplementary Table s5: Characteristics of the patients for whom FAST score was available

Patients with FAST score available came from the following centers: Angers (n=220), Antwerp (n=107), Berne (n=61), Grenoble (n=80), Perth (n=24), and Wenzhou (n=163).

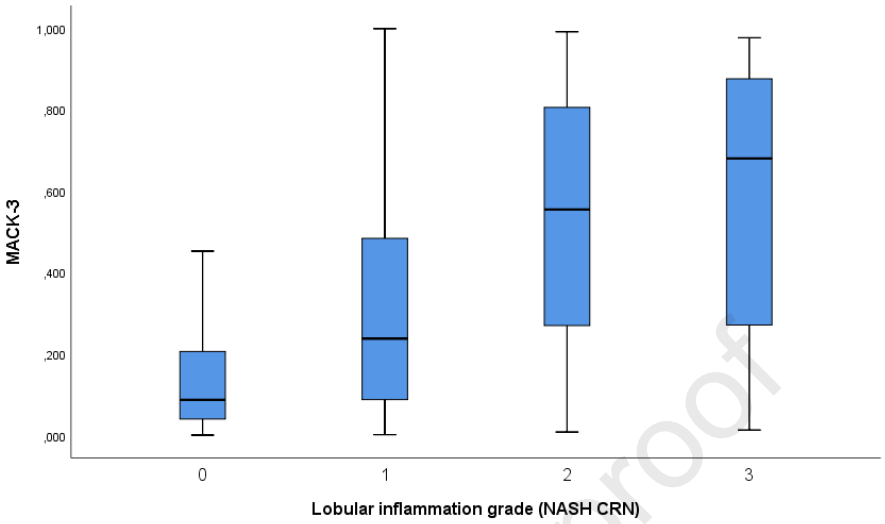
	All (n=1924)	**FAST available (n=655)**	**FAST not available (n=1269)**	***P***
Age (years)	49.2 ± 13.0	51.4 ± 14.2	48.1 ± 12.2	< .001
Male sex (%)	1065 (55.4)	418 (63.8)	647 (51.0)	< .001
BMI (kg/m^2)	32.4 ± 7.5	31.3 ± 6.5	33.0 ± 7.8	< .001
Bariatric patients (%)	335 (17.4)	8 (1.2)	327 (25.8)	< .001
Diabetes (%)	703 (36.5)	254 (38.8)	449 (35.4)	.148
Steatosis grade (%):				< .001
- 0	234 (12.2)	37 (5.6)	197 (15.5)	
- 1	717 (37.3)	249 (38.0)	468 (36.9)	
- 2	603 (31.1)	210 (32.1)	393 (31.0)	
- 3	370 (19.2)	159 (24.3)	211 (16.6)	
Ballooning (%):				< .001
- 0	661 (35.6)	111 (18.7)	550 (43.5)	
- 1	788 (42.4)	320 (53.9)	468 (37.0)	
- 2	410 (22.1)	163 (27.4)	247 (19.5)	
Lobular inflammation (%)				< .001
- 0	602 (32.4)	114 (19.2)	488 (38.6)	
- 1	958 (51.5)	383 (64.5)	575 (45.5)	
- 2	288 (15.5)	93 (15.7)	195 (15.4)	
- 3	11 (0.6)	4 (0.7)	7 (0.6)	
NAFLD Activity Score (NAS)	3.3 ± 1.9	3.8 ± 1.6	3.0 ± 1.9	< .001
NASH (%)	1001 (53.8)	406 (68.4)	595 (47.0)	< .001
Fibrosis stage (%):				< .001
- 0	678 (35.2)	151 (23.1)	527 (41.5)	
- 1	604 (31.4)	175 (26.7)	429 (33.8)	
- 2	287 (14.9)	152 (23.2)	135 (10.6)	
- 3	241 (12.5)	129 (19.7)	112 (8.8)	
- 4	114 (5.9)	48 (7.3)	66 (5.2)	
Fibrotic NASH (%)	444 (23.1)	238 (36.3)	206 (16.2)	< .001
AST (IU/L)	44 ± 35	48 ± 40	42 ± 32	< .001
ALT (IU/L)	66 ± 58	71 ± 60	64 ± 57	< .001
Gamma-GT (IU/L)	93 ± 130	112 ± 165	84 ± 107	< .001
Alkaline phosphatase (IU/L)	84 ± 35	86 ± 38	83 ± 33	.044
Albumin (g/L)	43.2 ± 4.7	44.1 ± 4.6	42.7 ± 4.7	< .001
Platelets (G/L)	244 ± 67	232 ± 64	250 ± 68	< .001
HOMA	6.7 ± 13.2	8.7 ± 18.3	5.7 ± 9.5	< .001
CK18 (IU/L)	346 ± 449	400 ± 563	318 ± 375	< .001
FIB4	1.28 ± 1.03	1.45 ± 1.11	1.20 ± 0.97	< .001
MACK-3	0.306 ± 0.279	0.360 ± 0.284	0.277 ± 0.272	< .001
Liver stiffness (kPa)	-	10.6 ± 8.9	-	-

Supplementary Figure s1: MACK-3 results as a function of histological grades of steatosis



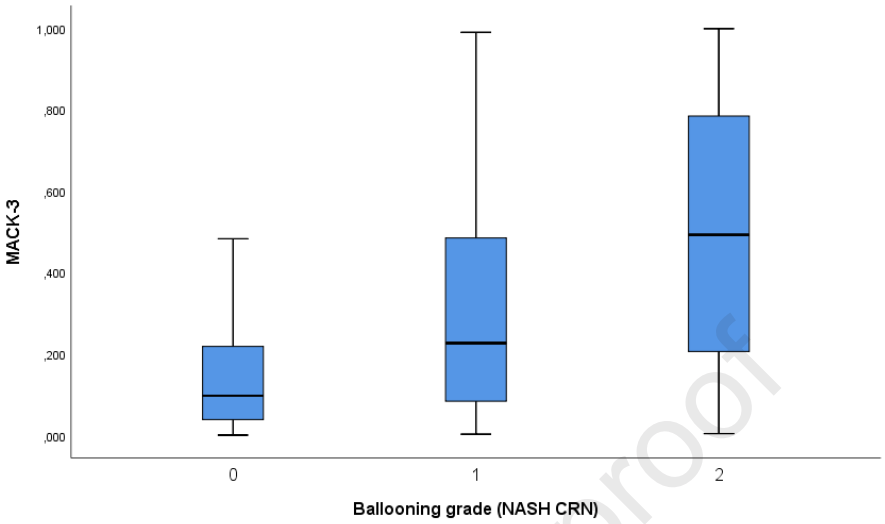
Grade	MACK-3	Comparison (*P*)		
		Vs grade 1	Vs grade 2	Vs grade 3
0	0.101 ± 0.164	< .001	< .001	< .001
1	0.235 ± 0.242	-	< .001	< .001
2	0.364 ± 0.273	-	-	< .001
3	0.478 ± 0.283	-	-	-

Supplementary Figure s2: MACK-3 results as a function of histological grades of lobular inflammation



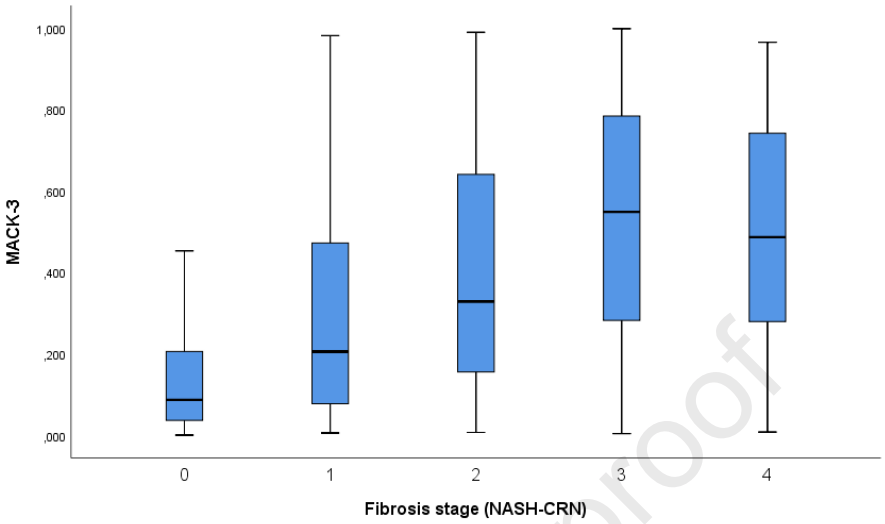
Grade	MACK-3	Comparison (*P*)		
		vs grade 1	vs grade 2	vs grade 3
0	0.159 ± 0.181	< .001	< .001	< .001
1	0.314 ± 0.265	-	< .001	.030
2	0.535 ± 0.298	-	-	.657
3	0.567 ± 0.371	-	-	-

Supplementary Figure s3: MACK-3 results as a function of histological grades of ballooning



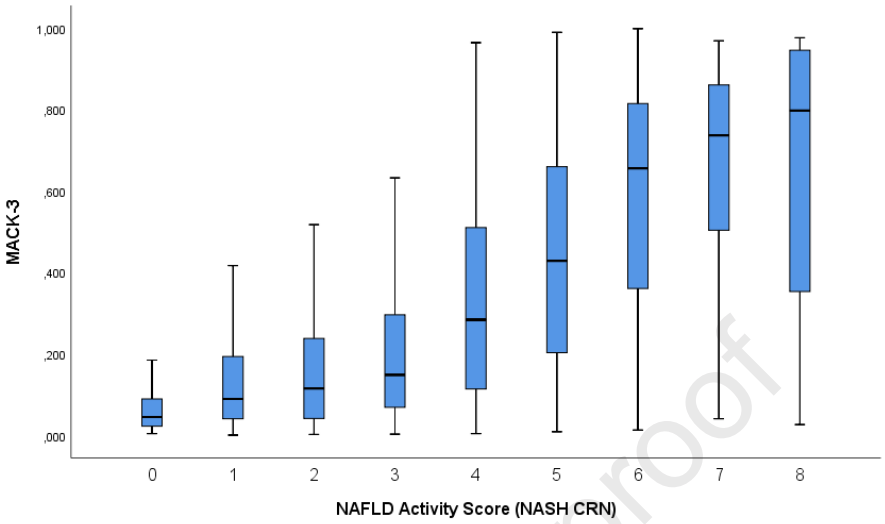
Grade	MACK-3	Comparison (*P*)	
		vs grade 1	vs grade 2
0	0.167 ± 0.187	< .001	< .001
1	0.312 ± 0.265	-	< .001
2	0.488 ± 0.306	-	-

Supplementary Figure s4: MACK-3 results as a function of histological stages of fibrosis



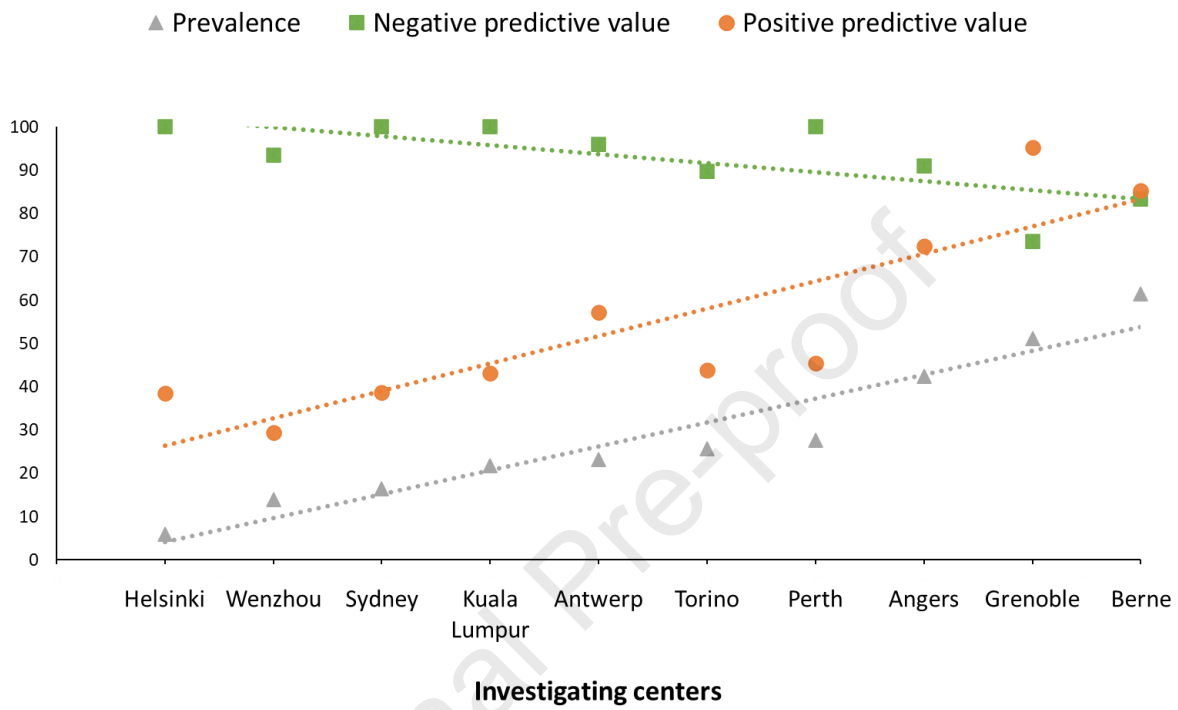
Stage	MACK-3	Comparison (*P*)			
		vs stage 1	vs stage 2	vs stage 3	vs stage 4
0	0.160 ± 0.184	< .001	< .001	< .001	< .001
1	0.299 ± 0.267	-	< .001	< .001	< .001
2	0.403 ± 0.291	-	-	< .001	.001
3	0.521 ± 0.279	-	-	-	.570
4	0.504 ± 0.265	-	-	-	-

Supplementary Figure s5: MACK-3 results as a function of the NAFLD Activity Score (NAS)

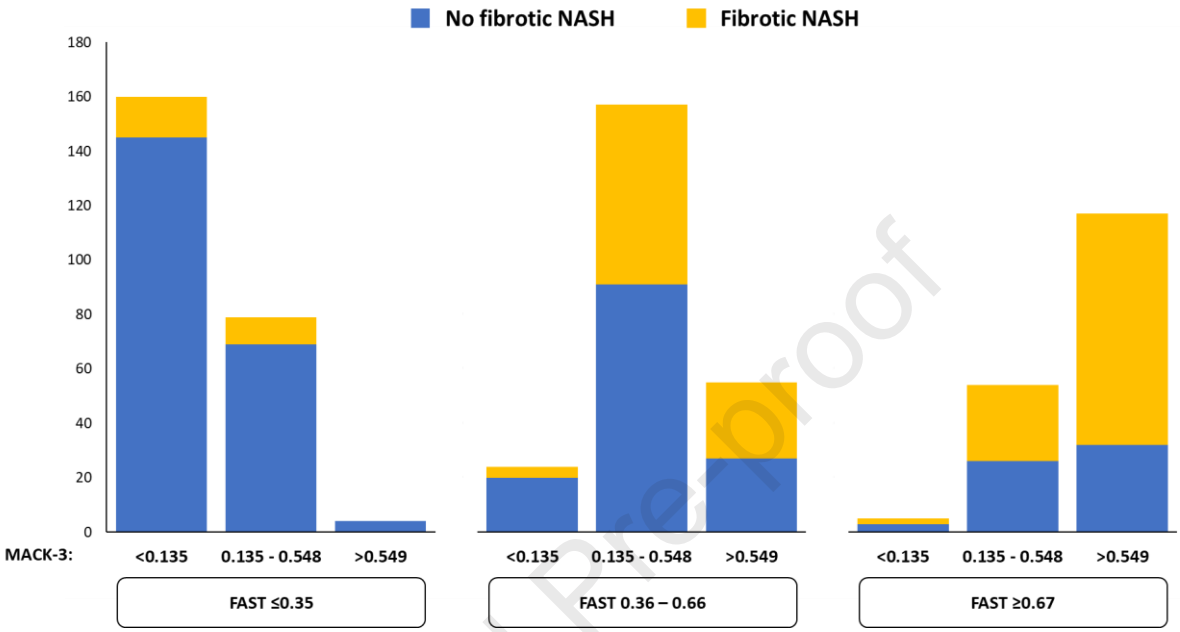


NAS	MACK-3	Comparison (*P*)							
		vs NAS 1	vs NAS 2	vs NAS 3	vs NAS 4	vs NAS 5	vs NAS 6	vs NAS 7	vs NAS 8
0	0.072 ± 0.080	< .001	< .001	< .001	< .001	< .001	< .001	< .001	.027
1	0.156 ± 0.178	-	.128	< .001	< .001	< .001	< .001	< .001	.053
2	0.186 ± 0.202	-	-	.007	< .001	< .001	< .001	< .001	.058
3	0.221 ± 0.213	-	-	-	< .001	< .001	< .001	< .001	.082
4	0.333 ± 0.257	-	-	-	-	< .001	< .001	< .001	.129
5	0.449 ± 0.278	-	-	-	-	-	< .001	< .001	.217
6	0.592 ± 0.270	-	-	-	-	-	-	.332	.461
7	0.628 ± 0.284	-	-	-	-	-	-	-	.600
8	0.649 ± 0.435	-	-	-	-	-	-	-	-

Supplementary Figure s6: Negative and positive predictive values of MACK-3 as a function of the prevalence of fibrotic NASH observed across the ten investigating centers



Supplementary Figure s7: Fibrotic NASH as a function of FAST and MACK-3 results



WHAT YOU NEED TO KNOW

BACKGROUND

- Drug development for NASH is hampered by a high 60-80% screening failure rate, mainly because of the absence of fibrotic NASH on the baseline liver biopsy.
- MACK-3, a blood test combining AST, HOMA and cytokeratin-18, has been recently developed specifically for the noninvasive diagnosis of fibrotic NASH.

FINDINGS

- Coming from a large multicentric international cohort including 1,924 patients, our results validate the good accuracy of MACK-3 for the noninvasive diagnosis of fibrotic NASH.
- MACK-3 decreases the screen failure rate in a similar rate than FAST, to only 35%.

IMPLICATIONS FOR PATIENT CARE

- MACK-3 allows better identification of candidates for NASH therapeutic trials