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(54) Title: METHODS AND KITS FOR DIAGNOSING ALCOHOLIC HEPATITIS

(57) Abstract: The present invention relates to methods and kits for diagnosing alcoholic hepatitis. In particular, the present invention relates to a method of diagnosing alcoholic hepatitis in a subject comprising i) determining the level of at least one cytokeratin-18 fragment in a blood sample obtained from the subject, ii) comparing the level determined at step i) with a predetermined reference value and iii) detecting differential between the level determined at step i) with the predetermined reference value indicates that the subject suffers or does not suffer from alcoholic hepatitis.

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METHODS AND KITS FOR DIAGNOSING ALCOHOLIC HEPATITIS

FIELD OF THE INVENTION:

The present invention relates to methods and kits for diagnosing alcoholic hepatitis.

BACKGROUND OF THE INVENTION:

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 Mallory-Denk bodies.(1) Due to coagulation derangements in patients with severe AH, a transjugular liver biopsy (TJLB) is often preferred over the percutaneous technique. However, TJLB is a highly specialized technique 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.

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)

SUMMARY OF THE INVENTION:

The present invention relates to methods and kits for diagnosing alcoholic hepatitis. In particular the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The diagnosis of alcoholic hepatitis (AH) often requires a transjugular liver biopsy (TJLB), a procedure not always readily accessible. The inventors analyzed plasma biomarkers to estimate the presence of histological features of AH among patients with clinical suspicion of AH. Using ELISA, they tested M65 and M30 (circulating fragments of cytokeratin-18) and their respective fraction carried by microvesicles (MVs). Test and validation cohorts prospectively included patients with clinical features of AH undergoing TJLB. Patients with histologically proven AH had higher levels of total and MV-bound M65, total and MV-bound M30 than those without ($p < 0.001$ for all tests). M65 and M30 both had AUROCs of 0.84 to estimate the presence of AH. For M65, a cutoff of 2000 IU/L had a positive predictive value of 91%, while a cutoff of 641 IU/L had a negative predictive value of 88%. In the validation cohort, AH was histologically confirmed in 48/68 (71%) patients. ABIC score was B or C in 69%. 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. In conclusion, plasma levels of cytokeratin-18 fragments are reliable non-invasive markers of AH.

Accordingly, the first object of the present invention relates to a method of diagnosing alcoholic hepatitis in a subject comprising i) determining the level of at least one cytokeratin-18 fragment in a blood sample obtained from the subject, ii) comparing the level determined at step i) with a predetermined reference value and iii) detecting differential between the level determined at step i) with the predetermined reference value indicates that the subject suffers or does not suffer from alcoholic hepatitis.

As used herein the term “cytokeratin-18” or “CK18” has its general meaning in the art and refers to the protein encoded by KRT18 gene (Gene ID 3875). Thus the term “cytokeratin-18 fragment” refers to any fragment released in the plasma by hepatocyte following apoptosis (e.g. caspase) or necrosis.

In some embodiments, the level of M30 cytokeratin-18 is determined in the blood sample. In some embodiments, the level of M65 cytokeratin-18 fragment is determined in the blood sample.

As used herein, the term “M30 cytokeratin-18 fragment” refers to the caspase cleaved fragment of human keratin 18 protein (or “cytokeratin-18,” “CK-18,” “keratin-18,” “K18”) encoded by the KRT18 gene, and is a serum indicator of cellular apoptosis. The fragment is specifically recognized by M30 antibody which detects a neoepitope mapped to positions 387 to 396 of a 21-kDa fragment of CK18 (CK18Asp396 neoepitope) that is only revealed after caspase cleavage of the protein and is postulated as a selective biomarker of apoptotic cell death.

As used herein, the term “M65 cytokeratin-18 fragment” refers to the soluble human keratin 18 protein (or “cytokeratin-18,” “CK-18,” “keratin-18,” “K18”) encoded by the KRT18 gene, and is a serum indicator of cellular death. The fragment is specifically recognized by M65 antibody which detects a common epitope present in the full-length protein as well as the 21-
5 kDa caspase cleaved fragment and is thus believed to measure, in addition to apoptosis, intact CK18 that is released from cells undergoing necrosis.

As used herein, the term “blood sample” refers to a whole blood sample, serum sample and plasma sample. A blood sample may be obtained by methods known in the art including venipuncture or a finger stick. Serum and plasma samples may be obtained by centrifugation
10 methods known in the art. The sample may be diluted with a suitable buffer before conducting the assay.

The measurement of the level of the cytokeratin-18 fragment in the blood sample is typically carried out using standard protocols known in the art. For example, the method may comprise contacting the blood sample with a binding partner capable of selectively interacting
15 with the cytokeratin-18 fragment in the sample. In some embodiments, the binding partners are antibodies, such as, for example, monoclonal antibodies or even aptamers. For example the binding may be detected through use of a competitive immunoassay, a non-competitive assay system using techniques such as western blots, a radioimmunoassay, an ELISA (enzyme linked immunosorbent assay), a “sandwich” immunoassay, an immunoprecipitation assay, a precipitin
20 reaction, a gel diffusion precipitin reaction, an immunodiffusion assay, an agglutination assay, a complement fixation assay, an immunoradiometric assay, a fluorescent immunoassay, a protein A immunoassay, an immunoprecipitation assay, an immunohistochemical assay, a competition or sandwich ELISA, a radioimmunoassay, a Western blot assay, an immunohistological assay, an immunocytochemical assay, a dot blot assay, a fluorescence
25 polarization assay, a scintillation proximity assay, a homogeneous time resolved fluorescence assay, a IAsys analysis, and a BIAcore analysis. The aforementioned assays generally involve the binding of the partner (ie. antibody or aptamer) to a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells);
30 polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like. An exemplary biochemical test for identifying specific proteins employs a standardized test format, such as ELISA test, although the information provided herein may apply to the development of other biochemical or diagnostic tests and is not limited to the development of an ELISA test (see,

e.g., Molecular Immunology: A Textbook, edited by Atassi et al. Marcel Dekker Inc., New York and Basel 1984, for a description of ELISA tests). Therefore ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies which recognize the cytokeratin-18 fragment. A sample containing or suspected of containing the cytokeratin-18 fragment is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Measuring the level of the cytokeratin-18 fragment (with or without immunoassay-based methods) may also include separation of the compounds: centrifugation based on the compound's molecular weight; electrophoresis based on mass and charge; HPLC based on hydrophobicity; size exclusion chromatography based on size; and solid-phase affinity based on the compound's affinity for the particular solid-phase that is used. Once separated, said one or two biomarkers proteins may be identified based on the known "separation profile" e.g., retention time, for that compound and measured using standard techniques. Alternatively, the separated compounds may be detected and measured by, for example, a mass spectrometer. Typically, levels of immunoreactive biomarker (e.g. MR-proADM, NT-proBNP or sTNFR1) in a sample may be measured by an immunometric assay on the basis of a double-antibody "sandwich" technique, with a monoclonal antibody specific for the cytokeratin-18 fragment (Cayman Chemical Company, Ann Arbor, Michigan). According to said embodiment, said means for measuring the cytokeratin-18 fragment level are for example i) buffer, ii) a monoclonal antibody that interacts specifically with the the cytokeratin-18 fragment, iii) an enzyme-conjugated antibody specific for the cytokeratin-18 fragment and a predetermined reference value of the cytokeratin-18 fragment.

Level of M30 fragment can be measured using any commercially available assay system (Peviva AB, Sweden), and performed using previously described assays that can be validated to good clinical laboratory practice (see, Cummings J., et al., Br J Cancer 92:532-8, 2005; and Cummings J., et al., Br J Cancer 95:42-8, 2006).

Level of M65 fragment can be measured using any commercially available assay system (Peviva AB, Sweden), and performed using previously described assays that can be validated to good clinical laboratory practice (see, Cummings J., et al., Br J Cancer 92:532-8, 2005; and Cummings J., et al., Br J Cancer 95:42-8, 2006).

A predetermined reference value can be relative to a number or value derived from population studies, including without limitation, such subjects having same or similar age range, subjects in the same or similar ethnic group, and subjects having the same severity of symptom. Such predetermined reference values can be derived from statistical analyses and/or risk prediction data of populations obtained from mathematical algorithms and computed indices of metabolic syndrome. In some embodiments, the predetermined reference values are derived from the level of the cytokeratin-18 fragment in a control sample derived from one or more subjects who were not subjected to the event. Furthermore, retrospective measurement of the level of the cytokeratin-18 fragment in properly banked historical subject samples may be used in establishing these predetermined reference values. The predetermined reference value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the predetermined reference value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the level of the marker in a group of reference, one can use algorithmic analysis for the statistic treatment of the measured levels of the marker in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When $AUC > 0.5$, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.6 and 0.7, the accuracy is poor. When AUC is between 0.7 and 0.8, the accuracy is fair. When AUC is between 0.8 and 0.9 the accuracy is good. When AUC is higher than 0.9, the accuracy is excellent. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as:

MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

In some embodiments, when the level of the cytokeratin-18 fragment (M65 or M30) is below 641 U/L it can be concluded that the subject does not suffer from alcoholic hepatitis. In some embodiments, when the level of the cytokeratin-18 fragment is above 2000 U/L, it is concluded that the subject suffers from alcoholic hepatitis. When the level of the cytokeratin-18 fragment is between 641 and 2000 U/L further investigations are thus needed to conclude that the subject suffers from alcoholic hepatitis. For example a transjugular liver biopsy can be performed.

Once the subject is diagnosed as suffering from alcoholic hepatitis, he can be administered with a therapeutically effective amount of corticosteroids, pentoxifylline, or N-acetylcysteine. As used, the term "corticosteroids" has its general meaning in the art and refers to class of active ingredients having a hydrogenated cyclopentoperhydrophenanthrene ring system endowed with an anti-inflammatory activity. Corticosteroid drugs typically include cortisone, cortisol, hydrocortisone (11 β ,17-dihydroxy, 21-(phosphonoxy)-pregn-4-ene, 3,20-dione disodium), dihydroxycortisone, dexamethasone (21-(acetyloxy)-9-fluoro-1 β ,17-dihydroxy-16 α -m-ethylpregna-1,4-diene-3,20-dione), and highly derivatized steroid drugs such as beconase (beclomethasone dipropionate, which is 9-chloro-11- β , 17,21, trihydroxy-16 β -methylpregna-1,4 diene-3,20-dione 17,21-dipropionate). Other examples of corticosteroids include flunisolide, prednisone, prednisolone, methylprednisolone, triamcinolone, deflazacort and betamethasone. corticosteroids, for example, cortisone, hydrocortisone, methylprednisolone, prednisone, prednisolone, betamethasone, beclomethasone dipropionate, budesonide, dexamethasone sodium phosphate, flunisolide, fluticasone propionate, triamcinolone acetonide, betamethasone, fluocinolone, fluocinonide, betamethasone dipropionate, betamethasone valerate, desonide, desoximetasone, fluocinolone, triamcinolone, triamcinolone acetonide, clobetasol propionate, and dexamethasone.

A further object relates to a kit suitable for performing the method of the present invention which comprises a binding partner specific for the cytokeratin-18 fragment of interest. In some embodiments, said binding partners are antibodies as described above. In some embodiments, these antibodies are labelled as described above. Typically, the kits described above will also comprise one or more other containers, containing for example, wash reagents, and/or other reagents capable of quantitatively detecting the presence of bound antibodies. Typically compartmentalised kit includes any kit in which reagents are contained in separate

containers, and may include small glass containers, plastic containers or strips of plastic or paper. Such containers may allow the efficient transfer of reagents from one compartment to another compartment whilst avoiding cross-contamination of the samples and reagents, and the addition of agents or solutions of each container from one compartment to another in a quantitative fashion. Such kits may also include a container which will accept the blood sample, a container which contains the antibody(s) used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, and like), and containers which contain the detection reagent.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: Dot plots showing plasma levels of two forms of cytokeratin-18 fragments, namely the monoclonal antibody M65 detecting full length and fragmented forms and the M30 antibody detecting the cleaved fragment in patients with and without biopsy-confirmed AH in the test and validation cohorts

Figure 2: proposed diagnostic algorithm of AH taking into account M65 levels

Figure 3: proposed diagnostic algorithm of AH taking into account M30 levels

EXAMPLE:

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 obtained in the test cohort were validated in a prospective cohort of patients previously included (from February 2010 to May 2011) at Hospital Clinic, Barcelona, Spain. Clinical suspicion of AH was based on the following criteria: excessive alcohol consumption (>60 g/day) prior to admission, moderately elevated aminotransferases with aspartate aminotransferase [AST] > alanine aminotransferase [ALT], high gamma glutamyl transpeptidase (GGT) and serum bilirubin levels. Severe AH was defined as an Age, Bilirubin, INR and Creatinine (ABIC) score ≥ 6.71 (ABIC B and C) at admission.(16) Exclusion criteria were: active infection with hepatitis B or C virus, hepatocellular carcinoma, active extra-hepatic cancer, prior liver transplantation and prior placement of a transjugular intrahepatic portosystemic shunt (TIPS).

This study was approved by the Institutional Review Boards of Paris North Hospitals, Paris 7 University, AP-HP (N° 11-112) and the Hospital Clinic of Barcelona. All patients included in this study gave written informed consent. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

5 *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. Expert liver
10 pathologists (P Bedossa and V Paradis at Hôpital Beaujon, Clichy; XX at Hospital Clinic, Barcelona, Spain) analyzed all biopsy specimens using previously published histologic criteria.(17) Pathologists were unaware of the results of plasma biomarkers analyses. The histological diagnosis of AH was based on the presence of hepatocellular injury (hepatocellular ballooning and presence of Mallory-Denk bodies), inflammatory infiltrate (predominantly
15 polymorphonuclear cells), and pericellular fibrosis.(17) The portal pressure was estimated based on the hepatic venous pressure gradient (HVPG), as described in detail previously.(18)

Measurement of Caspase-Generated CK-18 Fragments in the Blood

For patients of the test cohort, peripheral venous blood was drawn into citrate tubes the day of the liver biopsy, and was then centrifuged twice at 2500 g for 15 minutes at 18°C to
20 obtain platelet free plasma. For patients of the validation cohort, central venous blood was drawn into an 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
25 instructions. We also measured plasma levels of total (M65 EpiDeath 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 previously described, the difference between soluble CK-18
30 levels in initial and in filtered plasma reflects the fraction of soluble CK-18 carried by MVs.(11) 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 previously described in detail.(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-Fluoroisothiocyanate antibodies as well as their matched isotype controls were obtained from R&D Systems Europe.

Dosing of all biomarkers was performed by an engineer unaware of the clinical characteristics and histological diagnosis of the patients.

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 (ROC) 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., Chicago, IL, United States) 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, biological and hemodynamic characteristics of the patients are shown in Table 1. There was no difference in the ABIC and MELD scores and Maddrey's Discriminant Function (MDF) between patients with and without histologically confirmed AH in both cohorts. Severe disease, defined by an ABIC score of 6.71 or more, was present in 69/83 (83%) patients of the test cohort and 47/68 (69%) patients in the validation cohort.

In patients with no histologic features of AH, the diagnosis was alcoholic cirrhosis in 35/37 (95%) patients, steatosis with advanced fibrosis in 1 and features suggestive of biliary salt transporters in 1 in the test cohort. Two patients had inflammatory features suggestive of

superimposed viral or drug-induced hepatitis. In the validation cohort, the diagnosis was alcoholic cirrhosis in 15/20 (75%), hypoxic hepatitis in 3/20 (14%) advanced fibrosis in 1 and acute viral hepatitis in 1.

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 Figure 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 Figure 1). Levels of CCL20 and TREM-1 were not tested in the validation cohort as the area under the ROC 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 641U/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 Figure 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 Figure 3). 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

Diagnostic performance of tested biomarkers in patients with clinical suspicion of severe AH

When restricting the analyses to patients with suspicion of severe AH ($ABIC \geq 6.71$), we obtained similar results. 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.

Diagnostic performance of previously proposed tests

An abrupt increase in serum bilirubin of 5 mg/dL or more 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%) in the validation cohort with a serum bilirubin of 5mg/dL or more. The association of more than $10.75 \times 10^9/L$ white blood cells with more than

147.5 X 10⁹/L platelets has also been recently proposed to diagnose AH and less than 5.95 X10⁹/L white blood cells with less than 86 X 10⁹ platelets to 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 47/83 (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 57/68 (84%) patients.

Discussion:

This study shows that circulating levels of CK-18 fragments, and particularly the M65 epitope, can be useful in the non-invasive diagnosis of AH, and thus avoid TJLB in two-thirds of the 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 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 ≥ 6.71 . 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 suggests that M65 and M30

are not sensitive to preanalytical parameters and are thus biomarkers suitable 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, as 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 and/or treatment with corticosteroids. The kinetics of CK-18 fragments levels might carry valuable information about patient outcome.

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% in the test cohort. 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 non-invasive 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 non-invasive diagnostic tool of AH in centers where TJLB is not readily available. In patients with M65 level below 641 U/L AH can be 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 definitely need a TJLB. This pragmatic approach proposes a solution to the debate over the need for systematic liver biopsy to establish the diagnosis of AH.

Conclusions

In this prospective study, circulating fragments of CK-18 had a good diagnostic performance to estimate non-invasively the presence of AH in patients with clinical suspicion of this disease, including in patients with the severe form of the disease, in a test and a validation cohorts. They outperformed other analytical non-invasive tools for the diagnosis of AH.

Therefore, they may be a valuable diagnostic tool, especially in centers with difficult access to TJLB.

TABLES:

Table 1: Characteristics of the patients with and without biopsy-confirmed alcoholic hepatitis at the time of liver biopsy

Variable	Test cohort (n = 83)			Validation cohort (n = 68)		
	No AH (n = 37)	AH (n = 46)	p-value	No AH (n = 20)	AH (n = 48)	p-value
Age	51 (48-57)	55 (48-57)	0.38	57 (49-64)	52 (47-56)	0.08
Male : Female	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	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 (X 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

	96 (59-130)	149 (89-199)	0.001	134 (88-200)	100 (77-156)	0.10
Platelets (X 10 ⁹ /L)						
HVPG (mmHg)	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
MDF	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	30 (81 %)	39 (85%)	0.52	12 (60%)	35 (73%)	0.29
Documented bacterial infection	10 (27%)	8 (17%)	0.29			

Results are presented as median (interquartile range). Abbreviations: ABIC, Age-bilirubin-INR-creatinine; MDF, Maddrey's discriminant function; CRP, c-reactive protein; HVPG, hepatic venous pressure gradient; WBC, white blood cells. *, missing in 11/83 patients.

5 **Table 2: Plasma levels of tested biomarkers in patients with and without alcoholic hepatitis**

Biomarker	Test cohort (n = 83)			Validation cohort (n = 68)		
	No AH (n=37)	AH (n=46)	p-value	No AH (n=20)	AH (n=48)	p-value
M65	481 (166-991)	3857 (909-6227)	< 0.001	869 (475-869)	2654 (1266-6070)	< 0.001
MV M65	75 (23-154)	805 (158-2383)	< 0.001	119 (43-388)	492 (224-1161)	< 0.001
M30	252 (153-576)	2401 (606-4732)	< 0.001	451 (320-708)	938 (617-1936)	< 0.001
MV M30	37 (10-129)	736 (98-1928)	< 0.001	63 (15-158)	273 (121-438)	< 0.001

CCL20	93 (50-279)	336 (150-827)	< 0.001
TREM-1	183 (135-273)	220 (145-290)	0.31
Annexin V+	7531 (4447-	7096 (5464-	0.85
MV	12044)	10871)	
CD41+31-	1468 (759-	1691 (907-	0.69
MV	2815)	3226)	
CD41-31+	137 (45-384)	161 (72-280)	0.73
MV			
CD11a+	26 (9-81)	19 (5-72)	0.43
MV			
CD62e+	99 (49-335)	189 (96-403)	0.08
MV			

Results are expressed as median (interquartile range) and U/L (M30, MV M30, M65 and MV M65), pg/mL (CCL20 and TREM-1) or concentration per μ L (Annexin V+, CD11a+, CD62e+, CD41+31- and CD41+31+ MV).

Abbreviations: AH, alcoholic hepatitis; MV, microvesicles.

5

Table 3: Diagnostic performance of tested biomarkers for the presence of biopsy-confirmed alcoholic hepatitis

Biomarker	Cutoff	Sens (%)	Spec (%)	PPV (%)	NPV (%)	AUROC
	Test cohort (n = 83)					
M65	2000	67 [57-77]	92 [86-98]	91 [85-97]	69 [59-79]	0.84 [0.75-0.93]
M65	641	93 [88-99]	62 [52-73]	75 [66-85]	88 [82-95]	0.84 [0.75-0.93]
MV M65	161	76 [67-85]	81 [73-90]	83 [75-91]	73 [64-83]	0.82 [0.72-0.91]

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

Results are expressed with 95% confidence intervals. Cutoffs are shown in U/L (M30, MV M30, M65 and MV M65) and pg/mL (CCL20 and TREM-1).

Abbreviations: AH, alcoholic hepatitis; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity; MV, microvesicles.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method of diagnosing alcoholic hepatitis in a subject comprising i) determining the level of at least one cytokeratin-18 fragment in a blood sample obtained from the subject, ii) comparing the level determined at step i) with a predetermined reference value and iii) detecting differential between the level determined at step i) with the predetermined reference value indicates that the subject suffers or does not suffer from alcoholic hepatitis.
2. The method of claim 1 wherein the level of M30 cytokeratin-18 is determined in the blood sample.
3. The method of claim 1 wherein the level of M65 cytokeratin-18 fragment is determined in the blood sample.
4. The method of claim 1 wherein the blood sample is a plasma sample
5. The method of claim 1 wherein when the level of the cytokeratin-18 fragment (M65 or M30) is below 641 U/L it is concluded that the subject does not suffer from alcoholic hepatitis.
6. The method of claim 1 wherein when the level of the cytokeratin-18 fragment is above 2000 U/L, it is concluded that the subject suffers from alcoholic hepatitis.
7. The method of claim 1 wherein when the level of the cytokeratin-18 fragment is between 641 and 2000 U/L further investigations are thus needed to conclude that the subject suffers from alcoholic hepatitis.
8. The method of claim 7 wherein a transjugular liver biopsy is performed.
9. The method of claim 1 wherein the subject is administered with a therapeutically effective amount of corticosteroids, pentoxifylline, or N-acetylcysteine when it is concluded that the subject suffers from alcoholic hepatitis.

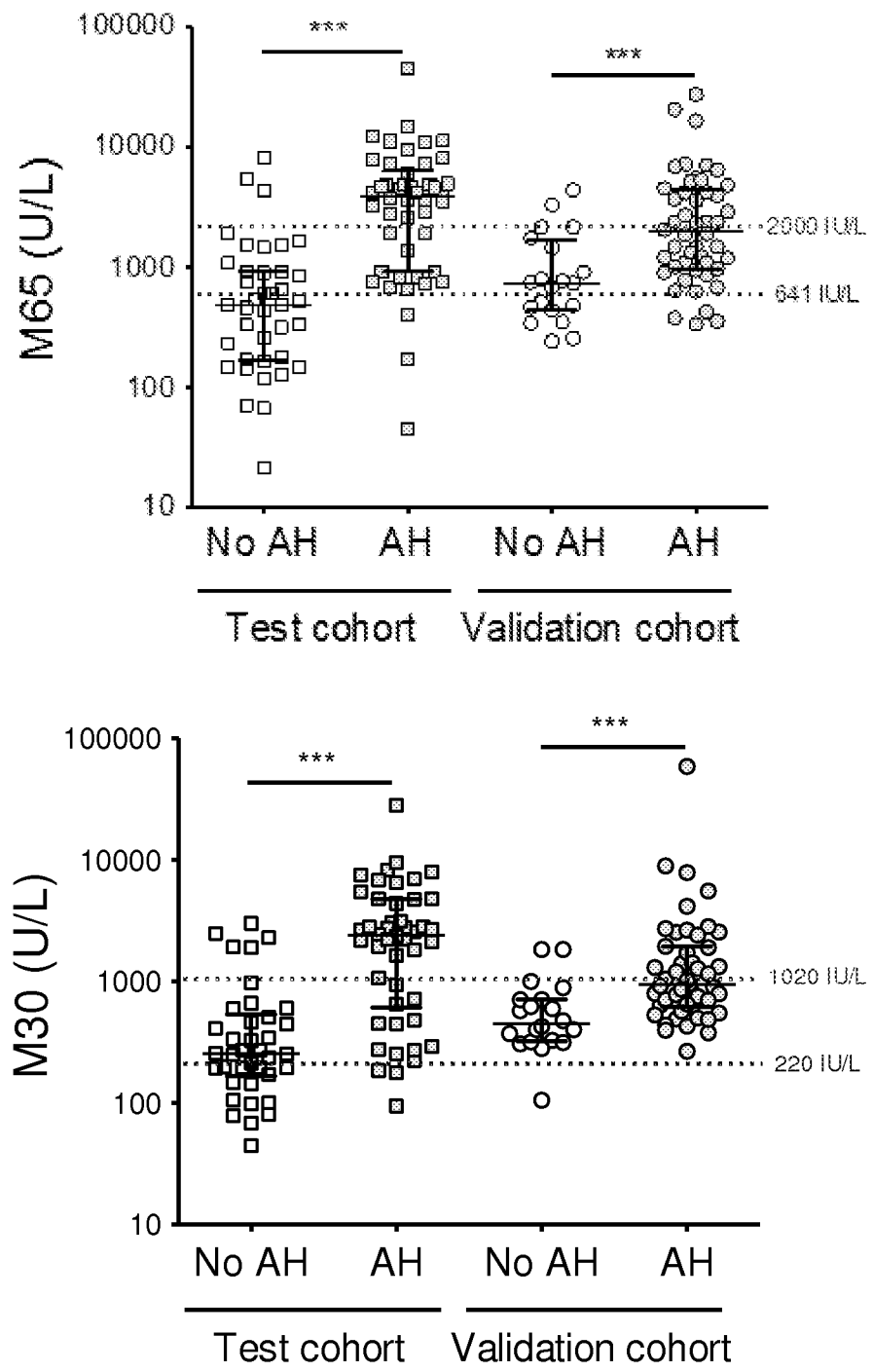


Figure 1

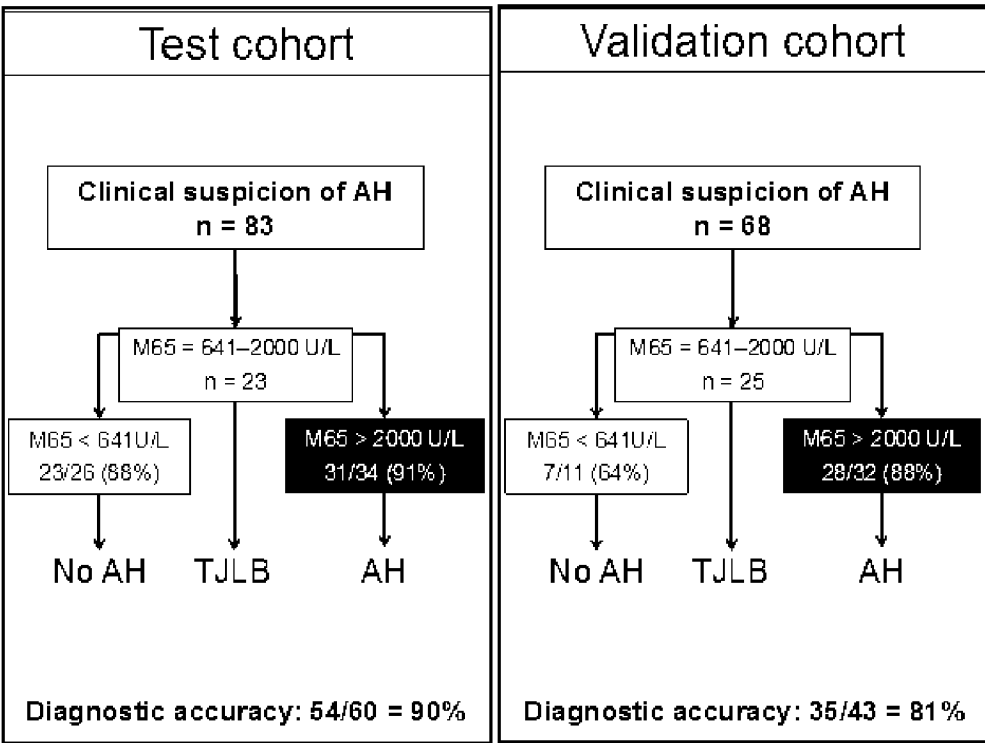


Figure 2

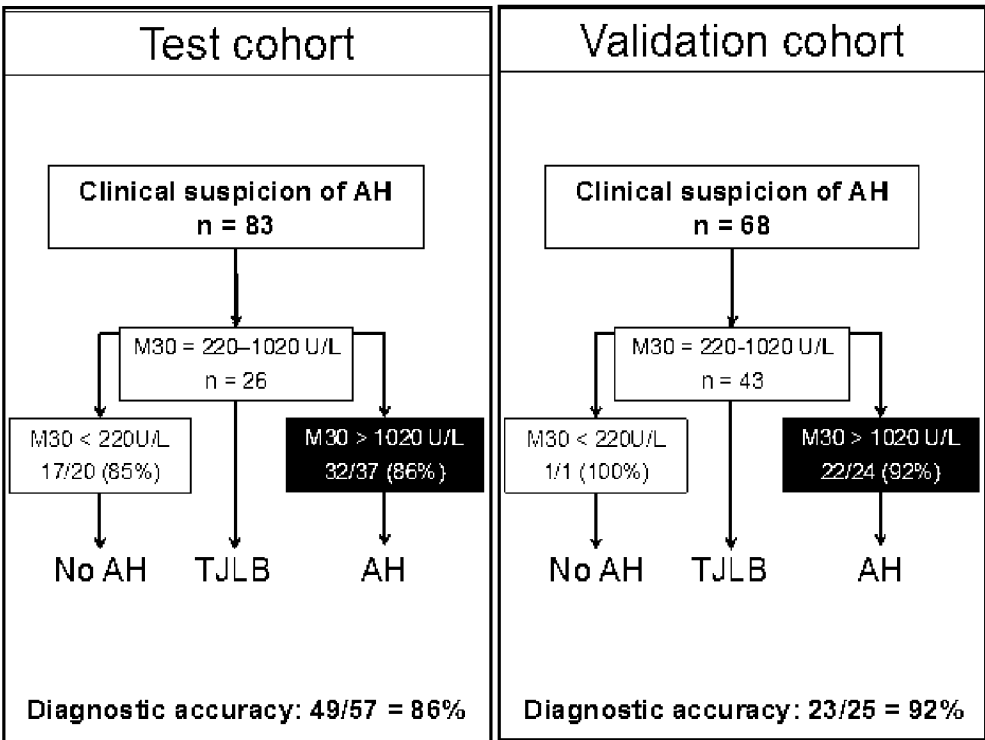


Figure 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/001708

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GONZALEZ-QUINTELA A ET AL: "Serum Levels of Keratin-18 Fragments [Tissue Polypeptide-Specific Antigen (TPS)] Are Correlated with Hepatocyte Apoptosis in Alcoholic Hepatitis", DIGESTIVE DISEASES AND SCIENCES, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NE, vol. 54, no. 3, 10 July 2008 (2008-07-10), pages 648-653, XP019673421, ISSN: 1573-2568 abstract, paragraph spanning pages 650 and 651, page 652, left-hand column; ----- -/--	1-9



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

11 April 2017

Date of mailing of the international search report

21/04/2017

Name and mailing address of the ISA/

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Pilch, Bartosz

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/001708

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZEWDU M C ET AL: "S1859 Serum Cytokeratin 18 and Adipocytokines in Alcoholic Hepatitis Versus Nonalcoholic Steatohepatitis", GASTROENTEROLOGY, ELSEVIER, AMSTERDAM, NL, vol. 138, no. 5, 1 May 2010 (2010-05-01), pages S-804, XP027026232, ISSN: 0016-5085 [retrieved on 2010-04-27] the whole document	1-9
X	GONZALEZ-QUINTELA A ET AL: "Serum cytokeratins in alcoholic liver disease: contrasting levels of cytokeratin-18 and cytokeratin-19", ALCOHOL, ELSEVIER, AMSTERDAM, NL, vol. 38, no. 1, 1 January 2006 (2006-01-01), pages 45-49, XP028003141, ISSN: 0741-8329, DOI: 10.1016/J.ALCOHOL.2006.01.003 [retrieved on 2006-01-01] abstract, paragraph 2.1.1 and 2.2, paragraph 3, fig 2B	1-9
X	S Mueller: "ORAL PRESENTATIONS 0144 M65 and M30 as highly sensitive markers of alcoholic steatohepatitis", 1 April 2014 (2014-04-01), XP055363615, Retrieved from the Internet: URL:http://www.sciencedirect.com/science/article/pii/S0168827814601446/pdf?md5=1ad ae8f8bfead4f3ab0af3001f04ba8a&pid=1-s2.0-S0168827814601446-main.pdf [retrieved on 2017-04-11] abstract	1-9
A	ARTURO GONZALEZ-QUINTELA ET AL: "Serum levels of cytokeratin-18 (tissue polypeptide-specific antigen) in liver diseases", LIVER INTERNATIONAL, vol. 26, no. 10, 1 December 2006 (2006-12-01), pages 1217-1224, XP055363436, GB ISSN: 1478-3223, DOI: 10.1111/j.1478-3231.2006.01380.x the whole document	1-9

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/001708

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YUSUF YILMAZ ED - MUSSAP MICHELE ET AL: "Cytokeratins in hepatitis", CLINICA CHIMICA ACTA, ELSEVIER BV, AMSTERDAM, NL, vol. 412, no. 23, 2 September 2011 (2011-09-02), pages 2031-2036, XP028309163, ISSN: 0009-8981, DOI: 10.1016/J.CCA.2011.09.002 [retrieved on 2011-09-08] paragraph 6 -----	1-9