The impact of liver disease aetiology and the stages of hepatic fibrosis on the performance of non-invasive fibrosis biomarkers: an international study of 2411 cases

G. Sebastiani*, L. Castera[†], P. Halfon[‡], S. Pol[§], A. Mangia[¶], V. Di Marco[**], M. Pirisi[††], M. Voiculescu[‡‡], M. Bourliere[§§] & A. Alberti*[,¶¶]

*VIMM-Venetian Institute of Molecular Medicine, Padova, Italy.
[†]Service d'Hépato-gastroentérologie, Hôpital Haut Lévêque, C. H. U. Bordeaux, Pessac, France
[‡]Laboratoire Alphabio, Hospital Ambroise Pare, Marseille, France.
[§]Liver Unit and Inserm U-567, Cochin Hospital, Paris, France.
[¶]Division of Gastroenterology, Hospital Casa Sollievo della Sofferenza, IRCCS, San Giovanni Rotondo, Italy.
[**]Cattedra e Unità Operativa Complessa di Gastroenterologia ed Epatologia, University of Palermo, Palermo, Italy.
[††]Dipartimento di Medicina Clinica e Sperimentale, Università del Piemonte Orientale, Novara, Italy.
[‡‡]Department of Internal Medicine and Nephrology, Fundeni Clinical Institute, Bucharest, Romania.
[§§]Hôpital Saint Joseph, Marseille, France.
[¶¶]Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Padova, Italy.

Correspondence to:
Prof A. Alberti, Venetian Institute for Molecular Medicine (VIMM), Via Orus 2, 35100 Padova, Italy.
E-mail: alfredo.alberti@unipd.it

SUMMARY

Background
Performance of non-invasive fibrosis biomarkers may be influenced by aetiology of chronic liver disease (CLD) and the stages of hepatic fibrosis, but large-scale studies are pending.

Aim
To investigate the effect of aetiogy and stages of hepatic fibrosis on the performance of fibrosis biomarkers.

Methods
A total of 2411 patients with compensated CLD (HCV = 75.1%, HBV = 10.5%, NASH = 7.9%, HIV/HCV = 6.5%) were consecutively enrolled in 9 centres. APRI, Forns'index, Lok index, AST-to-ALT ratio, Fib-4, platelets and Fibrotest-Fibrosure were tested against liver biopsy, considered the gold standard. The effect of the stages of hepatic fibrosis to diagnose significant fibrosis and cirrhosis (≥F2 and F4 respectively) was investigated through difference between advanced and non-advanced fibrosis stages (DANA). Performance was expressed as observed area under the ROC curve (ObAUROC) and AUROC adjusted for DANA (AdjAUROC).

Results
Performance of APRI and Fibrotest-Fibrosure was higher than other biomarkers. In all aetiologies, AdjAUROC was higher than ObAUROC. APRI showed its best performance in HCV monoinfected cases, with an AdjAUROC of 0.77 and 0.83 for ≥F2 and F4 respectively. In HBV and non-alcoholic steatohepatitis (NASH) patients, its performance was poor (AdjAUROC <0.70). Performance of Fibrotest-Fibrosure was good in all aetiologies for both ≥F2 and F4 (AdjAUROC >0.73), except for ≥F2 in NASH (AdjAUROC = 0.64). Performance of all biomarkers was reduced in HCV cases with normal ALT.

Conclusions
Aetiology is a major factor influencing the performance of liver fibrosis biomarkers. Even after correction for DANA, APRI and Fibrotest-Fibrosure exhibit the best performance. However, liver biopsy is not replaceable, especially to diagnose ≥F2 and in HCV carriers with normal ALT.

INTRODUCTION

Chronic infection with the hepatitis B (HBV) and C (HCV) viruses and non-alcoholic steatohepatitis (NASH) are the main causes of chronic and progressive liver disease worldwide, leading to cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC).[1–3] Moreover, due to common routes of transmission, up to one-third of patients infected with the human immunodeficiency virus (HIV) in the US and Europe are coinfected with HCV or HBV.[4, 5] These different aetiological forms of chronic liver disease (CLD) share a common histopathological pathway that is the formation and accumulation of fibrosis, which leads to progressive distortion of the hepatic architecture, that is the hallmark of evolution to cirrhosis.[6] Natural history studies indicate that advanced fibrosis and cirrhosis develop in about 20–40% of patients with chronic hepatitis B (CHB) or chronic hepatitis C (CHC) and in a similar proportion of those with NASH.[7–9] Staging of liver fibrosis by liver biopsy represents the gold standard for prognostic assessment and, in CHC and CHB, for deciding to initiate anti-viral therapy.[10] Liver biopsy, however, has a number of limitations, being invasive, costly, difficult to standardise and disliked by many patients.[11–13] Its universal use in CLDs is unpractical due to the huge number of patients who are often asymptomatic.[2, 14] Recently, increasing interest has been directed towards the use of non-invasive tools to assess liver fibrosis. These include simple markers based on routinary tests, like platelets, AST-to-platelet ratio index (APRI), Forns' index, Fib-4, Lok index and AST-to-ALT ratio (AAR), and other more sophisticated and patented tests, such as Fibrotest-Fibrosure.[15–21] In the midst of a large series of publications on these markers, investigated mainly in CHC, their implementation in clinical practice is still debated and large-scale studies in aetiological forms other than HCV are still limited.[12, 13, 22] Recently, it has been shown that the performance of these biomarkers may be influenced by the prevalence of liver fibrosis stages defining advanced and non-advanced fibrosis in the analysed patients cohort.[23, 24] Poynard and colleagues have therefore concluded that area under the receiving operating characteristic curve (AUROC), that is generally used to describe performance, should be standardised accordingly.[23] The aim of this large-scale, international, independent study was to investigate the effect of aetiology of liver disease and of the stages of hepatic fibrosis on the performance of several non-invasive biomarkers in patients with CLD.

METHODS

Study design

This was an International, multicentre retrospective study conducted in nine clinical centres across Europe. Patients with CLDs of any aetiology were enrolled, according to inclusion and exclusion criteria. Our aim was to investigate the effect of liver disease aetiology and the stages of hepatic fibrosis on the performance of a series of non-invasive biomarkers for liver fibrosis. Details of patients' demographics, aetiology of CLDs, laboratory results and liver biopsy were recorded in each Centre. The study was conducted according to the Declaration of Helsinki.

Inclusion and exclusion criteria

We included consecutive patients admitted between January 2003 and December 2008. The inclusion criteria were (i) a diagnosis of well-compensated CLD of any aetiology and (ii) availability of both liver biopsy and relevant parameters for the assessment of non-invasive biomarkers, performed on the same day. Exclusion criteria were (i) decompensated liver cirrhosis; (ii) comorbidities that could confound the interpretation of non-invasive biomarkers, including haemolysis, Gilbert's syndrome, thrombocytopenia not liver-related; (iii) history or evidence at entry of HCC; (iv) liver transplantation. All patients gave their informed consent to be included in the study. Out of 2805 consecutive patients with CLDs who had a liver biopsy and non-invasive biomarkers performed on the same day, we included 2411 patients. A total of 221 were excluded for decompensated liver cirrhosis, 56 for confounding factors for the non-invasive biomarkers, 105 for HCC and 14 for previous liver transplantation. Patients with chronic viral hepatitis were treatment-naïve.

Aetiologies of liver disease

HCV infection was established by a positive third-generation anti-HCV test (Ortho Diagnostic Systems, Raritan, NJ, USA), with serum HCV-RNA detected by polymerase chain reaction (PCR) (Amplicor HCV Monitor test, Roche Diagnostics, Indianapolis, IN, USA). The HCV genotype was determined by INNOLipa (Innogenetics, Bayer, Ghent, Belgium). Diagnosis of HBV infection was based on the presence of HBsAg for more than 6 months, and HBeAg/anti-HBe were determined using commercial assays (Roche Diagnostics, Basel, Switzerland). HBV-DNA

level was measured by real-time PCR. HIV infection was defined by HIV ELISA, confirmed by Western blot assay, with positive HIV-RNA. The diagnosis of NASH was based on liver histology. Secondary causes of steatohepatitis including significant alcohol consumption (≥100 g/week) and the use of hepatotoxic drugs were excluded. Among patients with either HCV or HBV infection, 487 (20.2%) subjects admitted occasional alcohol consumption and 29 (1.2%), moderate alcohol consumption.

Histological assessment

Liver biopsies were analysed in each centre by the local pathologist who was unaware of clinical data. Diagnosis of NASH was based on the common features including steatosis, inflammation (portal and lobular), hepatocyte ballooning and fibrosis, according to the scoring system recently developed by the NIH-sponsored NASH Clinical Research Network.[25] In patients with CHC, or CHB or HIV/HCV coinfection, fibrosis was scored according to METAVIR classification as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.[26–29] Staging of liver fibrosis in NASH patients was as follows: F0, normal connective tissue; F1, foci of perivenular or pericellular fibrosis in zone 3; F2, perivenular or pericellular fibrosis confined to zones 3 and 2, with or without portal/periportal fibrosis; F3, bridging or septal fibrosis; and F4, cirrhosis.[30]

Non-invasive biomarkers for liver fibrosis

The following non-invasive biomarkers were determined in all patients, according to published formulas: platelets, AAR, APRI, Forns' index, Lok index, Fib-4. Fibrotest-Fibrosure values were obtained through Biopredictive (Biopredictive, Paris, France) or by courtesy of Prof. Thierry Poynard. For all non-invasive biomarkers investigated, the cut-off values indicated in the original reports or in subsequent studies in various aetiologies of CLD were applied.[15–21, 27, 31–33]

Outcome measures

Performance of non-invasive biomarkers was assessed for the detection of: (i) significant fibrosis (≥F2 according to METAVIR or NASH classification) and (ii) cirrhosis (F4) as these threshold are generally considered clinically relevant to initiate anti-viral therapy and for specific management and follow-up protocols.[34–37]

Statistical analysis

Descriptive results were expressed as mean ± s.d. (standard deviation) or number (percentage) of patients with a condition. The *t*-test or non parametric Mann–Whitney test was used to compare quantitative data and the chi-squared test was applied for comparison of frequency data. All tests were two-tailed and *P*-values <0.05 were considered significant. The performance of the non-invasive methods for liver fibrosis was measured as sensitivity, specificity, positive and negative predictive value (PPV and NPV respectively), accuracy, positive and negative likelihood ratio (LR). Sensitivity, specificity, PPV, NPV and accuracy were expressed as percentage. The diagnostic value of the non-invasive biomarkers was expressed and compared using the AUROC and its corresponding 95% confidence intervals (CI). AUROCs were calculated including non-invasive markers' quantitative values using empirical non parametric method according to DeLong *et al.* and compared using the method of Hanley *et al.*[38, 39]

Standardisation of AUROCs according to the prevalence of fibrosis stages

As recently proposed by Poynard *et al.*, AUROCs were adjusted according to the prevalence of fibrosis stages using the DANA (Difference between advanced and non-advanced fibrosis).[23] The DANA is an index for standardising comparisons to transform any different prevalence profile into a homogeneous distribution of fibrosis stages from F0 to F4, as defined by a prevalence of 0.20 for each of the five METAVIR stages (standard prevalence). DANA was calculated according to the following formula: [(prevalence F2 × 2 + prevalence F3 × 3 + prevalence F4 × 4)/(prevalence F2 + prevalence F3 + prevalence F4)] – [prevalence F1/(prevalence F0 + prevalence F1)]. The adjusted AUROCs (adjAUROCs) were calculated as follows: AdjAUROC = observed AUROC (obAUROC) + (0.1056) × (2.5 – DANA).

RESULTS

Demographics, laboratory and histological features of the 2411 patients with CLDs

Overall, there were 1391 males and 1020 females with mean age of 46.9 ± 12.1 years. Of 1968 patients with CHC, either monoinfected or HIV coinfected, 1381 (70.2%) were infected with genotype 1 of HCV. Of 253 patients with CHB, 13 (5.1%) were coinfected with HDV and 46 (18.2%) were HBeAg positive. The main demographic, laboratory and histological features, and their

Table 1 | Demographic, laboratory and histological characteristics of 2411 patients with CLDs, also according to aetiological subgroups

	Total	HCV	HBV	NASH	HIV/HCV	P*			
n (%)	2411	1810 (75.1)	253 (10.5)	190 (7.9)	158 (6.5)				
Age (years)	46.9 12.1	46.9 ± 11.9	43.7 ± 14.7	51.2 ± 13.3	41.9 ± 5.6	0.6	0.01	0.001	0.004
Male gender (%)	1391 (57.7)	1014 (56.0)	184 (72.7)	142 (74.7)	111 (70.3)	0.27	<0.0001	<0.0001	0.001
BMI (kg/m^2)	24.9 ± 5.4	24.7 ± 3.7	24.2 ± 3.5	28.9 ± 5.1	24.4 ± 5.2	0.65	0.5	<0.0001	0.45
Platelets (10^3/mm^3)	211.9 ± 62.9	209.3 ± 60.4	180.2 ± 49.2	251.7 ± 81.2	184.3 ± 59.9	0.27	0.006	0.07	0.01
AST (IU/L)	54.3 ± 47.7	55.8 ± 41.2	71.0 ± 92.2	79.6 ± 58.5	79.9 ± 83.5	0.3	0.13	0.01	0.004
ALT (IU/L)	74.9 ± 58.0	76.8 ± 59.2	74.6 ± 50.3	72.3 ± 47.1	58.1 ± 45.8	0.2	0.4	0.31	0.11
Liver fibrosis stage according to METAVIR or NASH classification (%)									
F0	272 (11.3)	199 (11.0)	26 (10.3)	23 (12.1)	23 (14.6)	0.60	0.0004	0.15	0.002
F1	1027 (42.6)	791 (43.7)	81 (32.0)	69 (36.3)	82 (51.9)				
F2	586 (24.3)	440 (24.3)	74 (29.2)	50 (26.3)	25 (15.8)				
F3	287 (11.9)	217 (12.0)	27 (10.7)	22 (11.6)	20 (12.7)				
F4	239 (9.9)	163 (9.0)	45 (17.8)	26 (13.7)	8 (5.0)				

BMI, body mass index; NASH, non-alcoholic steatohepatitis; AST, aspartate aminotrasferase; ALT, alanine aminotrasferase.

Results given as mean ± s.d. or n (%). * T-test or chi-squared test between 'total' and 'HCV' in the first column, between 'total' and 'HBV' in the second column, between 'total' and 'NASH' in the third column and between 'total' and 'HIV/HCV' in the fourth column.

distribution by aetiological subgroups are summarised in Table 1. Overall, 1112 (46.1%) patients had significant fibrosis and 239 (9.9%) had cirrhosis. The mean length of liver specimen was 17.7 ± 8.4 mm and the mean number of portal tracts was 10.6 ± 5.9. Biopsy length was greater than 15 mm in 1466 (60.8%) and greater than 20 mm in 1027 (42.6%) patients. As compared with the whole population, patients with CHB and those with HIV/HCV coinfection were younger (*P* = 0.01 and *P* = 0.004 respectively), whereas patients with NASH were significantly older (*P* = 0.001). Male gender was more frequent in CHB and HIV/HCV coinfected patients and in cases with NASH (*P* < 0.0001, *P* = 0.001 and *P* < 0.0001 respectively). As expected, patients with NASH had a significantly higher BMI when compared with the whole population of the study (*P* < 0.0001). Platelets were significantly lower in patients with CHB and HIV/HCV coinfection with respect to the whole population (*P* = 0.006 and *P* = 0.01 respectively). Moreover, AST levels were significantly higher in patients with NASH and HIV/HCV coinfection as compared with the whole population (*P* = 0.01 and *P* = 0.004 respectively). The distribution of liver fibrosis stages also showed some significant differences: patients with CHB had more advanced fibrosis stages, and patients with HIV/HCV coinfection had less advanced fibrosis stages when compared with the whole population (signifi-

cant fibrosis being present in 57.7% and 33.5% of cases vs. 46.1%; *P* = 0.0004 and *P* = 0.002 respectively).

Effect of the stages of hepatic fibrosis on the performance of non-invasive biomarkers to diagnose significant fibrosis

The performance of non-invasive biomarkers to diagnose significant fibrosis and the effect of the stages of hepatic fibrosis are shown in Table 2. Overall, among the non-invasive biomarkers considered, Fibrotest-Fibrosure and APRI showed the best performance. For all non-invasive biomarkers, the correction of the ObAUROC for DANA resulted in a higher AdjAUROC, although this difference was not statistically significant. Fibrotest-Fibrosure was able to classify 100% of cases, a figure that was significantly higher than that of APRI, Forns' index and Fib-4 (*P* < 0.0001). Even when corrected for DANA, Fibrotest-Fibrosure and APRI showed the best performance for significant fibrosis, as indicated by an AdjAUROC of 0.76 and of 0.74 respectively.

Effect of the stages of hepatic fibrosis on the performance of non-invasive biomarkers to diagnose cirrhosis

The performance of non-invasive biomarkers to diagnose cirrhosis and the effect of the stages of hepatic

Table 2 | Performance of non-invasive biomarkers for significant fibrosis and correction for the stages of hepatic fibrosis in 2411 patients with CLDs

	APRI		Forns' index		Fib-4		Fibrotest-Fibrosure
Cut-off	0.5	1.5	4.2	6.9	1.45	3.25	0.49
Classified cases (%)	67.5		56.9		60.0		100
Sensitivity (%)	67.9	37.4	76.0	65.6	70.5	55.0	61.0
Specificity (%)	71	95.9	60.0	50.8	65.0	60.0	83.0
PPV (%)	66.7	85.1	77.2	72.1	73.4	66.2	79.2
NPV (%)	72	60.6	59.3	70.4	60.1	40.5	62.4
Accuracy (%)	64.3	69.5	68.5	70.9	67.3	72.0	71.3
LR+	2.34	9.12	1.9	1.33	2.01	1.63	3.59
LR−	0.45	0.65	0.4	0.68	0.45	0.58	0.47
DANA				1.90			
ObAUROC (95% CI)							0.7 (0.65–0.75)
	0.68 (0.62–0.74)		0.67 (0.61–0.73)		0.66 (0.61–0.71)		
AdjAUROC (95% CI)	0.74 (0.68–0.80)		0.73 (0.67–0.79)		0.72 (0.67–0.77)		0.76 (0.71–0.81)

AdjAUROC, adjusted area under the curve; APRI, AST-to-platelet ratio index; CI, confidence interval; DANA, difference between advanced and non-advanced fibrosis; LR, likelihood ratio; NPV, negative predictive value; ObAUROC, observed area under the curve; PPV, positive predictive value.

fibrosis are shown in Table 3. Overall, among the non-invasive biomarkers considered, APRI and Fibrotest-Fibrosure showed the best performance for cirrhosis. The performance of AAR was rather poor and significantly lower than that of platelets, APRI and Fibrotest-Fibrosure ($P < 0.0001$). APRI and Lok index were able to classify less number of cases than Fibrotest-Fibrosure, AAR and platelets (86.7% and 52.3% vs. 100% respectively, $P < 0.0001$) For all non-invasive biomarkers, the correction of the ObAUROC for DANA resulted in a higher AdjAUROC, although this difference was not statistically significant. Even when corrected for DANA, APRI and Fibrotest-Fibrosure showed the best performance for cirrhosis, as indicated by an AdjAUROC of 0.80 and of 0.79 respectively. Interestingly, both APRI and Fibrotest-Fibrosure showed a high NPV (>93%) to exclude cirrhosis.

Impact of aetiology of liver diseases on the performance of non-invasive biomarkers

A subgroup analysis according to different aetiologies was performed for the non-invasive biomarkers and their respective cut-off values that showed the best performance in the whole series of patients. APRI showed its best performance in CHC, with an AdjAUROC of 0.77 for significant fibrosis and an AdjAUROC of 0.83 for cirrhosis (Table 4). Although slightly inferior, the performance of APRI in HIV/HCV coinfected patients was not significantly different from that in monoinfected HCV cases, with an AdjAUROC of 0.74 for significant fibrosis and an AdjAUROC of 0.77 for cirrhosis. In CHB, APRI showed a significantly lower performance than in CHC, with an AdjAUROC of 0.69 for significant fibrosis and an AdjAUROC of 0.66 for cirrhosis ($P < 0.0001$). The performance of APRI was significantly lower for NASH vs. CHC for both significant fibrosis and cirrhosis ($P < 0.0001$). Interestingly, APRI showed a high NPV (>97%) to exclude presence of cirrhosis in both HCV monoinfected and HIV/HCV coinfected cases.

Fibrotest-Fibrosure showed its best performance in CHC, with an AdjAUROC of 0.77 for significant fibrosis and 0.79 for cirrhosis (Table 5). The performance of Fibrotest-Fibrosure in HIV/HCV coinfected patients was similar to that in HCV monoinfected cases. In CHB, Fibrotest-Fibrosure showed a slightly reduced performance than in CHC for diagnosis of cirrhosis, but this difference was not significant. Conversely, performance of Fibrotest-Fibrosure for significant fibrosis was significantly lower for NASH vs. CHC ($P < 0.0001$). On the other hand, the performance of Fibrotest-Fibrosure for cirrhosis was significantly higher in NASH cases vs. HBV cases ($P < 0.0001$). Interestingly, in all aetiologies except for CHB, Fibrotest-Fibrosure showed a very high NPV (>95%) to exclude presence of cirrhosis.

Table 3 | Performance of non-invasive biomarkers for cirrhosis and correction for the stages of hepatic fibrosis in 2411 patients with CLDs

	Platelets	APRI		Lok index		AAR	Fibrotest-Fibrosure
Cut-off	150	1	2	0.2	0.5	1	0.75
Classified cases (%)	100	86.7		52.3		100	100
Sensitivity (%)	53.3	74.0	41.3	76.0	56.5	43.4	54.1
Specificity (%)	85.7	82.6	93.7	40.0	60.7	58.3	90.1
PPV (%)	34.6	31.7	41.8	30.5	66.0	66.7	60.8
NPV (%)	92.9	96.7	93.6	88.5	40.3	35	93.8
Accuracy (%)	77.9	81.7	88.5	66.8	69.7	48.6	86.3
LR+	3.72	4.25	6.56	1.27	1.44	1.04	5.46
LR−	0.54	0.31	0.63	0.6	0.71	0.97	0.51
DANA				1.90			
ObAUROC (95% CI)	0.72 (0.66–0.78)	0.74 (0.69–0.79)		0.63 (0.57–0.69)		0.54 (0.48–0.60)	0.73 (0.68–0.78)
AdjAUROC (95% CI)	0.78 (0.72–0.84)	0.80 (0.75–0.85)		0.69 (0.63–0.75)		0.6 (0.54–0.66)	0.79 (0.74–0.84)

AdjAUROC, adjusted area under the curve; APRI, AST-to-platelet ratio index; CI, confidence interval; DANA, difference between advanced and non-advanced fibrosis; LR, likelihood ratio; NPV, negative predictive value; ObAUROC, observed area under the curve; PPV, positive predictive value.

Table 4 | Performance of APRI according to aetiological subgroups and correction for the stages of hepatic fibrosis in 2411 patients with CLDs

	Overall		HCV		HBV		NASH		HIV/HCV	
Cut-off (end-point)	1.5 (≥F2)	2 (F4)	1.5 (≥F2)	2 (F4)	1.5 (≥F2)	2 (F4)	1.5 (≥F2)	2 (F4)	1.5 (≥F2)	2 (F4)
Sensitivity (%)	27.4	41.3	45.6	67.4	36.9	20.6	33.3	20.5	60.0	50.0
Specificity (%)	95.9	93.7	95.1	93.7	98	83.6	82.1	92.4	71.4	80.4
PPV (%)	85.1	41.8	85.8	46.1	93.7	16.7	61.5	16.7	60.0	10.0
NPV (%)	60.6	93.6	72.8	97.2	50	77.9	58.9	90.4	71.4	97.3
Accuracy (%)	64.3	88.5	75.6	89.9	65.3	69.7	59.6	76.7	66.7	79.2
LR+	6.68	6.56	9.3	10.7	18.5	1.26	1.86	2.7	2.1	2.55
LR−	0.76	0.63	0.57	0.35	0.64	0.94	0.81	0.86	0.56	0.62
DANA	1.90		1.86		2.04		2.0		1.89	
ObAUROC (95% CI)	0.68 (0.62–0.74)	0.74 (0.69–0.79)	0.7 (0.65–0.75)	0.76 (0.71–0.81)	0.64 (0.58–0.70)	0.61 (0.55–0.66)	0.58 (0.52–0.64)	0.55 (0.49–0.61)	0.68 (0.62–0.74)	0.69 (0.63–0.75)
AdjAUROC (95% CI)	0.74 (0.68–0.80)	0.80 (0.75–0.85)	0.77 (0.72–0.82)	0.83 (0.78–0.88)	0.69 (0.63–0.76)	0.66 (0.60–0.71)	0.63 (0.57–0.69)	0.60 (0.54–0.66)	0.74 (0.68–0.80)	0.75 (0.71–0.83)

AdjAUROC, adjusted area under the curve; APRI, AST-to-platelet ratio index; CI, confidence interval; DANA, difference between advanced and non-advanced fibrosis; LR, likelihood ratio; NPV, negative predictive value; ObAUROC, observed area under the curve; PPV, positive predictive value.

Table 5 | Performance of Fibrotest-Fibrosure according to aetiological subgroups and correction for the stages of hepatic fibrosis in 2411 patients with CLDs

	Overall		HCV		HBV		NASH		HIV/HCV	
Cut-off (end-point)	0.48 (≥F2)	0.75 (F4)	0.48 (≥F2)	0.75 (F4)	0.48 (≥F2)	0.75 (F4)	0.48 (≥F2)	0.75 (F4)	0.48 (≥F2)	0.75 (F4)
Sensitivity (%)	55.7	54.1	56.2	53.7	54.2	42.1	40.9	75.0	75.0	66.7
Specificity (%)	78.4	90.1	78.9	90.1	83.3	91.4	76.7	94.7	64.5	79.5
PPV (%)	83.4	60.8	83.6	53.4	89	76	81.5	75	64	18.2
NPV (%)	67.5	93.8	68	95	52.6	86.4	62.3	96.2	88.6	97.2
Accuracy (%)	70.0	86.3	67.2	86.4	64.0	80.9	58.6	89.0	68.1	78.7
LR+	2.58	5.46	2.66	5.42	3.25	4.89	1.76	14.2	2.11	3.25
LR–	0.57	0.51	0.56	0.51	0.55	0.63	0.77	0.26	0.39	0.42
DANA	1.90		1.86		2.04		2.0		1.89	
ObAUROC (95% CI)	0.7 (0.65–0.75)	0.73 (0.68–0.78)	0.7 (0.65–0.75)	0.72 (0.67–0.77)	0.69 (0.63–0.75)	0.68 (0.63–0.73)	0.59 (0.54–0.64)	0.84 (0.78–0.80)	0.7 (0.64–0.76)	0.73 (0.68–0.78)
AdjAUROC (95% CI)	0.76 (0.71–0.81)	0.79 (0.74–0.84)	0.77 (0.72–0.82)	0.79 (0.74–0.84)	0.74 (0.68–0.80)	0.73 (0.68–0.78)	0.64 (0.59–0.64)	0.89 (0.83–0.95)	0.76 (0.70–0.82)	0.79 (0.74–0.84)

AdjAUROC, adjusted area under the curve; APRI, AST-to-platelet ratio index; CI, confidence interval; DANA, difference between advanced and non-advanced fibrosis; LR, likelihood ratio; NPV, negative predictive value; ObAUROC, observed area under the curve; PPV, positive predictive value.

Effect of other variables on the performance of non-invasive biomarkers

The influence of other variables on the performance of the non-invasive biomarkers, which exhibited the best performance, was also investigated. Inter-centre variability was marginal (data not shown). The effect of liver biopsy size on the performance of non-invasive biomarkers was also studied. As regards APRI, similar performance was observed for subjects with biopsy samples <20 mm as compared with those with specimens >20 mm: AdjAUROC = 0.72 (0.66–0.78, 95% CI) vs. 0.75 (0.70–0.80, 95% CI) for significant fibrosis, and 0.79 (0.75–0.83, 95% CI) vs. 0.80 (0.75–0.85, 95% CI) for cirrhosis respectively. On the same line, as regards Fibrotest-Fibrosure, similar performance was observed for subjects with biopsy samples <20 mm as compared with those with specimens >20 mm: AdjAUROC = 0.74 (0.68–0.80, 95% CI) vs. 0.76 (0.71–0.81, 95% CI) for significant fibrosis, and 0.79 (0.75–0.83, 95% CI) vs. 0.79 (0.74–0.84, 95% CI) for cirrhosis respectively.

Performance of non-invasive biomarkers in HCV patients with normal ALT and in elderly patients with CHC

To assess the performance of non-invasive biomarkers and the effect of DANA in patients who may have a peculiar distribution of liver fibrosis stages, a dedicated analysis was conducted on two subgroups of patients: (i) HCV patients with normal ALT, who are expected to have a lower prevalence of advanced fibrosis stages; (ii) HCV elderly patients (age ≥65 years), who are expected to have a higher prevalence of advanced fibrosis stages. Indeed, these two subgroups of patients represent the vast majority of HCV carriers in the general population in most Western Countries, with an indication to liver biopsy that is often debated and controversial.[40–42]

Overall, 595 (32.9%) HCV cases had normal ALT and 190 (10.5%) cases were over 65 years of age. The main demographic, laboratory and histological features of the two subgroups and comparison with the whole population study are summarised in Table S1. When compared with the whole study population, patients with normal ALT were significantly younger ($P = 0.001$) and had a lower prevalence of advanced fibrosis stages (significant fibrosis being present in 29.5% vs. 45.3%, $P < 0.0001$). Moreover, they present with lower frequency of male gender ($P < 0.0001$) and higher platelets ($P < 0.0001$) with respect to the whole population.

Overall, performance of all non-invasive biomarkers for significant fibrosis in patients with normal ALT was rather poor, with ObAUROC <0.65 for all the non-inva-sive markers tested (Table S2). When correction for DANA was applied, APRI and Fibrotest-Fibrosure showed the highest performance, which remained, however, only discrete (AdjAUROC of 0.71 for APRI and 0.70 for Fibrotest-Fibrosure). These figures were lower than those observed in the whole population, even if the difference was not statistically significant. On the same line, performance of all non-invasive biomarkers for cirrhosis was lower than in the whole population, the highest ObAUROC being 0.65 (Table S3). The performance of AAR was significantly lower than that of platelets, APRI and Fibrotest-Fibrosure ($P < 0.0001$). When correction for DANA was applied, APRI and Fibrotest-Fibrosure showed the highest performance, with an AdjAUROC of 0.73 for both markers.

As regards elderly patients with HCV, they had a significantly lower prevalence of male gender ($P < 0.0001$), higher BMI ($P = 0.04$), lower platelets ($P = 0.005$) and higher AST and ALT ($P = 0.01$ and $P < 0.0001$ respectively) (Table S1). As expected, elderly HCV patients present with a higher prevalence of advanced fibrosis stages (significant fibrosis being present in 71.0% vs. 45.3%, $P < 0.0001$) when compared with the whole population.

Overall, performance of all non-invasive biomarkers for significant fibrosis in HCV elderly patients was similar to that of the whole population (Table S4). Among the non-invasive biomarkers considered, Fibrotest-Fibrosure and APRI showed the best performance for diagnosis of significant fibrosis, as indicated by an AdjAUROC of 0.74 for both markers. As regards the diagnosis of cirrhosis, platelets showed the best performance, as indicated by an AdjAUROC of 0.87. Interestingly, platelets showed the highest NPV (>95%) to exclude cirrhosis (Table S5).

DISCUSSION

The results of this large-scale, multicentre, independent study showed that aetiology of CLDs is a major factor influencing the performance of a series of non-invasive biomarkers. Moreover, APRI and Fibrotest-Fibrosure had the best performance for the diagnosis of significant fibrosis and cirrhosis in various aetiologies of CLDs. This study represents one of the few and the largest in which a direct comparison of the performance of non-invasive biomarkers for liver fibrosis in aetiologies other than HCV has been carried out. Our study has also shown that the performance of non-invasive biomarkers for liver fibrosis may be distinct in special subgroups of patients who are expected to have a peculiar distribution of fibrosis stages, such as HCV carriers with normal ALT and elderly HCV patients. Although these two

subgroups of HCV patients are highly represented in the general population and the indication to liver biopsy remains controversial, few studies have specifically investigated the performance of non-invasive biomarkers in them. Moreover, our study represents the first large-scale, multicentre, independent study that evaluated the effect of the stages of hepatic fibrosis on the performance of the most validated non-invasive biomarkers for liver fibrosis. Indeed, it has been suggested that the prevalence of liver fibrosis stages may be a major factor of variability in assessing the diagnostic value of a fibrosis marker, usually evaluated and compared using AUROC, and it may be a cause of unsatisfactory results.[23] According to our results, the prevalence of liver fibrosis stages may have an effect on the AUROC, and should be taken into account. Indeed, in our experience, the AUROC was higher of 0.04–0.08 for all non-invasive biomarkers studied when corrected for DANA.

Staging of liver fibrosis has been recognised as of paramount importance for both prognosis and management of CLDs.[3, 4, 36, 43] Liver biopsy has always been regarded as the reference standard for liver fibrosis assessment.[10] However, it is invasive, costly and not feasible as screening tool for liver fibrosis in CLDs, which affect millions of people worldwide.[11, 44] In recent years, many efforts have been dedicated towards the identification of non-invasive tools for a more rapid, practical and less expensive initial screening for disease stage and risk of progression. Several non-invasive biomarkers for liver fibrosis have been described, including simple methods with virtually no cost, such as platelets, AAR, APRI, Fib-4, Lok index and Forns' index, and more elaborated patented panels of biomarkers, such as Fibrotest-Fibrosure.[15–21] In clinical practice, the implementation of non-invasive biomarkers for liver fibrosis is still limited by the scepticism shared by many clinicians on their performance in substitution of liver histology, especially in aetiologies other than CHC, where limited validation exists.[12, 13, 22, 45] Among the non-invasive biomarkers investigated in our study, APRI and Fibrotest-Fibrosure showed the best performance for diagnosis of significant fibrosis and cirrhosis in all aetiologies of CLDs, even when the AUROC was corrected for DANA. This finding is coherent with other studies that compared the performance of non-invasive biomarkers for liver fibrosis.[27, 46–49] Importantly, we showed that performance of APRI and Fibrotest-Fibrosure may significantly differ among various aetiologies of CLDs. As regards APRI, it showed its best performance in HCV monoinfected patients. Although the performance was slightly inferior, no sig-

nificant difference was observed in HIV/HCV coinfected cases. Moreover, we found a very high NPV (>97%) with a cut-off value of 2 to exclude cirrhosis in both monoinfected and coinfected cases that could be useful in clinical practice to select patients in whom the risk of having cirrhosis is marginal. It has been suggested that performance of APRI may be diminished in coinfected patients because of HIV-related or antiretroviral-related thrombocytopenia.[50–52] Conversely, our results are in line with a recent meta-analysis, which reported a tendency for lower performance of APRI in HIV/HCV coinfected patients that did not reach the statistical significance.[51] On the same line, other studies reported a similar performance of APRI in HIV/HCV coinfected patients.[53, 54] In CHB, the performance of APRI was somehow unsatisfactory for both significant fibrosis and cirrhosis, with an AUROC always <0.70, even when corrected for DANA. These results are in line with previous studies.[27, 55] Only one study reported a good performance of APRI for significant fibrosis, as indicated by an AUROC of 0.86.[56] The differences between the patient populations in this study could be responsible for this disagreement. As regards NASH, the performance of APRI was poor for both significant fibrosis and cirrhosis, as indicated by an AdjAUROC of 0.63 and 0.60 respectively. Similar results have been reported in a small-scale study.[57] Another study investigated the performance of APRI for prediction of cirrhosis in only 50 patients. The authors found a discrete performance, as indicated by an AUROC of 0.786 for cirrhosis.[58] Our results, based on 190 consecutive patients, could not confirm this initial report.

As regards Fibrotest-Fibrosure, it showed a good performance in all aetiologies of CLDs, except for the diagnosis of significant fibrosis in NASH. Moreover, in all aetiologies except for CHB, Fibrotest-Fibrosure showed a very high NPV (>95%) to exclude presence of cirrhosis, a result that could be useful in clinical practice to select patients in whom the risk of having cirrhosis is marginal. Similar to APRI, Fibrotest-Fibrosure showed its best performance in HCV monoinfected cases. The performance of Fibrotest-Fibrosure in patients with HIV/HCV coinfection was similar to that found for monoinfected HCV patients. One initial study of 130 HIV/HCV coinfected patients coming from the group that patented the test reported an excellent AUROC of 0.856 for the prediction of significant fibrosis.[29] However, a following study of 272 patients, which took into account also DANA, reported lower performances that were similar to our results, as indicated by an ObAUROC of 0.64 for significant fibrosis, that raised to 0.78 when corrected by

DANA, and 0.81 for cirrhosis.[52] The performance of Fibrotest-Fibrosure in CHB was investigated in few studies. The reported AUROC ranged from 0.76 to 0.85 for diagnosis of significant fibrosis and from 0.76 to 0.80 for diagnosis of cirrhosis that are somehow in line with the results of the present study.[27, 28] Interestingly, it has been recently suggested that Fibrotest-Fibrosure may have a role in the follow-up of HBV inactive carriers as well as better selection of patients who require a liver biopsy.[33, 59] As regards NASH, very few reports investigated the role of Fibrotest-Fibrosure.[60] In our experience, Fibrotest-Fibrosure had a high performance to diagnose cirrhosis, with a very high NPV (>96%) to exclude cirrhosis and an overall AdjAUROC of 0.89. As, to our knowledge, this is the first independent application of Fibrotest-Fibrosure in NASH patients, this result deserves attention and should be confirmed by other studies. It should be here underlined that Fibrotest-Fibrosure has the advantage of classifying all the cases, while APRI leaves up to 41% of cases unclassified.

Thanks to the large number of patients included in our cohort, we were also able to perform a subgroup analysis aimed at assessing the performance of non-invasive biomarkers and the effect of DANA in patients who may have a peculiar distribution of liver fibrosis stages, including HCV patients with normal ALT and HCV elderly patients. In clinical practice, these groups of patients are very frequent and may be the ones in whom liver biopsy is even more questionable, and a reliable non-invasive tool is highly needed.[40, 42] Indeed, regardless of ALT levels, the decision to initiate anti-viral therapy should be individualised based on the severity of liver disease assessed by liver biopsy, the potential of serious side effects, the likelihood of response and the presence of comorbidities.[40] Few studies have investigated the role of non-invasive biomarkers in patients with normal ALT.[61–66] Some studies suggested that performance of non-invasive biomarkers is in line with that in patients with elevated ALT.[61] Conversely, other reports suggested that performance of non-invasive biomarkers may be somewhat reduced in patients with normal ALT.[62, 63, 65, 66] Our study, based on a large series of cases in which the prevalence of significant fibrosis was in line with previous population studies,[14] indicates that the performance of non-invasive biomarkers is reduced in patients with normal ALT, even when AUROC is adjusted according to fibrosis stages distribution. Consequently, the clinical use and interpretation of these markers in this subgroup of patients should be cautious. In elderly HCV patients, the greater prevalence of liver disease is linked to higher morbidity and mortality, which are excellent reasons for improving the evaluation of liver fibrosis and to better identify indication to anti-viral therapies.[41, 42] A single report by Poynard and colleagues investigated the role of Fibrotest-Fibrosure in elderly patients with CHC, and a good performance of the patented panel was reported.[67] Interestingly, in our study, platelets showed a very good performance to diagnose cirrhosis in this subgroup of patients. Indeed, a cut-off of 150 000/mm^3 had an excellent NPV to exclude the presence of cirrhosis. A good performance of platelets in excluding cirrhosis has been already reported, but this is the first study to assess its performance in a large series of elderly HCV patients.[68] This finding could be useful in clinical practice as first line approach through a simple, inexpensive biomarker to select elderly patients in whom the risk of having cirrhosis is trivial. Elderly HCV patients are indeed more at risk of having advanced fibrosis, but the clinician is less prone to perform a liver biopsy in them as the procedure is likely to be more risky than in younger patients.[41] It has been shown that complications related to liver biopsy procedure are more severe in patients with underlying cardiac or pulmonary disease, who also have a higher operative risk than younger patients.[69] As a consequence of the fear of the physician towards liver biopsy, elderly patients may be at risk of undermanagement, even though they generally present with a more severe disease and a faster fibrosis progression.

The present study has several strengths. The use of a multicentre cohort of well-characterised patients from numerous centres across the world gave us the opportunity to obtain a very large population of patients of different aetiologies, and with a wide spectrum of liver disease, ranging from absence of fibrosis to well-compensated, subclinical cirrhosis. For many of the non-invasive biomarkers investigated, this study represents one of the few independent validations, particularly in aetiologies other than CHC.

We acknowledge several limitations to our analysis. Indeed, this is a retrospective study and potential selection bias of patients may derive. The mean length of the liver biopsy in this study was 17.7 mm, which is somehow suboptimal according to recently recommended threshold of 20 mm.[10] However, we have investigated the effect of liver biopsy size on the performance of non-invasive markers and we found a similar performance for subjects with biopsy samples of <20 mm as compared to those with specimens >20 mm. This result is in line with other reports, in which the length of liver biopsy did not

influence significantly the performance of the non-invasive methods investigated.[19, 70] Interestingly, according to other recommendations, a biopsy specimen of 15 mm length gives sufficient histological information.[71] No information about fragmentation of liver biopsy specimens was available in the present study. Indeed, it has been demonstrated that the number of fragments may have a significant impact on the AUROC of non-invasive markers.[72] Our study did not have a central pathologist for the interpretation of liver histology. Lack of evaluation by a single Pathologist of all biopsies could be seen as a weakness of our study, but it better describes what occurs in real life. Moreover, the use of morphometric quantification of liver fibrosis would have minimised subjectivity in the assessment of hepatic fibrosis.[73, 74] It should be here underlined that liver biopsy remains an imperfect gold standard due to intraobserver and interobserver variability, which mainly depends on the quality of liver specimen.[75–78] Moreover, it has been shown that even in the subpopulation with biopsy length ≥25 mm, there is still 35% of risk of false positive/negative of the biopsy result.[76] Mehta and colleagues have recently suggested that in the most favourable scenario, an AUROC >0.90 cannot be achieved when assessing significant fibrosis even for a perfect markers and that a correct way to validate non-invasive markers for liver fibrosis vs. liver biopsy would be to perform prospective studies using clinical outcomes.[79] Nevertheless, as liver biopsy remains the only direct way to assess liver histology, guidelines still recommend it for the staging of hepatic fibrosis.[3, 36, 43]

In conclusion, this large-scale, independent study suggests that the performance of non-invasive biomarkers can be highly influenced by aetiology of CLD. The stages of hepatic fibrosis may also influence the performance as expressed by AUROC, especially in subgroups of patients where peculiar distribution of liver fibrosis stages is expected. Finally, among a series of non-invasive biomarkers, APRI and Fibrotest-Fibrosure showed the best performance. Both these non-invasive biomarkers may be used in clinical practice to safely exclude cirrhosis in HCV monoinfected and HIV/HCV coinfected cases. The choice of the method may depend on the local availability and experience of the clinician. APRI could be the first choice in regions with limited healthcare resources. On the other hand, Fibrotest-Fibrosure has the advantage to classify all cases, while APRI leaves a significant percentage of cases unclassified. In patients with NASH, Fibrotest-Fibrosure may also be applied in clinical practice to exclude cirrhosis, while it cannot be satisfactorily used for the diagnosis of significant fibrosis. In elderly HCV patients, presence of cirrhosis may be excluded with high certainty with a specific cut-off of platelets. For the diagnosis of significant fibrosis, especially in HCV carriers with normal ALT, performance of non-invasive biomarkers is not such to substitute liver biopsy. Further prospective studies about application of the most promising non-invasive biomarkers for liver fibrosis, especially in aetiologies other than HCV and using clinical outcomes as reference standard, are needed.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Demographic, laboratory and histological characteristics of 595 HCV patients with normal ALT and of 190 HCV patients with age ≥65 years and comparison with the whole HCV population.

Table S2. Performance of non-invasive biomarkers for significant fibrosis and correction for the stages of hepatic fibrosis in 595 HCV cases with normal ALT.

Table S3. Performance of non-invasive biomarkers for cirrhosis and correction for the stages of hepatic fibrosis in 595 HCV cases with normal ALT.

Table S4. Performance of non-invasive biomarkers for significant fibrosis and correction for the stages of hepatic fibrosis in 190 HCV elderly cases.

Table S5. Performance of non-invasive biomarkers for cirrhosis and correction for the stages of hepatic fibrosis in 190 HCV elderly cases.

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