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SCIENTIFIC REVIEWS

NON-INVASIVE DIAGNOSTICS OF LIVER FIBROSIS

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Abstract. *Detecting new units of pathogenesis in the liver fibrosis due to alcoholism, chronic viral Hepatitis B and C, non-alcoholic fatty liver disease (NAFLD), autoimmune, parasitic and metabolic diseases and other, reveals perspective for new non-invasive serum biomarkers. In fibrosis, from the wide variety of markers enzymes, proteins and cytokines are mainly used. While direct biomarkers reflect the stage of fibrosis and fibrinogenesis, indirect markers allow assessment of the general liver functions. The combination of direct and indirect markers increases the diagnostic reliability and therefore these panels and indices are investigated quite intensively in recent years in order to decrease the number of liver biopsies without completely replace it, which is still regarded as the reference method.*

Key words: *liver fibrosis, liver biopsy, alcoholic liver disease, non-alcoholic fatty liver disease, chronic viral hepatitis B, chronic viral Hepatitis C*

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GENERAL INFORMATION ABOUT LIVER FIBROSIS

The trend of continuous increase in liver fibrosis (LF) due to chronic liver damage from alcohol, chronic viral Hepatitis B and C, non-alcoholic fatty liver disease (NAFLD), autoimmune, parasitic and metabolic diseases and less frequently from toxins, drugs (Methotrexate, Tolbutamide), iron, copper and other, requires demand for sensitive, specific, non-invasive biomarkers. Chronic liver diseases of various etiology are among the leading causes of morbidity and mortality in the world [1, 2, 3, 4]. The chronic liver disease progresses through various pathological stages that range from mild inflammation to liver fibrosis and cirrhosis. The assessment of the stage of liver disease is important for the diagnostics, during treatment, as well as for follow-up. Liver fibrogenesis is a dynamic process in which a chronic inflammation stimulates the production and accumulation of collagen and extracellular matrix proteins. Hepatic stellate cells are the first cells responsible for the preparation of these extracellular matrix proteins. This dynamic process may also include remodeling and regression of fibrous tissue through the breakdown of matrix proteins by protease enzymes [2, 4, 5]. In alcoholism, factors for liver fibrosis and cirrhosis are two pro-fibrotic agents, acetaldehyde and reactive oxygen species (ROS), derivatives of ethanol. Hepatocytes are the primary site of metabolism of ethanol, where these two products are synthesized in abundance, leave outside and enter the stellate cells to activate them. Here, acetaldehyde directly regulates the transcription of collagen and synthesis of the transforming growth factor-beta 1 (TGF-β1). The effect of ROS on hematopoietic stem cells (HSCs) induces the production of inflammatory mediators which contribute to fibrotic changes in the liver. In NAFLD and its subtype non-alcoholic steatohepatitis (NASH), which are encountered in the metabolic syndrome, obesity, type 2 diabetes, dyslipidemia, and insulin resistance, a central role in fibrosis play adipokines by stimulation of the phagocytic activity

and the secretion of cytokines by Kupffer cells and macrophages and resistin by increasing the expression of monocyte chemotactic protein 1 (MCP-1) and interleukin-8 (IL-8) produced by excessive visceral fat tissue [2, 5, 6]. In cholestasis, proliferating epithelial cells of the bile ducts synthesize connective tissue growth factor (CTGF), which stimulates the production of myofibroblasts and deposition of collagen, as well as inflammatory responses by neutrophils. In chronic viral Hepatitis B and C, the pathogenesis of fibrosis is multifactorial and involves a combination of oxidative stress, liver steatosis, elevated concentrations of iron, increased hepatocyte apoptosis, under the pressure of the viral proteins and viral replication. There is evidence that protein X of hepatitis virus B directly induces the secretion of TGF-β1 from the hepatocytes and thereby contributes to the activation of paracrine factors of stellate cells [2, 5, 7]. The diagnostics of LF is mainly based on: a) liver biopsy; b) imaging methods; and c) serum biomarkers.

Biopsy – Liver biopsy is the oldest and the most accurate method to assess the stage of liver fibrosis. It is considered the "gold standard" and continues to serve as a reference method against which other methods are compared. By biopsy, we obtain information not only about fibrosis but also for inflammation, necrosis, steatosis, deposits of iron or copper. Optimally biopsy contains 5-11 full portal spaces and reflects only 1/50000 of the volume of the liver [6, 7, 8]. Liver fibrosis is not a steady process, and biopsies from various areas show different stages of fibrosis. By biopsy, fibrosis, respectively cirrhosis, may be omitted in 10-30% of patients, so it is difficult to distinguish between early and advanced cirrhosis. There is a risk of complications ranging from mild pain in the abdomen (in about 20%) to severe intraperitoneal bleeding (occurs in 0.5%) and deaths (frequency 0.009-0.12%). Liver biopsy may be poorly tolerated by patients, especially if it must be repeated. Recently transjugular liver biopsy has been used, which is safer and better tolerated but is available only in specialized centers [5, 8, 9]. Therefore, in the last decade, particular attention is paid to serum biomarkers.

Biomarkers

Biomarkers are defined by the Hulka et al. as "cellular, biochemical or molecular changes that are measurable in biological media such as blood serum, tissues or cells". According to more recent data, the biomarker is an indicator of normal biological processes, pathogenic processes or pharmacological response to a therapeutic intervention (Fig. 1). The biomarker may be a specific cell, a molecule, a gene, a product of a gene, an enzyme or a hormone (Fig. 2 and 3) [10, 11, 12, 13, 14]. It can be used for prognosis, cause, diagnosis, progression, regression, or outcome of treatment of a certain disease. Biomarkers are classified by Perera and Weinstein [14, 15, 16]. The development of serum markers is in constant evolution, offering an attractive alternative to liver biopsy for patients and physicians. In recent years, the interest in the identification and description of liver fibrosis by means of non-invasive surrogate markers is on the rise. The advantages of serum biomarkers are many [13, 15, 17]: a) missing invasion; b) no complications; c) small variability; d) can be carried out repeatedly; e) low cost; f) may be performed ambulatory; g) have good sensitivity and specificity; h) serve to evaluate the effect of therapy (immunosuppressive therapy); i) they applicable to monitoring the disease progression or regression; j) they are not susceptible to false positive results, for example, in patients with inflammation associated with other diseases; k) they are useful in assessing the stage of fibrosis in patients without clear indication for liver biopsy, such as patients with chronic Hepatitis B or C with normal ALT; l) simple, easily accessible, reliable and validated in different types of liver diseases. Although there is no ideal marker, several markers have been identified as potential useful indicators of fibrosis when used in conjunction with one another. Noninvasive biomarkers also have limitations: a) their main disadvantage is low accuracy for detecting intermediate stages of fibrosis; b) in some of them there may be a lack of hepatic specificity (i.e. serum levels of hyaluronate may be influenced by the presence of renal failure); c) some of them may have been increased in extrahepatic fibrogenesis; d) there is a need for validation based on an international survey; e) they have limited value in assessing the development of complications such as esophageal varices and variceal bleeding. A key question in assessing new non-invasive biomarkers is their validation against the available gold standard (i.e liver biopsy) [11, 17, 18].

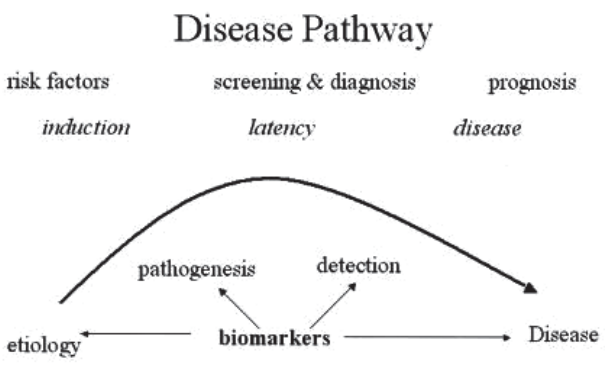

Fig. 1. Schematic presentation of the role of biomarkers in the pathological process [13]

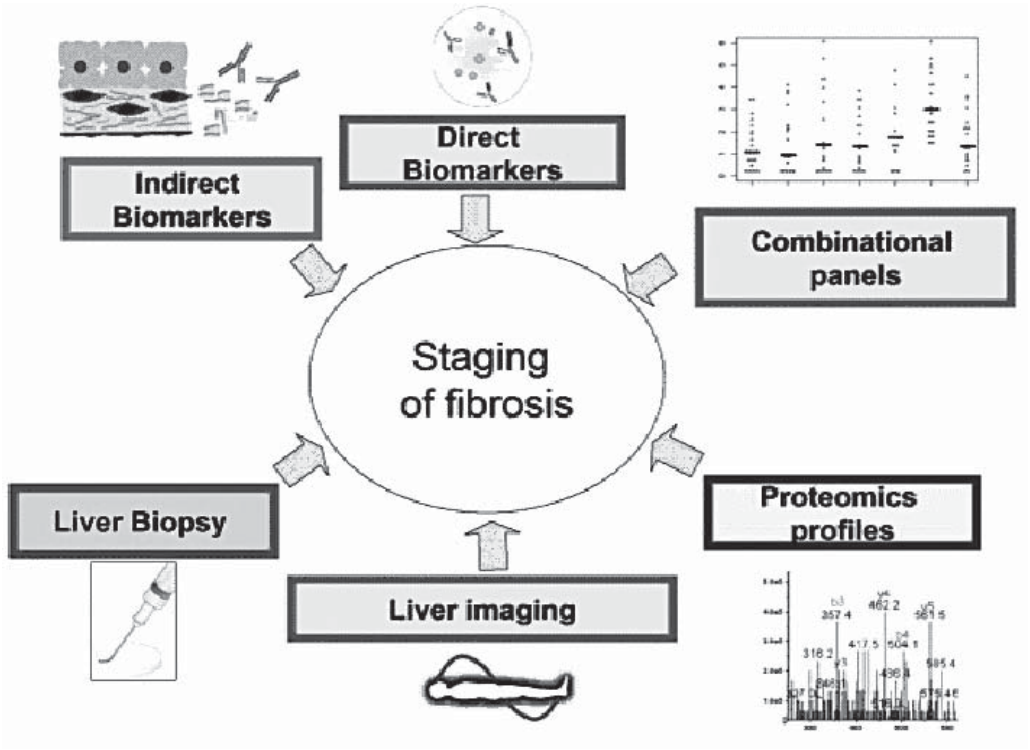

Fig. 2. Schematic presentation of the liver fibrosis and biomarkers [7]

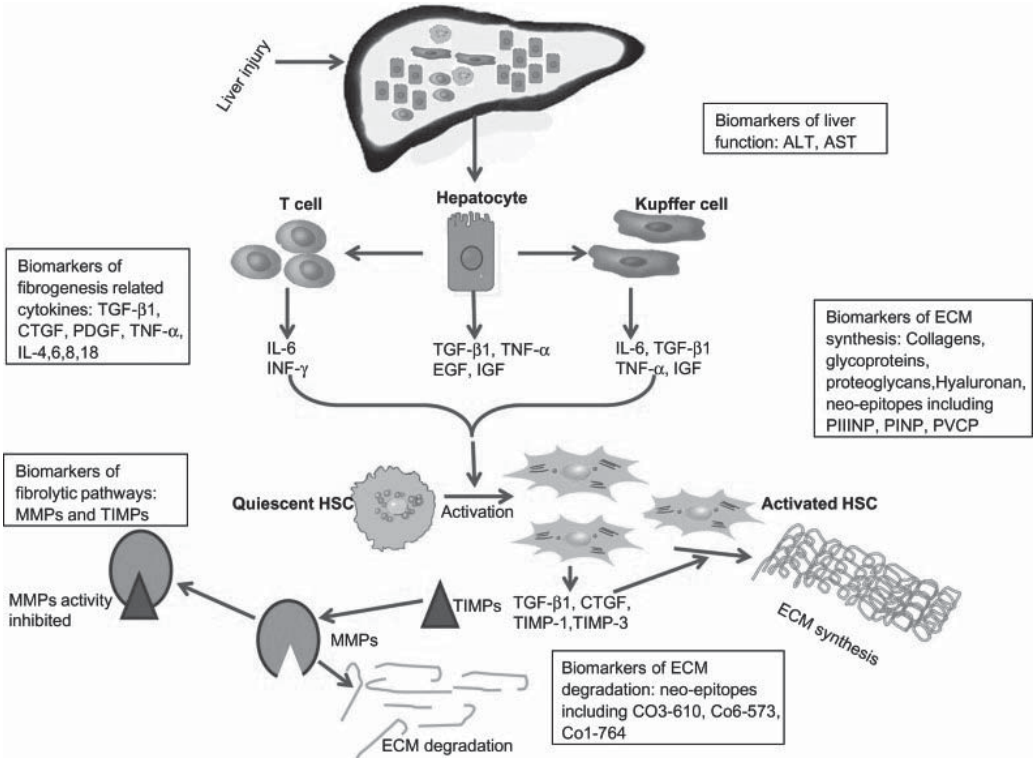

Fig. 3. Types of biomarkers in liver fibrosis according to pathogenesis [18]

Classification of biomarkers for liver fibrosis

Biomarkers are classified by Perera and Weinstein. There are two main categories: a) Class I biomarkers for fibrosis or direct biomarkers. They directly correlate with fibrogenesis and fibrinolysis and evaluate the development of the extracellular matrix (ECM). These are extracellular matrix components synthesized and secreted by myofibroblasts, stellate cells,

Kupffer cells, macrophages, Th-2 cells, neutrophils and other cells; b) Class II biomarkers or indirect markers of fibrosis. They reflect changes in liver function and are molecules that are released into the blood when there is an inflammation of the liver without correlation with the state of the ECM. Direct and indirect markers can be used alone or more frequently in combination. To assess the clinical reliability of markers we often use the area under the curve in ROC analysis (AUC). Below we present briefly some of the serum biomarkers which are subject to investigation in the last years and thus having clinical application in the diagnostics [7, 11, 12, 14, 18].

DIRECT MARKERS

These are the various components of the ECM. Direct markers show variable effectiveness in predicting liver fibrosis. They reflect two main processes - fibrogenesis and fibrinolysis (Fig. 2 and 3) [2, 3, 17, 19, 20].

1. Procollagens: procollagen I carboxy-terminal (PCICP), procollagen III amino-terminal (PCIIINP) and procollagen IV (PCIV) [5, 9, 12, 15]. These markers are an indicator for deposition of collagen fibers in the extracellular matrix. PCICP is a major component of the connective tissue. PSIIINP is another major component of the connective tissue, which has been widely studied. Its relative concentration in the basal membrane is greater during hepatic fibrogenesis because of an increase in its serum levels. Serum levels of PCIIINP show a stage of liver fibrosis. During cirrhosis, serum levels of PCIIINP correlate with serum bilirubin. In acute hepatitis, serum levels of PIIINP correlate with aminotransferase levels. It increases in viral Hepatitis B and C, alcoholic liver disease and NAFLD. Its levels correlate with severity of the liver disease. Moreover, reduction of PCIIINP correlates with patient response to treatment with interferon. The major limitation of using PCIIINP determination is that it is not specific for hepatic fibrosis and increases also in acromegaly, pulmonary fibrosis, chronic pancreatitis, and rheumatic diseases [7, 16, 18]. Moreover, it shows a lower diagnostic efficacy compared to collagen IV and hyaluronic acid. Collagen IV is a major component of ECM. Unlike the type I and type III collagens, which are processed by proteolysis, this molecule has been deposited intact and its presence in the serum directly affects its degradation. Therefore, investigation of collagen IV is more often used in clinical practice. It increases in liver diseases of different etiologies and its levels correlate significantly with the stage of liver fibrosis. The combination of collagen IV and PIIINP testing increases sensitivity and specificity. Moreover, the ratio of collagen I/III is also changed from 1:1 in a healthy liver to 1:2 in fibrosis and cirrhosis.

2. Hyaluronic acid (HA): This is a mucopolysaccharide glycosaminoglycan, with high molecular weight, a polymer that is present in the joints and liver. It is a component of ECM and is located in the synovial fluid. It is synthesized by the liver stellate cells. It is the most validated marker that most accurately predicts advanced fibrosis in chronic Hepatitis C and B, steatosis and alcoholic liver disease [9, 17, 18]. Due to its high negative predictive value (98-100%) it may be used alone in clinical practice for the exclusion of advanced fibrosis. In patients treated with immunosuppressive drugs there is reduction of serum levels of HA. High levels of HA may be due to increased synthesis and decreased elimination. In patients with nonalcoholic fatty liver disease, HA is selected for best fibrosis marker with specificity and sensitivity of 88-95% and 86-100%, respectively. HA is involved in several panels [6, 8, 9, 16].

3. MMPs (Matrix Metalloproteinases): The fibrinolysis or degradation of EMC, is an action which is primarily due to the family of metalloproteinase enzymes. This is a family of structurally related proteolytic enzymes that mediate the breakdown of ECM and basement membranes. The most commonly studied human metalloproteinases are MMP-1, or collagenase, MMP-2 or gelatinase-A, MMP-3 or stromelysin and MMP-9 or gelatinase-B. MMP-1 and MMP-2 are synthesized and secreted by activated stellate cells, MMP-9 are products of Kupffer cells. MMP-1 correlates inversely with histological severity, including necrosis and fibrosis. During liver fibrinogenesis, the expression of MMP-2 is significantly increased. The ratio MMP-1/TIMP1 correlates with the degree of inflammation. MMP-9 demonstrates inverse correlation with histological severity of chronic Hepatitis C [7, 15].

4. TIMPs (tissue inhibitors of matrix metalloproteinases): TIMPs are secreted proteins that interact with and modulate MMPs activation and operation. TIMP-1 controls the activity of most MMPs and TIMP-2 specifically inhibits MMP-2. The level of TIMP-1 significantly correlates with fibrosis, with a sensitivity of 100% but have low specificity. In chronic Hepatitis C the increase in TIMP-1 and TIMP-2 is associated with progression of fibrosis [7, 9, 15].

INDIRECT MARKERS

These are routine serum indicators. Serum levels of markers depend on their rate of purification, which is affected by dysfunction of endothelial cells, impaired

biliary excretion or renal function. They reflect primarily the liver function. In clinical practice, it is still adopted to carry out an initial screening of liver fibrosis and/or cirrhosis with simple laboratory tests (Fig. 2 and 3) [20, 21, 22, 23, 24].

1. AST/ALT ratio or AAR: Serum ALT is one of the oldest markers for assessment of liver function, in particular, the damage of the hepatocytes. Although the serum levels of ALT are affected by many factors, including gender, body mass index, hepatotoxic drugs, and others, this marker is still used because of its good sensitivity and specificity [23, 24, 25]. In some forms of acute and chronic hepatitis and/or steatosis the ratio AST/ALT is ≤ 1, while in alcoholic hepatitis AST/ALT is often > 2. The negative predictive value of the AAR is 81.3 to 96%, according to data from 2008 [7, 9, 24, 25].

2. AST/platelets ratio or APRI was developed by Wai et al. in 2003 [24, 25]. With a ratio of APRI > 1.5 and AUC of 0.80-0.89 is an indication of advanced fibrosis (respectively F3-F4) and cirrhosis in patients with chronic Hepatitis C and NAFLD and liver transplantation. In a meta-analysis involving more than 8700 patients the summary of AUC values of APRI for significant fibrosis (F2 or more), severe fibrosis (F3-F4) and cirrhosis (F4) were 0.77, 0.80 and 0.83, respectively. APRI finds clinical application as a marker for significant fibrosis in patients with Hepatitis C co-infected with HIV. Data from a meta-analysis indicate that APRI can identify hepatitis C-fibrosis only in moderate degree of accuracy (63.74%, $p < 0.01$) and with a sensitivity and specificity of 89% and 75% [16, 17, 24, 25].

3. PGA index is a combination of prothrombin index, GGT, and apolipoproteins A1. The index PGA is proposed by Poynard at al. in 1977, as a marker for assessing alcoholic liver disease [7, 8, 18]. It has recently been modified as PGAA index by adding α2-macroglobulins. This supplement improves the clinical efficiency of PGAA test. The test is associated with inflammation and fibrosis in liver disease. With this extension, the accuracy of the factor increases from 65% for PGA to 70% for PGAA. The increase in serum correlates directly to the degree of fibrosis.

4. Fibrospect II test combines three parameters: hyaluronic acid, TIMP-1, and α2-macroglobulin [25, 26]. The test can differentiate mild F0-F1 from severe fibrosis F2-F4 [122]. This is confirmed in patients with chronic Hepatitis C, where AUC is 0,831 for detection of significant fibrosis F2-F4 [123]. The index has been validated [26].

5. SHASTA index includes three indicators: hyaluronic acid, AST, and albumin [7, 8]. In a study of patients with chronic Hepatitis C and co-infected with HIV the index showed sensitivity of > 88%, negative predictive value > 94% and specificity of 100% and positive predictive value of 100% for detecting severe fibrosis (> F3).

6. Index of Forns: This index is described by Forns et al. in 2002 and is calculated based on the number of platelets, the level of cholesterol and GGT. Some authors include age. With the index of Forns, it is possible to distinguish mild fibrosis (F0-F1) of severe fibrosis (F2-F4) but it is less accurate in the differentiation of fibrosis F2 from F4. The index shows good diagnostic value (AUC: 0.81-0.86) in patients with Hepatitis C and a negative predictive value of 96% to exclude F2 or more severe fibrosis. Index of Forns has been validated in cohorts as predictive index for response to immunosuppressive therapy [7, 8].

7. FIB-4: This index includes the number of platelets, ALT, AST and age. FIB-4 well discriminates both severe fibrosis (AUC 0.85) and cirrhosis (AUC 0.91). Recently, this marker has been assessed in patients with chronic hepatitis B and 71% sensitivity and 73% specificity for diagnosing ≥F2 fibrosis is found. It is reliable in determining the NAFKD and shows sensitivity and specificity of advanced fibrosis (F3-F4) of 74-85% and 65-71%, respectively [6, 7, 11].

8. Fibrotest or Fibrosure test (in Europe and America, respectively): This test is the most widely validated indirect serum marker in Hepatitis B and C and NAFLD [62, 63]. Five parameters are used: total bilirubin, haptoglobin, GGT, α2-macroglobulin and apolipoprotein-A1. Furthermore, it may include age and gender. In a detailed review including 9 studies with 1679 patients, it has established an excellent discrimination for identifying cirrhosis (summarized AUC = 0.90) and to a lesser extent for identification of significant (≥ F2) fibrosis (AUC = 0.81). However, the conclusion is that non-invasive tests are not ready to replace liver biopsy yet. Later in a study with 6378 subjects, the mean standardized AUC for diagnose significant (≥ F2) fibrosis is 0.84 [95% CI: 083-0.86] without differences between distinct etiologies of chronic liver disease. It is interesting that in a novel study with 2411 patients, the effectiveness of FibroTest is good in all chronic hepatitis regardless of etiology for the detection of both ≥ F2 and finding F4 (standardized AUC > 0.73), except in ≥ F2 in NAFLD (standardized AUC = 0.64). For prediction of significant fibrosis (F ≥ 2), severe fibrosis (F ≥ 3) and cirrhosis (F4), AUC of the test are 0.903, 0.907 and 0.866, respectively. The test is an indicator not only of the stage of fibrosis but also for necrosis and inflammatory activity [6, 7, 17, 27].

9. Fibroindex has been developed by Koda et al. for patients with hepatitis C [7, 15, 19]. It includes the number of platelets, AST and concentration of IgG. It showed good diagnostic accuracy and high positive predictive values for significant fibrosis. Changes in the test correlate significantly with variations in the stage of fibrosis before and after administration of the antiviral therapy. FibroIndex shows high predictive values for significant fibrosis, including in the subgroup of cases with HCV and normal ALT activity. The sensitivity and specificity for detection of fibrosis in patients with HCV are 78% and 74%. In a comparative study it has been found that AUC of FibroIndex fibrosis is with sensitivity and specificity of 0.83 and 0.82, respectively.

10. FibroMeter test (FM) combines indicators patient age, platelet count, prothrombin index, AST, γ2-macroglobulins, HA and urea nitrogen. The applicability and effectiveness of FM are validated in the diagnostics of various chronic diseases, including chronic Hepatitis B and C, alcoholic liver disease and NAFLD. FM has 2 major diagnostic purposes: to establish the stage of fibrosis, which corresponds to the index of the histological change and the amount of fibrosis associated with morphometric determinations of the fibrous zone [6, 7, 8].

11. ELF test (known as Enhanced liver fibrosis and as European liver fibrosis test): Since 1997, a group of European scientists led by Rosenberg, and funded by Bayer Health care, have been seeking serum markers of liver fibrosis [6, 8, 28, 29, 30]. The program lasts for more than a decade and comes to identifying the ELF panel and is currently marketed in Europe. Recently, it has been studied intensively, especially in Europe. More than 1000 patients with liver biopsy have been followed in 13 centers across Europe. Patients have had chronic liver disease (CLD), as over 40% have had chronic Hepatitis B or C. Serum samples have been taken during liver biopsy. ELISA method was used for performing the ELF test. The results show that the ELF test is an algorithm of three biomarkers that can be used to determine the stage of hepatic fibrosis with good precision [29, 30, 31]. The calculation is based on age, HA, PIIINP and TIMP. In the original calculation the age is included and the value is called OELF, but later the age is excluded and the test is known as ELF. The sensitivity of ELF for detection of stage 3 or 4 of fibrosis is 90% and negative predictive value of 92% and AUC 0.804. It is suitable for assessment of fibrosis in chronic Hepatitis B and C, autoimmune liver disease, ALD and NAFLD. AUC in different studies is in the range of 0.773 to 0.98 for Hepatitis C and NAFLD. Researchers all over the world continue to conduct validation studies of ELF in independent groups for further evaluation of the results. The combination of the three markers more accurately reflects the severity of fibrosis. The average established AUC is 0.8 which level of performance is considered the threshold for acceptance in clinical practice. ELF markers are at least as good, if not better than liver histology at predicting clinical outcomes. It is of great importance that this test can detect patients with mild or moderate fibrosis, which is usually asymptomatic. Each of the three biomarkers included in the ELF has its informative value [16, 17, 18, 28, 29, 31, 32]. The advantage of this test is that it is automated and offered by different companies on the market.

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