ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 8, Issue 2, 2015

ISSN - 0974-2441

Review Article

DIAGNOSTIC METHODS FOR NON-ALCOHOLIC FATTY LIVER DISEASES ALTERNATIVE TO LIVER BIOPSY: A REVIEW

SURENDRA H BODAKHE*, SANJAY KUMAR GUPTA

Department of Pharmacology, Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur, Chhattisgarh - 495 001, India. Email: drbodakhe@gmail.com

Received: 23 January 2015, Revised and Accepted: 03 February 2015

ABSTRACT

Non-alcoholic fatty liver diseases (NAFLD) are the most occurring chronic liver diseases caused by hepatic manifestation of metabolic syndromes like insulin resistance in Type-2 diabetes, obesity and hyperlipidemic condition. Indian men have a high prevalence of NAFLD. Genetic factor is also an important consideration for NAFLD. The core aim of this study is to focus on non-invasive biochemical markers for diagnosis of NAFLD as an alternative to liver biopsy. Biopsy is the gold standard for NAFLD; however a variety of studies powerfully suggest that due to the limitation and risk of biopsy, as well as improvement of the diagnostic accuracy of biochemical markers, biopsy should no longer be considered obligatory. Thus, due to the limitation of liver biopsy, numerous biomarkers has been developed recently, which may provide more accurate results than an inferior quality liver biopsy. Some important biomarkers are caspase-cleaved cytokeratin-18, breath biomarker, circulating microRNAs, abdominal ultrasound, adipocytokines, etc.

Keywords: Adipocytokines, Cytokeratin-18, Liver biopsy, MicroRNAs, Non-alcoholic fatty liver diseases, Non-invasive biomarkers.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one cause of a fatty liver, occurring due to deposition of fat (steatosis) in the liver, not by chronic intake of alcohol in excess [1]. NAFLD is associated with insulin resistance and metabolic syndrome (obesity, combined hyperlipidemia, Type 2 diabetes mellitus and high blood pressure) [1,2]. Genetic variation in apolipoprotein C3 gene is also responsible for NAFLD [2]. Non-alcoholic steatohepatitis (NASH) is the most extreme form of NAFLD this being regarded as a major cause of cirrhosis of the liver of unknown cause [3].

The consensus conference statements recommend liver biopsy in the management of almost all patients with the most frequent chronic liver disease (CLD): Chronic hepatitis C virus and hepatitis B virus, NAFLD, and AFLD with its risk of NASH, but also underline the necessity of developing reliable non-invasive tests [4].

Common findings in NAFLD are elevated liver enzymes and a liver ultrasound showing steatosis [1]. Non-invasive diagnostic tests have been developed, such as Fibro Tests that estimates liver fibrosis [5], and Steato Test (ST) that estimates steatosis [6]. Apoptosis has been shown to be the mechanism of hepatocytes destruction and caspase-cleaved cytokeratin-18 (CK18) (M30-Apoptosense ELISA) in serum/plasma is often elevated in patients with NASH [7,8]. Hypothyroidism is more prevalent in NASH patients, which would be detected by determining the thyroid- stimulating hormone (TSH) [9]. Aminotransferase levels are also found to elevate in non-alcoholic fatty liver diseases [10].

Initially an ideal diagnosis can only be achieved by liver biopsy and histopathological analysis, but recently, a number of non-invasive biomarkers are developed that can differentiate the stages of fibrosis with accuracy similar as liver biopsy without any complication. Fibrosis and cirrhosis occur from NASH and NASH from simple steatosis [9, 11].

Liver biopsy

Liver biopsy is a medical test involving sampling of cells or tissues of liver from living subjects to diagnose the presence or extent of liver disease and to monitor the progress of treatment. The first liver aspirate was performed by the German physician Paul Ehrlich in 1883 and percutaneous liver biopsy was first reported in the 1920s. Biopsy is often required for the diagnosis of jaundice, NAFLD and other liver diseases [12]. Biopsy can be performed percutaneously, intravenously or directly during abdominal surgery. The sample is examined by microscope, and may be processed further by immunohistochemistry, determination of iron and copper content, and microbiological culture if tuberculosis is suspected [13].

Liver biopsy is usually a safe procedure, but it is invasive. Difficulties of biopsy are rare but potentially lethal. The majority of complications (60%) occur within 2 hrs, and 96% occur within 24 hrs following the procedure. Approximately, 2-3% of patients undergoing liver biopsy require hospitalization for the management of an adverse event [14,15]. About 30% of patients experience significant pain during the procedure. Significant bleeding after a liver biopsy occurs in 1-2 out of 100 patients who are biopsie [17,18]. Bleeding usually becomes apparent within 3-4 hrs. It often stops on its own, but if persists, a blood transfusion may be needed. Surgery or angiography may be required if the bleeding is severe or does not stop on its own. Intra-peritoneal hemorrhage is the most serious consequence of bleeding. Fatal complications have been reported in up to 0.01-0.3% of biopsied patients [16-18]. However bleeding is now unlikely with ultrasound guided liver biopsy in the last 5 years.

Biopsy results show major variability (up to 40% in the diagnosis of fibrosis) which can lead to an incorrect or poor diagnosis, the results depend on the representatively of the punctured sample [19]. In 2002, the consensus conferences in France and in the USA raised the possibility of treating patients with chronic hepatitis without liver biopsy. These conferences also underlined the necessity of developing reliable non-invasive tests that might be an alternative to liver biopsy in NAFLD [20]. Biopsy will likely remain particularly important in the diagnosis of unexplained liver disease. Non-invasive tests for liver fibrosis in alcoholic, non-alcoholic and viral liver diseases are likely to become more widely used [21].

Non-invasive biomarkers

Biopsy is not most preferred technique to diagnose the liver diseases because of some demerits such as excessive bleeding, intra-peritonial hemorrhage, sampling error and availability of highly accurate and more

précised non-invasive biomarkers. These non-invasive biomarkers aid to give alternative of liver biopsy which is advantageous due to its low cost and safer than liver biopsy [16-18].

Therefore, familiarity of these biomarkers necessary to diagnose the NAFLD and other liver diseases. However, these biomarkers have also not been extensively validated because the risk of false positive or negative in large community based populations. There is none of available test has established a continuous and linear correlation with fibrosis stage and fibrosis grades. Liver biopsy also has sometime false positive or negative result [19].

ST and fatty liver index (FLI)

These both markers are able to recognize the presence of NAFLD. ST and FLI are accurate and easy to use markers for the diagnosis of steatosis and allowed differentiation between mild, moderate and severe steatosis. It is found that ST and FLI useful for the discrimination between insignificant (S0 and S1) and a significant steatosis (S2 and S3) [22].

The degree of hepatic steatosis in NAFLD patients was assessed according to the Kleiner criteria: Grade 0: Fat droplets <5% hepatocytes (S0); Grade 1: Fat droplets in 5-33% hepatocytes (S1); Grade 2: Fat droplets in 33-66% hepatocytes (S2) and Grade 3: Fat droplets in >66% hepatocytes (S3) [23].

FLI is an algorithm for the prediction of steatosis based on biochemical factors. ST is a simple blood test, combining 10 blood components, developed in order to provide an estimate of quantitative steatosis in NAFLD [11]. FLI is a composite index, which combines four parameters: Body mass index (BMI), waist circumference, triglycerides and gamma-glutamyl transpeptidase (GGT) in a mathematical algorithm, developed to detect the presence of steatosis [24].

Steato Test™ combines 10 blood components: Alpha-2 macroglobulin (g/L), haptoglobin (g/L), apolipoprotein A (g/L), total bilirubin (µmol/L), GGT (IU/L), alanine-aminotransferase (ALT) (IU/L), aspartate-aminotransferase (AST) (IU/L), total cholesterol (mmol/L), triglycerides (mmol/L), fasting glucose (mmol/L) with age, gender and BMI. However alpha-2 macroglobulin is found to confusing whether it reflect steatosis state or not. ST scores range from 0.00 to 1.00 with higher scores indicating a greater probability of significant lesions. The use of non-invasive methods for diagnosis of NAFLD is adequate and accurate. ST and FLI are precise and easy to use for diagnosis of steatosis and for differentiation between mild, moderate and severe steatosis. FLI contains routine lab tests, is cheaper and has similar efficiency to ST for detection of steatosis and its degree. The complementary use of two biological methods could improve the diagnostic accuracy [22].

Hyaluronic acid and laminin in liver fibrosis

Serum hyaluronic acid (HA) and Laminin (LN) levels increase with the development of liver fibrosis and could be used as a non-invasive biomarkers to discriminate between patients with liver fibrosis and healthy individuals. A cut-off point of 59.5 ng/ml HA and 52.0 ng/ml LN for the discrimination of patients with liver fibrosis from healthy controls and 102.0 ng/ml HA and 92.5 ng/ml LN for the discrimination of patients with mild from severe fibrosis showed acceptable area under this curve, sensitivity and specificity. An increase in serum HA and LN concentrations above the predictive value is associated with liver fibrosis [25].

Liver fibrosis is a process involving production and deposition of various components that constitute the extracellular matrix. Some of these components are non-collagenous glycoproteins such as LN, HA, and proteoglycans [26].

HA is a glycosaminoglycan distributed in the extracellular spaces. In the liver, HA is mostly synthesized by the hepatic stellate cells and degraded by the sinusoidal endothelial cells. In recent years, some studies proposed HA and LN as indices of the extent of liver fibrosis in CLD [27,28].

One of the main glycoproteins of the basement membrane is LN, which is synthesized by hepatocytes and sinusoidal cells [29], stellate cells or lipocytes produce the largest amount of serum LN. An increased serum LN level in advanced stages of fibrosis has observed [30]. LN serum concentrations could be a sensitive screening test for hepatic fibrotic disease and portal hypertension if the test were assessed together with the serum level of HA [31].

Breath biomarkers

Breath biomarkers have the potential to offer information that is similar to conventional clinical tests or they are entirely unique. Preliminary data support the use of breath biomarkers in the study of liver disease, in particular NAFLD [32].

Evaluation of whether breath ethanol, ethane, sulfur compounds and acetone would be associated with hepatic histopathology amongst morbidly obese patients presenting for bariatric surgery. It is found that breath ethanol, ethane and acetone can be useful biomarkers in patients with NAFLD. In particular, breath ethanol can be associated with hepatic steatosis, and breath acetone can be associated with NASH. Mitochondrial dysfunction plays a central role in the general pathogenesis of NAFLD, increasing the risk of developing steatosis and subsequent hepatocellular inflammation. Hepatic mitochondrial function is assessed by a non-invasive [12] C-methionine breath test in patients with histologically proven NAFLD [33]. Breath composition is altered in liver diseases. Ion-molecule reaction-mass spectrometry (IMR-MS) combined with a new statistical modality improves the diagnostic accuracy of breath analysis in liver diseases. IMR-MS is a promising tool for liver diagnostics by breath analysis [34].

Circulating microRNAs

MicroRNAs are small non-coding RNAs that control translation and transcription of many genes. They are receiving growing attention because of numerous reports on their dysregulation in human diseases and their potential as diagnostic and therapeutic targets. Because of their stability and presence in almost all body fluids, microRNAs constitute a novel class of non-invasive biomarkers [35-37].

Circulating MicroRNAs miR-122, miR-34a, miR-16 and miR-21 are commonly deregulated in liver fibrosis and hepatocellular carcinoma. Serum levels of miR-34a and miR-122 may represent novel non-invasive biomarkers of diagnosis and histological disease severity in patients with NAFLD. Circulating levels of these miRNAs correlate with hepatic histological disease severity in patients with NAFLD and can potentially serve as circulating markers for disease stage assessment [38]. The highly abundant liver-specific miR-122 is of particular interest because of miR-122 is known to regulate metabolic pathways in the liver, including cholesterol biosynthesis [39-42].

Plasma proteomic profiling via an N-terminal isotope tagging strategy

Proteomics is used to determine the expression and dynamics of many proteins, and is especially suitable for exploration of disease biomarkers. Comparison of proteomes of disease and control plasma samples has been shown to be a suitable approach for discovering plasma biomarkers of liver diseases [43]. Quantitative proteomic techniques based on stable isotope labeling are increasingly being applied in biomarker studies [44,45].

The N-terminal acetyl isotope (d0/d6) labeling technique shows great potential in biomarker detection with its high-throughput identification and accurate quantification of proteins, as well as being a simple procedure. Leading edge liquid chromatography/Fourier transform ion cyclotron resonance MS (LCFTICR-MS) technology's superior sensitivity and powerful measurement can also contribute to plasma biomarker detection [46].

Plasma proteomic profiling via an N-terminal isotope tagging strategy coupled with l LCFTICR-MS measurement to detect liver fibrosis

staging. Pooled plasma from different liver fibrosis stages, which were assessed in advance by the liver biopsy, was quantitatively analyzed. A total of 72 plasma proteins were found to be dysregulated during the fibrogenesis process, and this finding constituted a valuable candidate plasma biomarker bank for follow-up analysis. Quantitative proteomics via the N-terminal acetyl isotope labeling technique provides an effective and useful tool for screening plasma candidate biomarkers for liver fibrosis [47].

Abdominal ultrasound

Abdominal ultrasound is widely used for screening fatty liver disease in asymptomatic patients with an incidental elevation of liver enzymes. Ultrasound is very sensitive, but it cannot detect small amounts of hepatic steatosis, and it is not quantitative [48-51].

Adipocytokines

The term "adipokines or adipocytokines" comprises a group of polypeptide hormones, which are expressed predominantly, although not entirely, by adipose tissue in a regulated manner. These molecules are secreted into the circulation and regulate the functions of different tissues through local, central and/or peripheral actions [52]. The major adipocytokines are;

Adiponectin

Adiponectin is an abundant adipocyte-derived hormone with well-established anti-inflammatory and insulin sensitizing properties. The significance of adiponectin in protecting obesity-related NAFLD has been increasingly recognized. Adiponectin is an insulin-sensitizing adipokine possessing multiple beneficial effects on obesity-related medical complications and thus, NAFD. This adipokine is secreted from adipocytes into the circulation as three oligomeric isoforms, including trimer, hexamer and the high molecular weight oligomeric complex. Each oligomeric isoform of adiponectin possesses distinct biological properties and activates different signalling pathways in various target tissues [53].

Leptin

Leptin, an adipocytokine produced primarily in visceral adipocytes, is the gene product of the ob gene, identified in the mid-1990s. Leptin receptors are found primarily in the hypothalamus, but also in vascular endothelial cells. Leptin acts by binding to specific receptors in the hypothalamus to alter the expression of several neuropeptide especially neuropeptide-Y that regulate neuroendocrine function, energy intake and expenditure. Leptin levels correlate with percentage body fat and are correspondingly higher in women. In the genetically obese mouse model,a mutated gene results in little or no leptin production. These obese mice lose weight with leptin replacement, and hence, leptin is clearly involved in obesity [54].

There is a large body of evidence implicating leptin in the scheme of NAFLD overall. It is generally accepted that insulin resistance is almost always present in NAFLD, particularly in NASH. Serum leptin in multivariate analysis is related to insulin levels, fat mass, lean mass and correlates strongly with insulin resistance in humans [55-58]. In a biopsy proven cases of NASH, leptin levels were twice those found in controls matched for BMI, gender and age. The leptin is likely involved in the pathogenesis of insulin resistance, and hence steatosis [59]. Thus, increase leptin level may be associated with insulin resistance (Type-2 diabetic mellitus), obesity and NAFLD.

Retinol binding protein (RBP-4)

RBP-4 is newly identified adipokine derived mainly from the adipose tissue, in the pathogenesis of metabolic syndrome (insulin resistance), as well as NAFLD, which is considered a hepatic manifestation of this syndrome. Obesity, insulin resistance and dyslipidemia are the most significant risk factors of NAFLD, but the role of adipokines in the pathogenesis of this disease is not clear. The ability of RBP-4 to differentiate the children with advanced liver steatosis from those with mild steatosis is not significant [60].

Decreasing levels of RBP-4 were associated significantly with increasing levels of serum triglyceride. High levels of RBP-4 were associated significantly with low necro-inflammatory activity, a low NAFLD activity score and a low fibrosis score. Furthermore, serum RBP-4 levels decreased significantly as disease severity increased; there is a stepwise decrease in RBP-4 from children with steatosis to borderline NASH to definitive NASH. Thus, an inverse relationship between RBP-4 levels and degree of liver damage. RBP4, therefore, might be a potential novel non-invasive marker of severity of pediatric NAFLD [61]. The liver is the major source of RBP-4 secretion in rodents and in humans [62] few other studies displayed no differences between controls and NAFLD adults in the level of RBP-4 [63,64].

Resistin

Resistin is a polypeptide hormone belonging to adipokines [65], reduces insulin sensitivity in adipocytes, skeletal muscles and hepatocytes by suppression of insulin stimulated glucose uptake. Moreover resistin expression has been demonstrated in mononuclear leucocytes and in inflamed tissues, suggesting a pro-inflammatory properties for resistin or its regulation by inflammatory mediators including interleukin-1 (IL-1), IL-6 and tumor necrosis factor –α (TNF-α) [66]. Previous studies have shown that expression of resistin is detectable in liver tissue and is up-regulated by chronic damage, indicating that resistin can be used as an indicator of severity of cirrhosis in CLD of patients [67].

Serum levels of CK18 M30

Caspase-cleaved CK18 is considered as a biomarker of NAFLD using a combination of the M30 and M65 ELISAs. M30 and M65 Apoptosense ELISA is an ELISA developed for the detection of soluble caspase-cleaved keratin-18. CK18 M30 levels were significantly higher in patients with NAFLD versus controls; this marker is able to differentiate the degree of severity from simple steatosis to fibrosis and cirrhosis [68]. Activation of caspase 3 results in cleavage of CK18 giving Rise to the morphological changes of apoptosis. Once the cell undergoes secondary necrosis, CK18 fragments are released and can be detected in serum. Caspase-cleaved CK18 fragments (CK18 M30) is measured in serum using ELISAs.

Markers of hepatocellular apoptosis may be particularly useful; as free fatty acid accumulates in the cell, lysosomes and mitochondria undergo permeabilisation and activation of the caspase cascade [69]. Increasing evidence suggests hepatocytes apoptosis is a key mediator of liver injury in NAFLD. Plasma CK18 fragments were markedly increased in patients with NASH compared with patients with simple steatosis or normal biopsies [70]. Markers of oxidative stress, including lipid peroxidation products may also be useful markers of disease. These substances are volatile and not always easily measured in serum [71]. M30 and M65 are relatively newly described the sandwich ELISA assays that determine in either plasma or serum different circulating forms of the protein CK18 [72,73].

Haptoglobin (Hp) and C-reactive protein (CRP)

Hp is a protein that in humans is encoded by the Hp gene. In blood plasma, Hp binds free hemoglobin released from erythrocytes with high affinity and thereby inhibits its oxidative activity [74,75]. CRP is a protein found in the blood, the levels of which rise in response to inflammation. Its physiological role is to bind to phosphocholine expressed on the surface of dead or dying cells in order to activate the complement system via the C1Q complex [76]. CRP is synthesized by the liver [77], in response to factors released by the adipocytes [78]. It is a member of the pentraxin family of proteins [77]. It is not related to C-peptide or protein C. Up regulation of CRP and down regulation of Hp associated with NAFLD. Western blotting validated that CRP is dramatically higher in the serum of alcoholic fatty liver compared to healthy controls and other animals with liver disease of NAFL or liver fibrosis [79].

Advanced magnetic resonance imaging (MRI) spectroscopy

With recent advances in technology, advanced MRI methods such as diffusion-weighted and perfusion-weighted MRI, magnetic resonance (MR) Elastography, chemical shift-based fat-water separation, and

MR spectroscopy can now be applied to liver imaging. MRI plays an increasingly important role in the assessment of patients with CLD because of the lack of ionizing radiation and the possibility of performing multi parametric imaging. This technique is also useful for determination of iron deposition in NAFLD patients. Some general limitations of the methods include limited availability, complex acquisition, processing, and a learning curve [80].

Iron overload in the liver can result from a variety of causes but is most commonly encountered in patients with genetic hemochromatosis, transfusional hemosiderosis, and a chronic inflammatory state. Increased iron stores are toxic to the liver and are well known to be carcinogenic in patients with hemochromatosis [81].

OTHER DIAGNOSTIC METHODS

Other techniques are relevant to blood tests as erythrocyte sedimentation rate, glucose, albumin and renal function. Hypothyroidism is more common in NASH patients, which would be detected by determining the TSH, Serum TSH level was significantly higher in non-alcoholic fatty liver than in normal liver patient [82]. However, multiple logistic regression analysis showed that TSH level was not the independent risk factor of NAFLD [83]. In the past few years, transient elastography (Fibroscan R, Echosens, France) has been increasingly used as a non-invasive tool for the assessment of liver fibrosis by measuring liver stiffness. Transient elastography has been shown to be highly reproducible, easy and rapid with minimal inter- and intra-observer variability [84,85]. It is also found TNF-alpha and interleukin level increase with liver steatosis to fibrosis [83]. AST to aminotransferase to platelet ratio index is significantly higher in the advanced fibrosis group. AST to ALT ratio is also higher with progression of diseases [86,87].

SUMMARY AND CONCLUSION

NAFLD is one of the most common causes of CLD. The spectrum of the disease ranges from simple steatosis, followed by NASH i.e. fatty liver with inflammation, may progress to fibrosis, cirrhosis and its complications. NAFLD is diagnosed by liver biopsy and non-invasive biochemical markers. Biopsy still used as the gold standard for diagnosis of NAFLD, but due to several limitation and its invasive procedure, specific biomarkers can be used to diagnose liver diseases. However neither biomarkers nor biopsy alone is sufficient to take a definitive decision in a given patient.

Limitation of liver biopsy, such as pain occur in 30% patients, excessive bleeding in 2% population and intra-peritonial hemorrhage in few patients. Biopsy is still useful, but not as the first line estimate of the CLD, but are employed preferably when validated non-invasive methods such as ST and FLI are not applicable. In some countries like France, this strategy is already extensively used and approved by the health authorities. Biopsy could be valuable when several liver injuries are suspected in the same patients and in less frequent CLD.

A non-invasive diagnostic approach is essential for the evaluation of the severity of liver disease, treatment decisions and assessment of drug efficacy. These biomarkers are necessary to diagnose the NAFLD and other liver diseases but none of them reliable alone. Several definitive biomarkers are leptin, resistin, adiponectin, RBP-4, LN, HA, Hb, CRP, breath biomarkers, MicroRNAs, caspase cleaved CK-18 M30, AST: ALT and AST: Platelet ratio is support to diagnose the NAFLD and other liver related diseases.

None of the technique is appropriate to determine the accurate and linear correlation with different grade and severity of diseases; therefore, specific non-invasive biomarkers are required to assess the diseases accurately. Recently proteomic and glycomic technique is established to assess the dynamic and conformational change of serum protein in NAFLD patients. Advanced MRI is also helpful for diagnosis of liver fibrosis and detection of iron overload in NAFLD patients, which are higher many times.

REFERENCES

1. Adams LA, Angulo P. Treatment of non-alcoholic fatty liver disease. Postgrad Med J 2006;82(967):315-22.
2. Petersen KF, Dufour S, Hariri A, Nelson-Williams C, Foo JN, Zhang XM, *et al.* Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. N Engl J Med 2010;362(12):1082-9.
3. Clark JM, Diehl AM. Nonalcoholic fatty liver disease: An underrecognized cause of cryptogenic cirrhosis. JAMA 2003;289(22):3000-4.
4. Poynard T, Morra R, Ingiliz P, Imbert-Bismut F, Thabut D, Messous D, *et al*. Assessment of liver fibrosis: Noninvasive means. Saudi J Gastroenterol 2008;14(4):163-73.
5. Halfon P, Munteanu M, Poynard T. FibroTest-ActiTest as a non-invasive marker of liver fibrosis. Gastroenterol Clin Biol 2008;32 6 Suppl 1:22-39.
6. Ratziu V, Massard J, Charlotte F, Messous D, Imbert-Bismut F, Bonyhay L, *et al.* Diagnostic value of biochemical markers (FibroTest-FibroSURE) for the prediction of liver fibrosis in patients with non-alcoholic fatty liver disease. BMC Gastroenterol 2006;6:6.
7. Feldstein AE, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: A multicenter validation study. Hepatology 2009;50(4):1072-8.
8. Musso G, Gambino R, Cassader M, Pagano G. Meta-analysis: Natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. Ann Med 2011;43(8):617-49.
9. Liangpunsakul S, Chalasani N. Is hypothyroidism a risk factor for non-alcoholic steatohepatitis? J Clin Gastroenterol 2003;37(4):340-3.
10. Kim CH, Younossi ZM. Nonalcoholic fatty liver disease: A manifestation of the metabolic syndrome. Cleve Clin J Med 2008;75(10):721-8.
11. Poynard T, Ratziu V, Naveau S, Thabut D, Charlotte F, Messous D, *et al*. The diagnostic value of biomarkers (SteatoTest) for the prediction of liver steatosis. Comp Hepatol 2005;4:10.
12. Grant A, Neuberger J. Guidelines on the use of liver biopsy in clinical practice. British Society of Gastroenterology. Gut 1999;45 Suppl 4:IV1-IV11.
13. Silva MA, Hegab B, Hyde C, Guo B, Buckels JA, Mirza DF. Needle track seeding following biopsy of liver lesions in the diagnosis of hepatocellular cancer: A systematic review and meta-analysis. Gut 2008;57(11):1592-6.
14. Janes CH, Lindor KD. Outcome of patients hospitalized for complications after outpatient liver biopsy. Ann Intern Med 1993;118(2):96-8.
15. Pasha T, Gabriel S, Therneau T, Dickson ER, Lindor KD. Cost-effectiveness of ultrasound-guided liver biopsy. Hepatology 1998;27(5):1220-6.
16. Gilmore IT, Burroughs A, Murray-Lyon IM, Williams R, Jenkins D, Hopkins A. Indications, methods, and outcomes of percutaneous liver biopsy in England and Wales: An audit by the British Society of Gastroenterology and the Royal College of Physicians of London. Gut 1995;36(3):437-41.
17. Strassburg CP, Manns MP. Approaches to liver biopsy techniques – Revisited. Semin Liver Dis 2006;26(4):318-27.
18. Froehlich F, Lamy O, Fried M, Gonvers JJ. Practice and complications of liver biopsy. Results of a nationwide survey in Switzerland. Dig Dis Sci 1993;38(8):1480-4.
19. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, *et al.* Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. Am J Gastroenterol 2002;97(10):2614-8.
20. Agence Nationale d'Accréditation et d'Evaluation en Santé (ANAES). Consensus conference. Treatment of hepatitis C. Gastroenterol Clin Biol 2002;26:B303-20.
21. Manning DS, Afdhal NH. Diagnosis and quantitation of fibrosis. Gastroenterology 2008;134(6):1670-81.
22. Radu CI, Crişan DA, Grigorescu M. How can we predict steatosis using biochemical markers (SteatoTest and Fatty liver index)? Hum Vet Med 2011;3(2):89-96.
23. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41(6):1313-21.
24. Bedogni G, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A, *et al.* The fatty liver index: A simple and accurate predictor of hepatic steatosis in the general population. BMC Gastroenterol 2006;6:33.

25. Parsian H, Rahimipour A, Nouri M, Somi MH, Qujeq D. Assessment of liver fibrosis development in chronic hepatitis B patients by serum hyaluronic acid and laminin levels. Acta Clin Croat 2010;49(3):257-65.
26. Stauber RE, Lackner C. Noninvasive diagnosis of hepatic fibrosis in chronic hepatitis C. World J Gastroenterol 2007;13(32):4287-94.
27. Leroy V. Other non-invasive markers of liver fibrosis. Gastroenterol Clin Biol 2008;32 6 Suppl 1:52-7.
28. Halfon P, Bourlière M, Pénaranda G, Deydier R, Renou C, Botta-Fridlund D, *et al.* Accuracy of hyaluronic acid level for predicting liver fibrosis stages in patients with hepatitis C virus. Comp Hepatol 2005;4:6.
29. Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM, Martin GR. Laminin – A glycoprotein from basement membranes. J Biol Chem 1979;254(19):9933-7.
30. Niemelä O, Risteli J, Blake JE, Risteli L, Compton KV, Orrego H. Markers of fibrogenesis and basement membrane formation in alcoholic liver disease. Relation to severity, presence of hepatitis, and alcohol intake. Gastroenterology 1990;98(6):1612-9.
31. Körner T, Kropf J, Gressner AM. Serum laminin and hyaluronan in liver cirrhosis: Markers of progression with high prognostic value. J Hepatol 1996;25(5):684-8.
32. Solga SF, Alkhuraishe A, Cope K, Tabesh A, Clark JM, Torbenson M, *et al.* Breath biomarkers and non-alcoholic fatty liver disease: Preliminary observations. Biomarkers 2006;11(2):174-83.
33. Banasch M, Ellrichmann M, Tannapfel A, Schmidt WE, Goetze O. The non-invasive (13)C-methionine breath test detects hepatic mitochondrial dysfunction as a marker of disease activity in non-alcoholic steatohepatitis. Eur J Med Res 2011;16(6):258-64.
34. Millonig G, Praun S, Netzer M, Baumgartner C, Dornauer A, Mueller S, *et al.* Non-invasive diagnosis of liver diseases by breath analysis using an optimized ion-molecule reaction-mass spectrometry approach: A pilot study. Biomarkers 2010;15(4):297-306.
35. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, *et al.* Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105(30):10513-8.
36. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: A new potential biomarker for cancer diagnosis and prognosis. Cancer Sci 2010;101(10):2087-92.
37. Brase JC, Wuttig D, Kuner R, Sültmann H. Serum microRNAs as non-invasive biomarkers for cancer. Mol Cancer 2010;9:306.
38. Cermelli S, Ruggieri A, Marrero JA, Ioannou GN, Beretta L. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. PLoS One 2011;6(8):e23937.
39. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, *et al.* miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. Cell Metab 2006;3(2):87-98.
40. Krützfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, *et al.* Silencing of microRNAs *in vivo* with 'antagomirs'. Nature 2005 1;438:685-9.
41. Xu H, He JH, Xiao ZD, Zhang QQ, Chen YQ, Zhou H, *et al.* Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. Hepatology 2010;52(4):1431-42.
42. Lewis AP, Jopling CL. Regulation and biological function of the liver-specific miR-122. Biochem Soc Trans 2010;38(6):1553-7.
43. Beretta L. Comparative analysis of the liver and plasma proteomes as a novel and powerful strategy for hepatocellular carcinoma biomarker discovery. Cancer Lett 2009;286(1):134-9.
44. Ong SE, Mann M. Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol 2005;1(5):252-62.
45. Tao WA, Aebersold R. Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. Curr Opin Biotechnol 2003;14(1):110-8.
46. Wu S, Kaiser NK, Meng D, Anderson GA, Zhang K, Bruce JE. Increased protein identification capabilities through novel tandem MS calibration strategies. J Proteome Res 2005;4(4):1434-41.
47. Li S, Liu X, Wei L, Wang H, Zhang J, Wei H, *et al.* Plasma biomarker screening for liver fibrosis with the N-terminal isotope tagging strategy. Sci China Life Sci 2011;54(5):393-402.
48. Saadeh S, Younossi ZM, Remer EM, Gramlich T, Ong JP, Hurley M, *et al.* The utility of radiological imaging in nonalcoholic fatty liver disease. Gastroenterology 2002;123(3):745-50.
49. Mottin CC, Moretto M, Padoin AV, Swarowsky AM, Toneto MG, Glock L, *et al.* The role of ultrasound in the diagnosis of hepatic steatosis in morbidly obese patients. Obes Surg 2004;14(5):635-7.
50. Charatcharoenwitthaya P, Lindor KD. Role of radiologic modalities in the management of non-alcoholic steatohepatitis. Clin Liver Dis 2007;11(1):37-54, viii.
51. Mishra P, Younossi ZM. Abdominal ultrasound for diagnosis of nonalcoholic fatty liver disease (NAFLD). Am J Gastroenterol 2007;102(12):2716-7.
52. Fantuzzi G. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol 2005;115(5):911-9.
53. Wang Y, Zhou M, Lam KS, Xu A. Protective roles of adiponectin in obesity-related fatty liver diseases: Mechanisms and therapeutic implications. Arq Bras Endocrinol Metabol 2009;53(2):201-12.
54. Friedman JM, Leibel RL, Siegel DS, Walsh J, Bahary N. Molecular mapping of the mouse ob mutation. Genomics 1991;11(4):1054-62.
55. Chitturi S, Abeygunasekera S, Farrell GC, Holmes-Walker J, Hui JM, Fung C, *et al.* NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. Hepatology 2002;35(2):373-9.
56. Pagano G, Pacini G, Musso G, Gambino R, Mecca F, Depetris N, *et al.* Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: Further evidence for an etiologic association. Hepatology 2002;35(2):367-72.
57. Thomas T, Burguera B, Melton LJ 3rd, Atkinson EJ, O'Fallon WM, Riggs BL, *et al.* Relationship of serum leptin levels with body composition and sex steroid and insulin levels in men and women. Metabolism 2000;49(10):1278-84.
58. Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BL, Murphy LJ. Plasma resistin, adiponectin and leptin levels in lean and obese subjects: Correlations with insulin resistance. Eur J Endocrinol 2003;149(4):331-5.
59. Chitturi S, Farrell G, Frost L, Kriketos A, Lin R, Fung C, *et al.* Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: A manifestation of lipotoxicity? Hepatology 2002;36(2):403-9.
60. Romanowska A, Lebensztejn DM, Skiba E, Tarasów E, Kaczmarski M. Retinol binding protein-4 as a serum biomarker of intrahepatic lipid content in obese children – Preliminary report. Acta Biochim Pol 2011;58(1):35-8.
61. Nobili V, Alkhouri N, Alisi A, Ottino S, Lopez R, Manco M, *et al.* Retinol-binding protein 4: A promising circulating marker of liver damage in pediatric nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol 2009;7(5):575-9.
62. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, *et al.* Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 2005;436(7049):356-62.
63. Milner KL, van der Poorten D, Xu A, Bugianesi E, Kench JG, Lam KS, *et al.* Adipocyte fatty acid binding protein levels relate to inflammation and fibrosis in nonalcoholic fatty liver disease. Hepatology 2009;49(6):1926-34.
64. Cengiz C, Ardicoglu Y, Bulut S, Boyacioglu S. Serum retinol-binding protein 4 in patients with nonalcoholic fatty liver disease: Does it have a significant impact on pathogenesis? Eur J Gastroenterol Hepatol 2010;22(7):813-9.
65. Steppan CM, Lazar MA. The current biology of resistin. J Intern Med 2004;255(4):439-47.
66. Reilly MP, Lehrke M, Wolfe ML, Rohatgi A, Lazar MA, Rader DJ. Resistin is an inflammatory marker of atherosclerosis in humans. Circulation 2005;111(7):932-9.
67. Bertolani C, Marra F. The role of adipokines in liver fibrosis. Pathophysiology 2008;15(2):91-101.
68. Fitzpatrick E, Mitry RR, Quaglia A, Hussain MJ, DeBruyne R, Dhawan A. Serum levels of CK18 M30 and leptin are useful predictors of steatohepatitis and fibrosis in paediatric NAFLD. J Pediatr Gastroenterol Nutr 2010;51(4):500-6.
69. Ribeiro PS, Cortez-Pinto H, Solá S, Castro RE, Ramalho RM, Baptista A, *et al.* Hepatocyte apoptosis, expression of death receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. Am J Gastroenterol 2004;99(9):1708-17.
70. Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. *In vivo* assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology 2006;44(1):27-33.
71. Sanyal AJ. Mechanisms of disease: Pathogenesis of nonalcoholic fatty liver disease. Nat Clin Pract Gastroenterol Hepatol 2005;2(1):46-53.
72. Hägg M, Bivén K, Ueno T, Rydlander L, Björklund P, Wiman KG, *et al.* A novel high-through-put assay for screening of pro-apoptotic drugs. Invest New Drugs 2002;20(3):253-9.
73. Kramer G, Erdal H, Mertens HJ, Nap M, Mauermann J, Steiner G, *et al.* Differentiation between cell death modes using measurements of different soluble forms of extracellular cytokeratin 18. Cancer Res 2004;64(5):1751-6.

74. Graversen JH, Madsen M, Moestrup SK. CD163: A signal receptor scavenging haptoglobin-hemoglobin complexes from plasma. Int J Biochem Cell Biol 2002;34(4):309-14.
75. Madsen M, Graversen JH, Moestrup SK. Haptoglobin and CD163: Captor and receptor gating hemoglobin to macrophage lysosomes. Redox Rep 2001;6(6):386-8.
76. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. Structure 1999;7(2):169-77.
77. Pepys MB, Hirschfield GM. C-reactive protein: A critical update. J Clin Invest 2003;111(12):1805-12.
78. Lau DC, Dhillon B, Yan H, Szmitko PE, Verma S. Adipokines: Molecular links between obesity and atheroslcerosis. Am J Physiol Heart Circ Physiol 2005;288(5):H2031-41.
79. Liu SL, Cheng CC, Chang CC, Mai FD, Wang CC, Lee SC, *et al.* Discovery of serum biomarkers of alcoholic fatty liver in a rodent model: C-reactive protein. J Biomed Sci 2011;18:52.
80. Taouli B, Ehman RL, Reeder SB. Advanced MRI methods for assessment of chronic liver disease. AJR Am J Roentgenol 2009;193(1):14-27.
81. Harrison SA, Bacon BR. Relation of hemochromatosis with hepatocellular carcinoma: Epidemiology, natural history, pathophysiology, screening, treatment, and prevention. Med Clin North Am 2005;89(2):391-409.
82. Liangpunsakul S, Chalasani N. Is hypothyroidism a risk factor for non-alcoholic steatohepatitis? J Clin Gastroenterol 2003;37(4):340-3.
83. Zhang J, Sun H, Chen L, Zheng J, Hu X, Wang S, *et al.* Relationship between serum TSH level with obesity and NAFLD in euthyroid subjects. J Huazhong Univ Sci Technolog Med Sci 2012;32(1):47-52.
84. Fung JY, Lai CL, Yuen MF. Clinical application of transient elastography (Fibroscan) in liver diseases. Med Bull 2009;14:22-5.
85. Fan XF, Deng YQ, Ye L, Li YD, Chen J, Lu WW, *et al.* Effect of Xuezhikang Capsule on serum tumor necrosis factor-alpha and interleukin-6 in patients with nonalcoholic fatty liver disease and hyperlipidemia. Chin J Integr Med 2010;16(2):119-23.
86. Kruger FC, Daniels CR, Kidd M, Swart G, Brundyn K, van Rensburg C, *et al.* APRI: A simple bedside marker for advanced fibrosis that can avoid liver biopsy in patients with NAFLD/NASH. S Afr Med J 2011;101(7):477-80.
87. Bodakhe SH, Ram A. Hepatoprotective properties of *Bauhinia variegata* bark extract. Yakugaku Zasshi 2007;127(9):1503-7.