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A Prospective Study of the Utility of Plasma Biomarkers to Diagnose Alcoholic Hepatitis

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The diagnosis of alcoholic hepatitis (AH) often requires a transjugular liver biopsy (TJLB), a procedure that is not always readily accessible. We analyzed plasma biomarkers to estimate the presence of histological features of AH among patients with clinical suspicion of AH. Using enzyme-linked immunosorbent assay, we tested M65 and M30 (circulating fragments of cytokeratin-18) and their respective fraction carried by microvesicles (MVs), CCL20 and TREM1. Leukocyte, platelet, and endothelial-derived MVs were quantified by way of flow cytometry. Test and validation cohorts prospectively included patients with clinical features of AH undergoing TJLB. In the test cohort, 46 of 83 (55%) patients showed histological features of AH. Age, bilirubin, INR, and creatinine (ABIC) score was B or C in 83%. Patients with histologically proven AH had higher levels of total and MV-bound M65 and total and MV-bound M30 and CCL20 than those without ($P < 0.001$ for all tests). Levels of TREM-1 and of subpopulations of MVs were not different between groups. M65 and M30 both had an area under the receiver operating characteristics curve of 0.84 to estimate the presence of AH. For M65, a cutoff of 2000 IU/L had a positive predictive value of 91%, whereas a cutoff of 641 IU/L had a negative predictive value of 88%. In the validation cohort, AH was histologically confirmed in 48 of 68 (71%) patients. ABIC score was B or C in 69% of patients. For M65, the above cutoffs had a diagnostic accuracy of 81%. Even better results were obtained in patients with suspicion of severe AH (ABIC B or C) in both cohorts. *Conclusion:* Plasma levels of cytokeratin-18 fragments are reliable noninvasive markers of AH. Using the proposed cutoffs for M65, two thirds of TJLB can be avoided, which can be useful in centers where this technique is not readily available. (HEPATOLOGY 2017;66:555-563).

Alcoholic hepatitis (AH) is an acute-on-chronic type of liver injury that occurs in patients with heavy drinking. Current therapeutic options (i.e., corticosteroids, pentoxifylline, *N*-acetylcysteine) are still associated with a high mortality, with a 90-day mortality of 20%-50% in patients with severe disease.[1-3] The best approach to the diagnosis of AH is a matter of controversy.

The presence of AH can be suspected based on clinical and biochemical data, but a definitive diagnosis often requires histological confirmation based on hepatocyte ballooning, lobular neutrophil infiltration, and

Abbreviations: ABIC, age, bilirubin, international normalized ratio, and creatinine; AH, alcoholic hepatitis; AUROC, area under the receiver operating characteristics curve; CK-18, cytokeratin-18; MV, microvesicles; NPV, negative predictive value; PPV, positive predictive value; TJLB, transjugular liver biopsy.

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Mallory-Denk bodies.[1] Due to coagulation derangements in patients with severe liver disease, a transjugular liver biopsy (TJLB) is often preferred over the percutaneous technique. However, TJLB is a highly specialized technique that is not readily accessible in many centers. In routine clinical practice as well as in recent randomized clinical trials, the diagnosis of AH is frequently based on a clinical diagnosis without histological confirmation.[3,4] However, histologic findings of AH are absent in 10%-50% of patients with clinical suspicion of AH.[5-7] Biomarkers capable of differentiating decompensated alcoholic cirrhosis with and without superimposed AH is an urgent need in clinical hepatology.

The aims of this study were to identify such noninvasive biomarkers for the diagnosis of AH and to test their diagnostic accuracy. We focused on biomarkers associated with the histological characteristics of AH. Mallory-Denk bodies are cytoplasmic inclusions representing rearrangements of the cell cytoskeleton resulting from, but not exclusive to, ethanol injury.[8] Their presence is considered highly suggestive of AH. A main constituent of Mallory-Denk bodies is cytokeratin-18 (CK-18). Following apoptosis and necrosis, CK-18 fragments can be released in the plasma by hepatocytes and may thus reflect hepatocyte cell death.[9] Two forms of CK-18 fragments can be detected: the monoclonal antibody M65 detects both full length and fragmented forms while a different antibody (M30) detects the cleaved fragment.[10] Part of soluble CK-18 is in fact carried by microvesicles (MVs), *i.e.* extracellular vesicles released in extracellular space following cell activation or apoptosis.[11] Another histologic hallmark of AH is lobular neutrophil infiltration. CCL20 is a proinflammatory chemokine. Its levels are increased in the serum of patients with AH and are associated with key clinical features of the disease, including fibrosis stage, portal hypertension severity, endotoxaemia, and hepatic neutrophil infiltration.[12] TREM-1 is a receptor that is expressed and released by polymorphonuclear cells after exposure to bacteria membrane components.[13,14] AH is associated with high circulating lipopolysaccharide levels, suggesting that TREM-1 levels might be useful for AH diagnosis.[15] Activated leukocytes as well as endothelial cells in AH can also release MVs.[11] We therefore assessed the potential usefulness of circulating levels of M65 and M30 and their respective MV-bound fraction, CCL20, TREM-1, and different subpopulations of MVs for the noninvasive diagnosis of AH.

Patients and Methods

PATIENTS

Consecutive patients undergoing TJLB for clinical suspicion of AH in two centers were included in the study. The test cohort prospectively included patients at Hôpital Beaujon (Clichy, France) from June 2013 to March 2015. The results from the test cohort were validated in a prospective cohort of patients previously included (from February 2010 to May 2011) at Hospital Clinic in Barcelona, Spain. Clinical suspicion of AH was based on the following criteria: excessive alcohol consumption (>60 g/day) before admission, moderately elevated aminotransferases with aspartate aminotransferase > alanine aminotransferase and high levels of gamma glutamyl transpeptidase and serum bilirubin. Severe AH was defined as an age, bilirubin,

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international normalized ratio, and creatinine (ABIC) score $\geq$6.71 (ABIC B and C) at admission.[16] Exclusion criteria were: hepatocellular carcinoma, active extrahepatic cancer, prior liver transplantation, and prior placement of a transjugular intrahepatic portosystemic shunt.

This study was approved by the Institutional Review Boards of Paris North Hospitals, Paris 7 University, AP-HP (no. 11-112), and Hospital Clinic in Barcelona. All patients included in this study gave written informed consent. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

HISTOLOGIC, CLINICAL, AND HEMODYNAMIC ASSESSMENT

Demographic, clinical, and analytical parameters were prospectively collected in both centers. All liver biopsy specimens in both cohorts were obtained by the transjugular approach within 48 hours of admission. Liver specimens were formalin-fixed and paraffin-embedded, and 3-μm slides were stained with hematoxylin and eosin and Masson trichrome. Three expert liver pathologists analyzed all biopsy specimens using previously published histological criteria.[17] The histological diagnosis of AH was based on the presence of hepatocellular injury (hepatocellular ballooning and presence of Mallory-Denk bodies), inflammatory infiltrate (predominantly polymorphonuclear cells), and pericellular fibrosis.[17] The pathologists were unaware of the results of plasma biomarkers analyses. The portal pressure was estimated based on the hepatic venous pressure gradient as described in detail previously.[18]

Measurement of Plasma Biomarkers Levels

For patients of the test cohort, peripheral venous blood was drawn into citrate tubes the day of the liver biopsy and was centrifuged twice at 2500*g* for 15 minutes at 20°C to obtain platelet-free plasma. For patients of the validation cohort, central venous blood was drawn into an ethylene diamine tetraacetic acid (EDTA) tube the day of the liver biopsy, and was then centrifuged at 500*g* for 10 minutes to obtain plasma. Samples were then stored frozen at −80°C until use. Plasma samples were used for measurement of CCL20 (DY360, R&D Systems Europe, Lille, France), and TREM1 (DY1278B, R&D Systems Europe, Lille, France) according to the manufacturer's instructions. We also measured plasma levels of total (M65 Epi-Death ELISA, Peviva, Bromma, Sweden) and caspase-cleaved (M30-Apoptosense ELISA, Peviva, Bromma, Sweden) CK-18. Moreover, we determined the proportion of soluble CK-18 carried by MVs by measuring circulating CK-18 levels after filtration of the plasma through two 0.2-μm filters (Ceveron MFU 500, Technoclone, Austria). As described previously, the difference between soluble CK-18 levels in initial and in filtered plasma reflects the fraction of soluble CK-18 carried by MVs.[11] Dosing of the biomarkers was performed by an engineer unaware of the clinical characteristics and histological diagnosis of the patients. All assays were performed in duplicate with determination of the coefficient of variation between samples from the same patient. The results were considered adequate when the coefficient of variation was less than 12%. Otherwise, samples were measured again.

Circulating levels of platelet (CD41+), leuko-endothelial (CD31+/41−), pan-leukocyte (CD11a+), and endothelial (CD62e+) MVs were determined on a Gallios flow cytometer (Beckman Coulter, Villepinte, France) using a technique described previously.[11] Anti–CD11a- phycoerythrin, anti–CD31-phycoerythrin, and anti–CD41-phycoerythrin-cyanin7 antibodies as well as their matched isotype controls were obtained from Beckman-Coulter. Anti–CD62e-fluroisothiocyanate antibodies as well as their matched isotype controls were obtained from R&D Systems Europe.

STATISTICAL ANALYSIS

Quantitative variables were expressed as median (interquartile range) and categorical variables as absolute and relative frequencies. Comparisons of independent quantitative variables between groups were performed using the Mann-Whitney test. Comparisons of qualitative variables between groups were performed using the chi-square test and Fisher's exact test, as appropriate. Receiver operating characteristics curves were generated to assess the diagnostic performance of plasma biomarkers for the presence or absence of AH and to establish diagnostic cutoffs. Previously proposed clinical tools for the diagnosis of AH were tested, namely the serum bilirubin level[19] and the white blood cell and platelet counts.[20] All tests were two-sided, and significance was set at $P < 0.05$. Statistical analyses and figures were performed using the SPSS statistical package 20.0 software (SPSS Inc.,

TABLE 1. Characteristics of Patients With and Without Biopsy-Confirmed AH at Time of Liver Biopsy

	Test Cohort (n = 83)			Validation Cohort (n = 68)		
Characteristic	No AH (n = 37)	AH (n = 46)	*P*	No AH (n = 20)	AH (n = 48)	*P*
Age, y	51 (48-57)	55 (48-57)	0.38	57 (49-64)	52 (47-56)	0.08
Men:Women, n	31:6	37:9	0.70	15:5	37:11	0.85
Serum bilirubin, μmol/L	72 (59-142)	132 (64-304)	0.06	47 (32-102)	104 (45-336)	0.03
Serum creatinine, μmol/L	67 (55-86)	61 (53-74)	0.44	80 (57-96)	80 (62-97)	0.98
INR	1.9 (1.5-2.1)	1.7 (1.5-2.1)	0.63	1.6 (1.2-1.8)	1.6 (1.3-1.8)	0.79
Serum albumin, g/L	23 (21-26)	22 (19-26)	0.47	29 (25-31)	26 (23-32)	0.44
AST, IU/L	73 (54-126)	102 (74-135)	0.02	99 (64-214)	114 (67-150)	0.94
AST:ALT ratio	2.0 (1.5-2.3)	2.3 (1.7-3.0)	0.05	2.2 (1.5-4.0)	2.6 (1.7-3.7)	0.46
GGT, IU/L	106 (43-192)	270 (149-439)	<0.001	136 (73-346)	199 (126-430)	0.37
CRP, mg/L*	14 (5-24)	27 (12-53)	0.01	—	—	—
WBC, $\times 10^9$/L	5.9 (4.4-10.2)	11.7 (6.7-15.4)	<0.001	7.0 (5.5-10.6)	8.4 (6.8-12.6)	0.10
Platelets, $\times 10^9$/L	96 (59-130)	149 (89-199)	0.001	134 (88-200)	100 (77-156)	0.10
HVPG, mm Hg	20 (17-24)	19 (15-21)	0.11	17 (13-21)	19 (15-23)	0.43
MELD score	19 (16-22)	20 (16-26)	0.25	15 (12-21)	19 (14-26)	0.13
Maddrey's discriminant function	47 (31-66)	46 (29-76)	0.85	—	—	—
ABIC score	7.4 (6.8-8.4)	7.7 (7.1-8.7)	0.13	7.8 (6.4-8.7)	7.3 (6.7-8.7)	0.84
ABIC score B or C, n (%)	30 (81)	39 (85)	0.52	12 (60%)	35 (73%)	0.29
Documented bacterial infection, n (%)	10 (27)	8 (17)	0.29	—	—	—

Results are presented as the median (interquartile range) unless noted otherwise.
*Missing in 11 patients.
Abbreviations: ABIC, Age-bilirubin-INR-creatinine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; MELD, Model for End-Stage Liver Disease; CRP, c-reactive protein; HVPG, hepatic venous pressure gradient; INR, international normalized ratio; WBC, white blood cells.

Chicago, IL) and GraphPad Prism 5 software, respectively.

Results

PATIENT CHARACTERISTICS

Eighty-three patients with clinical suspicion of AH were included in the test cohort and 68 patients were included in the validation cohort. Among them, 46 (55%) and 48 (71%) had biopsy-confirmed AH, respectively. The clinical, laboratory and hemodynamic characteristics of the patients are shown in Table 1. There was no difference in the ABIC and Model for End-Stage Liver Disease scores and Maddrey's discriminant function between patients with and without histologically confirmed AH in both cohorts. Severe disease, defined by an ABIC score of ≥6.71, was present in 69 of 83 (83%) patients of the test cohort and 47 of 68 (69%) patients in the validation cohort. The clinical features of these patients are presented in Supporting Table S1. In the test cohort, four of 83 patients had been exposed to presumptive corticosteroid

TABLE 2. Plasma Levels of Tested Biomarkers in Patients With and Without AH

	Test Cohort (n = 83)			Validation Cohort (n = 68)		
Biomarker	No AH (n = 37)	AH (n = 46)	*P*	No AH (n = 20)	AH (n = 48)	*P*
M65, U/L	481 (166-991)	3857 (909-6227)	<0.001	869 (475-869)	2654 (1266-6070)	< 0.001
MV M65, U/L	75 (23-154)	805 (158-2383)	<0.001	119 (43-388)	492 (224-1161)	0.001
M30, U/L	252 (153-576)	2401 (606-4732)	<0.001	451 (320-708)	938 (617-1936)	< 0.001
MV M30, U/L	37 (10-129)	736 (98-1928)	<0.001	63 (15-158)	273 (121-438)	< 0.001
CCL20, pg/mL	93 (50-279)	336 (150-827)	<0.001	—	—	—
TREM-1, pg/mL	183 (135-273)	220 (145-290)	0.31	—	—	—
Annexin V+ MV, concentration per μL	7531 (4447-12,044)	7096 (5464-10,871)	0.85	—	—	—
CD41+31- MV, concentration per μL	1468 (759-2815)	1691 (907-3226)	0.69	—	—	—
CD41-31+ MV, concentration per μL	137 (45-384)	161 (72-280)	0.73	—	—	—
CD11a+ MV, concentration per μL	26 (9-81)	19 (5-72)	0.43	—	—	—
CD62e+ MV, concentration per μL	99 (49-335)	189 (96-403)	0.08	—	—	—

Results are presented as the median (interquartile range).

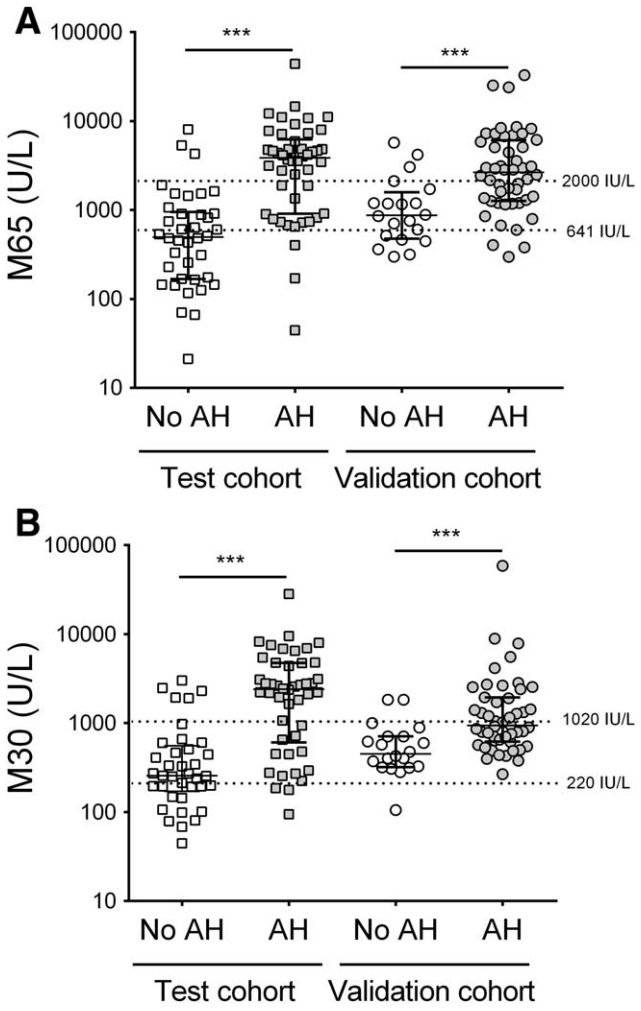

FIG. 1. Dot plots showing plasma levels of two forms of CK-18 fragments, namely (A) the monoclonal antibody M65 detecting full length and fragmented forms and (B) the M30 antibody detecting the cleaved fragment in patients with and without biopsy-confirmed AH in the test and validation cohorts. Data are presented as the median (horizontal bar) and interquartile range (error bar). $^{***}P < 0.001$.

treatment for 1, 1, 2, and 4 days prior to TJLB. In the validation cohort, none of the patients received corticosteroids before TJLB.

In 37 patients without histologic features of AH, in the test cohort, the diagnosis was alcoholic cirrhosis in 35 (95%) patients, steatosis with advanced fibrosis in one patient, and cholestasis with features suggestive of biliary salt transporters gene mutation in one patient. Two patients had inflammatory features suggestive of superimposed viral or drug-induced hepatitis. In the validation cohort, the diagnosis was alcoholic cirrhosis or advanced fibrosis in 14 of 20 (70%) patients, hypoxic hepatitis in three of 20 (14%) patients, alcoholic foamy degeneration in two of 20 (10%) patients, and acute infectious mononucleosis hepatitis in one patient.

In patients without histologic features of AH, in the test cohort, potential causes of decompensation were bacterial infection in 10 patients, recent (<2 weeks) gastrointestinal bleeding in six patients, and acute kidney injury in one patient. In the validation cohort, five patients had bacterial infection and three patients had recent gastrointestinal bleeding. No cause other than excessive alcohol consumption was found in the other patients. None of the patients had hepatocellular carcinoma.

DIAGNOSTIC PERFORMANCE OF TESTED PLASMA BIOMARKERS IN THE TWO COHORTS

In the test cohort, median plasma levels of M65, MV-M65, M30, MV-M30, and CCL20 were significantly higher in patients with biopsy-confirmed AH than in those in whom TJLB ruled out this diagnosis (Table 2 and Fig. 1; $P < 0.001$ for all tests). There was no significant difference in the plasma levels of TREM1 and of Annexin V–positive, platelet, leukocyte, or endothelial MVs between patients with and without AH. The diagnostic performance to estimate the presence of AH of each plasma biomarker is shown in Table 3.

In the validation cohort, we observed similar results (Tables 2 and 3 and Fig. 1). Levels of CCL20 and TREM-1 were not tested in the validation cohort as the area under the receiver operating characteristics curve (AUROC) were lower than those generated by M65 and M30 in patients from the test cohort.

Using upper and lower cutoffs of M65 at 2000 and 641 U/L, the presence of AH could be ruled in or ruled out in over two thirds of the patients, with a diagnostic accuracy of 90% and 81% in the test and validation cohorts, respectively (Table 3 and Fig. 2). Using upper and lower cutoffs of M30 at 1020 and 220 U/L, the presence of AH could also be ruled in or ruled out in two thirds of the patients with a diagnostic accuracy of 86% and 92% in the test and validation cohorts, respectively (Table 3 and Fig. 3). The clinical characteristics of patients misclassified with M65 cutoffs of 2000 and 641 U/L are presented in Supporting Table S2. We did not identify a clinical characteristic of patients misclassified with M65 cutoffs of 2000 and 641 U/L common to the test and validation cohorts (Supporting Table S2).

TABLE 3. Diagnostic Performance of Tested Biomarkers for the Presence of Biopsy-Confirmed AH

Biomarker	Cutoff	Sensitivity, %	Specificity, %	PPV, %	NPV, %	AUROC
Test cohort (n = 83)						
M65, U/L	2000	67 (57-77)	92 (86-98)	91 (85-97)	69 (59-79)	0.84 (0.75-0.93)
M65, U/L	641	93 (88-99)	62 (52-73)	75 (66-85)	88 (82-95)	0.84 (0.75-0.93)
MV M65, U/L	161	76 (67-85)	81 (73-90)	83 (75-91)	73 (64-83)	0.82 (0.72-0.91)
M30, U/L	1020	70 (60-79)	86 (79-94)	86 (79-94)	70 (60-79)	0.84 (0.76-0.93)
M30, U/L	220	93 (88-99)	46 (35-57)	68 (58-78)	85 (77-93)	0.84 (0.76-0.93)
MV M30, U/L	143	72 (62-81)	84 (76-92)	85 (77-92)	70 (61-80)	0.84 (0.76-0.93)
CCL20, pg/mL	141	83 (74-91)	65 (55-75)	75 (65-84)	75 (66-84)	0.72 (0.61-0.84)
TREM-1, pg/mL	211	54 (44-65)	68 (57-78)	68 (57-78)	54 (44-65)	0.55 (0.43-0.68)
Validation cohort (n = 68)						
M65, U/L	2000	58 (47-70)	80 (70-90)	88 (80-95)	33 (44-56)	0.78 (0.67-0.90)
M65, U/L	641	92 (85-98)	35 (24-46)	77 (67-87)	64 (52-75)	0.78 (0.67-0.90)
MV M65, U/L	161	85 (77-94)	55 (43-67)	82 (73-91)	61 (50-73)	0.75 (0.62-0.87)
M30, U/L	1020	46 (34-58)	90 (83-97)	92 (85-98)	41 (29-53)	0.79 (0.67-0.91)
M30, U/L	220	72 (61-82)	5 (0-10)	100 (100-100)	100 (100-100)	0.79 (0.67-0.91)
MV M30, U/L	143	65 (53-76)	75 (65-85)	86 (78-94)	47 (35-59)	0.80 (0.68-0.92)

Values in parentheses are 95% confidence intervals.

DIAGNOSTIC PERFORMANCE OF TESTED BIOMARKERS IN PATIENT SUBPOPULATIONS

When restricting the analyses to patients with suspicion of severe AH (ABIC score ≥6.71), we obtained similar results as shown in Supporting Tables S3 and S4 and in Supporting Figures S1 and S2. Using the same cutoff values of M65, the presence of AH could again be ruled-in or ruled-out in over two-thirds of the patients with a diagnostic accuracy of 92 and 89% in the test and validation cohorts, respectively. M30 had a diagnostic accuracy of 85 and 95% in the test and validation cohorts, respectively.

In the test cohort, there were 18/83 patients with recent bacterial infection, defined by positive blood, urine, or ascites culture, signs of pneumonia, or positive white blood cell count (>250 polymorphonuclear

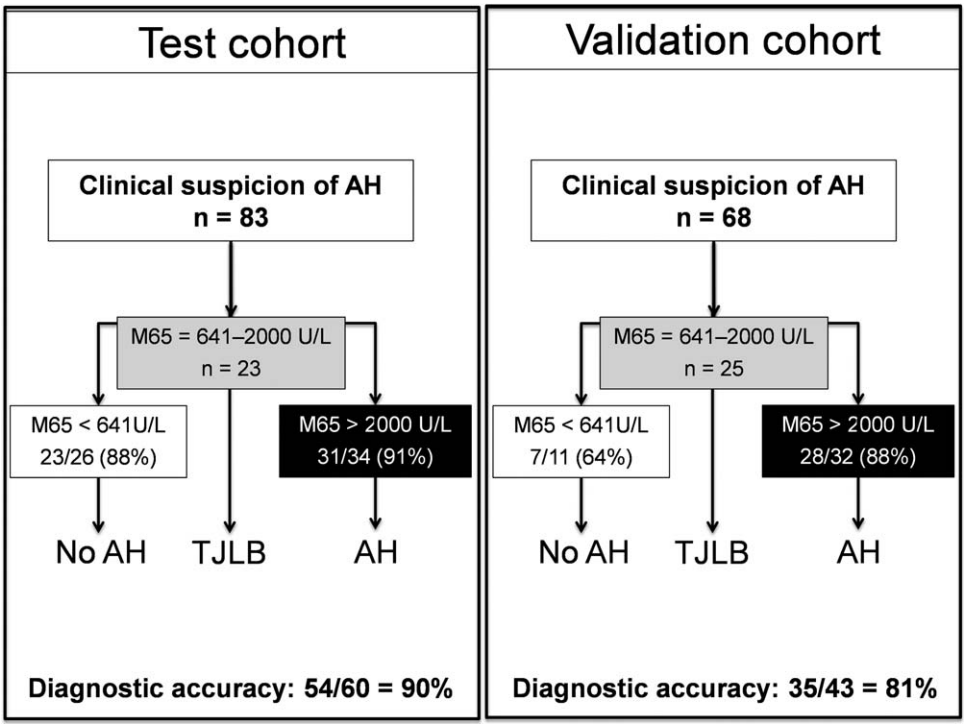

FIG. 2. Proposed diagnostic algorithm of AH taking into account M65 levels.

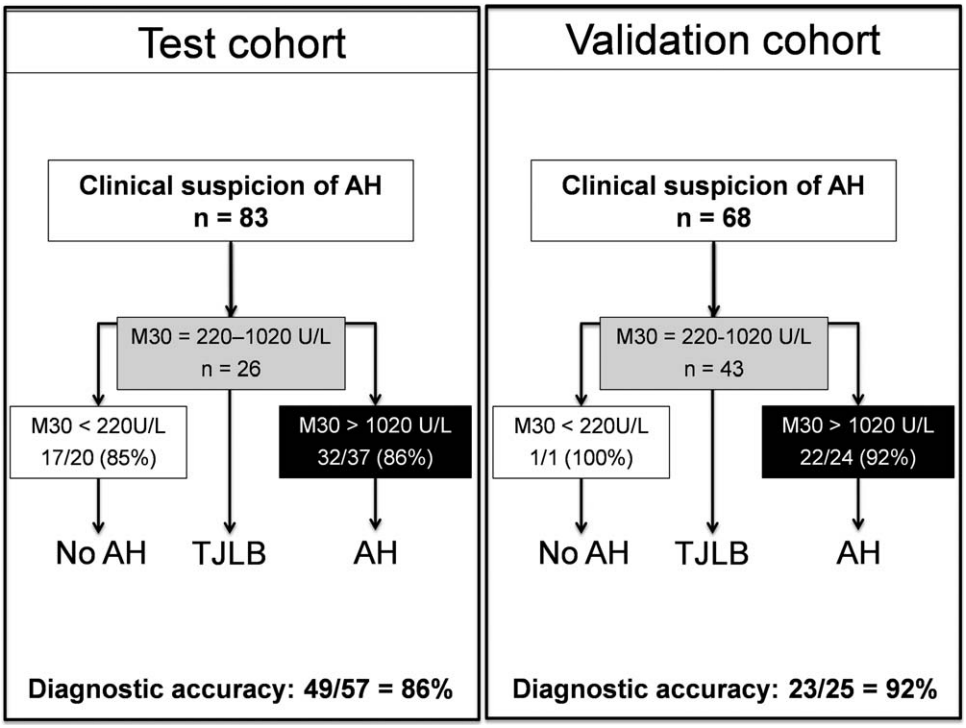

FIG. 3. Proposed diagnostic algorithm of AH taking into account M30 levels.

cells/μL) in the ascitic fluid. When excluding these patients from the analyses, the diagnostic accuracy of CK-18 fragments was slightly better than in the entire cohort, as shown in Supporting Figures S4 and S5. Data on bacterial infection were not prospectively collected in the validation cohort.

In the test cohort, five of 83 patients had replicative hepatitis B or C virus infection. When excluding these patients from the analyses, both CK-18 fragments had similar diagnostic accuracy to the whole test cohort (89% for M65; 87% for M30; data not shown). In the validation cohort, none of the patients had hepatitis B or C virus infection.

DIAGNOSTIC PERFORMANCE OF PREVIOUSLY PROPOSED TESTS

An abrupt increase in serum bilirubin of $\geq$5 mg/dL has been proposed as a diagnostic criterion of AH.[19] However, AH was present in only 31 of the 48 patients (65%) in the test cohort and 27 of the 34 (79%) patients in the validation cohort with a serum bilirubin of 5 mg/dL or more. It has also been proposed recently that the association of more than 10.75×10^9/L white blood cells with more than 147.5×10^9/L platelets should diagnose AH and that less than 5.95×10^9/L white blood cells with less than 86×10^9 platelets should rule out AH.[20] These combined parameters had a positive predictive value (PPV) of 86% (19/22) and negative predictive value (NPV) of 67% (10/15) in the test cohort. According to the latter diagnostic algorithm, TJLB remained necessary in 47 of 83 (57%) of patients. In the validation cohort, the PPV was 63% (5/8) and the NPV was 0% (0/3), and TJLB remained indicated in 57 of 68 (84%) patients.

Discussion

This study shows that circulating levels of CK-18 fragments, and particularly the M65 epitope, can be useful in the noninvasive diagnosis of AH, and thus avoid TJLB in two thirds of patients.

The main finding of this study is a high diagnostic accuracy of CK-18 fragments to estimate the presence of AH in patients with clinical suspicion of this disease. Several groups previously reported elevated circulating levels of CK-18 fragments in patients with excessive alcohol consumption. Some authors found high tissue-polypeptide–specific antigen, a CK-18 epitope, in the serum of patients with active alcohol consumption, including some with proven AH.[21,22]

Other groups observed high circulating levels of M65 and M30 epitopes in patients with alcoholic liver disease and advanced fibrosis[23] and in patients with clinically presumed AH but without histological assessment.[24] A correlation between serum levels of M65 and the degree of apoptosis on liver biopsy has also been observed in heavy drinkers.[25] However, the performance of M65 and M30 for the diagnosis of AH in patients with clinical suspicion of this diagnosis remained unexplored. A strength of this study is the reproducibility of its results. First, we obtained similar results in the test and validation cohorts despite differences in the proportion of patients in whom biopsy confirmed the diagnosis of AH. In the test cohort, the proportion of patients with histologically confirmed AH was in the lower range of previously reported rates.[5-7] This is likely explained by the need to definitely rule in or rule out AH in patients with ongoing alcohol drinking and severe liver dysfunction who are potential candidates for early liver transplantation, a treatment proposed to highly selected candidates with corticosteroid-resistant AH at our center, and by the ease of access to TJLB.[26] It must be noted that there was no significant difference in the proportion of patients with proven bacterial infection between patients with and without biopsy-proven AH. An illustration of the robustness of our findings is the similar or even better diagnostic accuracy of M65 and M30 in patients with suspicion of severe AH, namely with an ABIC score of $\geq$6.71, or in patients without bacterial infection or in patients without viral infection. Moreover, we obtained these results using the same cutoffs in the test and validation cohorts, despite differences in handling and processing of the plasma samples. This finding suggests that M65 and M30 are not sensitive to preanalytical parameters and therefore are suitable biomarkers for routine practice. Roughly, one third of both CK-18 fragments were carried by circulating MV. Their quantification produced a good diagnostic accuracy to estimate the presence of AH. However, it did not increase the diagnostic yield of total M65 and M30 dosing. Therefore, because the determination of the fraction of CK-18 fragments carried by MVs is a less straightforward procedure, we think that our results do not support its use in this clinical setting. Future studies could address the outcome of CK-18 fragments after alcohol consumption interruption, treatment with corticosteroids, or both. The kinetics of CK-18 fragment levels could carry valuable information about patient outcomes.

Another major finding of this study is that these biomarkers show better performance than traditional biochemical and hematological parameters in estimating the presence of AH. The current paradigm that a serum bilirubin level of 5 mg/dL or more adequately identifies patients with AH among heavy drinkers[19] was not observed in this study as AH was histologically ruled out in roughly a third of the patients with this level of hyperbilirubinemia in both cohorts. Regarding the recently proposed the use of white blood cell and platelet counts,[20] in our test cohort, the proposed upper limit cutoffs had an interesting PPV of 86%, but a low NPV of 64%. The diagnostic performance was much lower in the validation cohort in which the results were not reproduced. Moreover, using the proposed algorithm, TJLB remained indicated in the majority of patients from both cohorts. Recently, the use of the AshTest[27,28] (BioPredictive, Paris, France), has also been proposed as a noninvasive tool for the diagnosis of severe AH. Although we were not able to measure this test in our cohort, the AUROC of AshTest in the original publication was lower than the AUROC we obtained with M65 and M30 in both cohorts.

Based on our results, we propose the use of M65 as a noninvasive diagnostic tool for patient with suspected AH in centers where TJLB is not readily available. In patients with M65 levels below 641 U/L, AH can be reasonably ruled out. In those with M65 levels above 2000 U/L, AH is very likely. Patients with M65 levels between 641 and 2000 U/L should be referred for TJLB. This pragmatic approach proposes a potential solution to the debate over the need for systematic liver biopsy to establish the diagnosis of AH.

In conclusion, this prospective study reveals that circulating fragments of CK-18 have a good diagnostic performance to estimate the presence of AH noninvasively in patients with clinical suspicion of this disease, including patients with the severe form of the disease, in test and validation cohorts. This is particularly true in patients without recent bacterial infection. Circulating fragments of CK-18 outperformed other analytical noninvasive tools for the diagnosis of AH. Therefore, they may be a valuable diagnostic tool, especially in centers with difficult access to TJLB.

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