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Review Article

Usefulness of Collagen Type IV (cIV) in The Detection of Significative Fibrosis in Nonalcoholic Fatty Liver Disease

Stefano JT[1], Guedes LV[2], de Souza AAA[2], Vanni DS[2], Alves VAF[3], Carrilho FJ[1,2], Largura A[4,5], Arrese M[6] and Oliveira CP[1,2]*

[1]Laboratório de Gastroenterologia Clínica e Experimental (LIM-07) do Departamento de Gastroenterologia e Hepatologia do Hospital das Clínicas HCFMUSP da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil

[2]Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil

[3]Departamento de Patologia (LIM-14), Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil

[4]Laboratório Biovel Análises e Pesquisas Clínicas, Cascavel, PR, Brasil

[5]Hospital Universitário do Oeste do Parana (UNIOESTE), Cascavel, PR, Brasil

[6]Departamento de Gastroenterología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile and Centro de Envejecimiento y Regeneracion (CARE), Departamento de Biologia Celular y Molecular, Facultad de Ciencias Biologicas Pontificia Universidad Catolica de Chile, Santiago, Chile

***Corresponding author:** Claudia Oliveira, Laboratório de Gastroenterologia Clínica e Experimental (LIM-07) do Departamento de Gastroenterologia e Hepatologia do Hospital das Clínicas HCFMUSP da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil

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Abstract

Background and Aims: Our aim was to validate a new noninvasive marker panel to assess significant and advanced fibrosis in Non-Alcoholic Fatty Liver Disease (NAFLD) patients.

Method: We conducted a study of 126 biopsy-proven NAFLD patients. The diagnosis of NAFLD was based on histological criteria and fibrosis stages were determined according to NASH CRN criteria. Clinical and laboratorial data were collected in the interval between three months before or after liver biopsy. Histological fibrosis stages were classified as: significant fibrosis (≥F2) and advanced fibrosis (F3-F4). Five serum biomarkers [Hyaluronic Acid (HA), collagen type IV (cIV), Procollagen type III (PC III), Laminin (LN) and Cholylglycine (CG)] were assessed by chemiluminescence immunoassays.

Results: The majority of patients were female (61.61%), with a mean age of 55.7±9.13 years and mean BMI was 32.1±5.9. Prevalence of diabetes mellitus, dyslipidemia, arterial hypertension and metabolic syndrome was 68.75%, 82.29%, 63,54% and 81.05%, respectively. Patients with cIV above 30 ng/mL had a 5.57-times (IC: 1.86-16.69) of having significant fibrosis and 7.61-times (IC: 2.27-25.54) chance of having advanced fibrosis versus patients with values below 30 ng/mL. HA, PIIIP, LN and CG did not detect the presence of significant and advanced fibrosis. The AUROC for the detection of significant (0.718) and advanced fibrosis (0.791) was better for cIV than the other serum biomarkers.

Conclusion: Type 4 collagen, a simple serum biomarker, could predict the presence of significant and advanced fibrosis in NAFLD patients and it wouldbe a useful tool in routine clinical practice.

Keywords: Nonalcoholic fatty liver disease; Serum biomarker; Liver fibrosis, type 4 collagen

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is a pathological clinical condition that encompasses a large spectrum of diseases and a broad spectrum of manifestations, from simple steatosis to Non-Alcoholic Steatohepatitis (NASH), whose severity may vary according to the degree of fibrosis; cirrhosis; and Hepatocellular Carcinoma (HCC) [1]. Histologically, NAFLD can be differentiated into simple steatosis or NASH according to the absence or presence of signs of hepatocellular damage, such as hepatocyte ballooning and necroinflammation, which are present in NASH [2,3], in addition to the exclusion of secondary causes of liver disease and significant alcohol consumption [1]. Currently, NAFLD is the most common cause of liver disease in the Western population, with an estimated prevalence ranging from 6.3 to 33% in the general population, depending upon the group studied and the diagnostic method used. However, the prevalence of NASH is lower, ranging from 3 to 5% in the general population [4].

Liver biopsy is still considered the gold standard for liver tissue evaluation, allowing for the ascertainment not only of the degree of fibrosis but also of other important parameters, such as inflammation, necrosis, steatosis, and the presence of hepatic iron in the sample obtained [5]. However, liver biopsy is an invasive procedure with associated risks, sometimes causing pain, hemorrhage, and even death, among other complications6. Issues regarding the quality of the liver samples and interpretation of the results are also concerns. The quality of a liver biopsy is generally related to the size and number of portal spaces evaluated [7,8]. In addition, the results of the pathological anatomical evaluation can vary according to the subjective interpretation of the individual pathologist. Due to these limitations, non-invasive methods for liver fibrosis evaluation have been studied intensely and have improved in recent decades. These methods can be divided into two categories, namely, indirect markers, which can be assessed by routine clinical exams (e.g., aminotransferases and platelet count) [9], and direct markers, which include serum levels of substances involved in the molecular pathogenesis of fibrosis, such as matrix metalloproteinases, hyaluronic acid, and cytokines [Tumor Necrosis Factor-alpha (TNF-α) and Transforming Growth Factor beta (TGF-β)] [9]. Fujii et al. Revealed that noninvasive laboratory tests are useful to predict advanced fibrosis in NAFLD patients [10]. Some published studies have evaluated noninvasive tests for NAFLD. Our group has participated in international studies to validate these methods [11-14].

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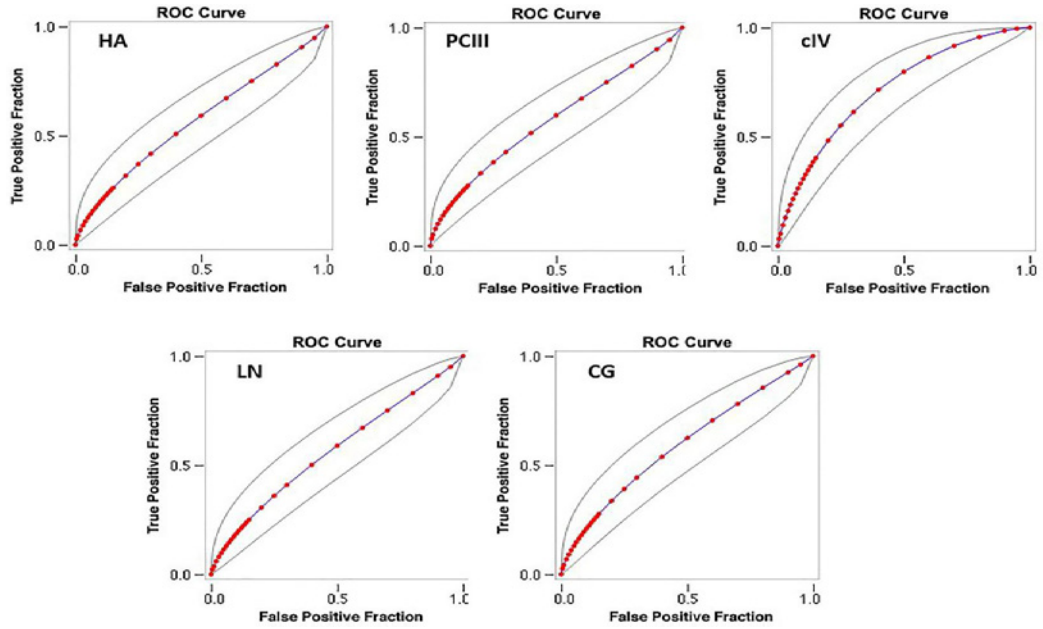

Figure 1: ROC curve for the detection of significant fibrosis (F2-F4).
HA: Hyaluronic acid, cIV: Collagen type IV, PC III: Procollagen type III, LN: Laminin, CG: Cholylglycine.

Due to the current high prevalence of obesity and Metabolic Syndrome (MtS), NAFLD is now the most frequent liver disease and the leading cause of liver enzyme abnormalities in Western countries. It is predicted that NASH will become the leading cause of advanced liver disease, liver transplantation and HCC in the next 10 to 20 years. Our aim was to assess the frequency of liver fibrosis in patients with NAFLD using a new noninvasive marker panel [Hyaluronic Acid (HA), collagen type IV (cIV), procollagen type III (PC III), Laminin (LN) and Cholylglycine (CG)].

Patients and Methods

We conducted a retrospective cross-sectional study of adult patients (≥18 years) with biopsy-proven NAFLD and included consecutive patients (n=126) who attended specialist fatty liver clinics at the University of Sao Paulo School of Medicine, Sao Paulo, Brazil. The diagnosis of NAFLD was based on histological criteria, and fibrosis stages were determined according to NASH CRN criteria [15]. Clinical and laboratory data were collected between three months before and three months after liver biopsy. According to liver biopsy results, histological fibrosis stages were classified as significant fibrosis (F2-F4) or advanced fibrosis (F3-F4). Electronic medical records of patients undergoing liver biopsy were retrospectively studied. The use of plasma or serum for biomarker analysis in the present study was approved by the Hospital das Clínicas Ethics Committee (294.198/2013).

Clinical and laboratory assessment

Clinical and laboratory data were collected between three months before and three months after liver biopsy, and electronic medical records of patients undergoing liver biopsy were retrospectively studied. Patients with evidence of other liver diseases (autoimmune hepatitis, viral hepatitis, drug-induced liver injury, hemochromatosis, cholestatic liver disease, or Wilson's disease) were excluded. In addition, subjects consuming excessive amounts of alcohol (alcohol intake >20 g/day for women; >30 g/day for men) at the time of biopsy or in the past were excluded. The inclusion criterion was that the patient had biopsy-proven NAFLD.

Relevant clinical details, including sex, age, weight, and height, were obtained at the time of biopsy. The body mass index was calculated by the formula weight (kg)/height (m^2). Patients were identified as having diabetes if they had been diagnosed with diabetes according to the American Diabetes Association criteria [16] or if they were using an oral hypoglycemic drug or insulin. The presence of MtS components was evaluated according to the National Cholesterol Education Program Adult Treatment Plan III (ATP III) guidelines [17].

Histologic analysis

Liver biopsies were performed and conducted as per routine clinical care for the investigation of abnormal liver function tests [elevated alanine Aminotransferase (ALT), aspartate Aminotransferase (AST), or Gamma-Glutamyl Transferase (GGT)] or to stage disease severity in patients with radiological evidence of fatty liver. Percutaneous liver biopsies were performed as per unit protocol at the site and were assessed by an experienced local hepatopathologist. The liver tissue was fixed in 4% formaldehyde and processed for hematoxylin-eosin and Masson trichrome staining for histological analysis. All specimens were scored by an experienced liver pathologist with expertise in NAFLD. Histological scoring was performed according to the Non-Alcoholic Steatohepatitis (NASH) Clinical Research Network criteria [15]. The NAFLD activity score was graded from 0 to 8, including scores for steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2). NASH was defined as steatosis with hepatocyte ballooning and inflammation ± fibrosis. Fibrosis was staged from F0 to F4. Clinical and laboratorial data were collected in the interval between three months before or

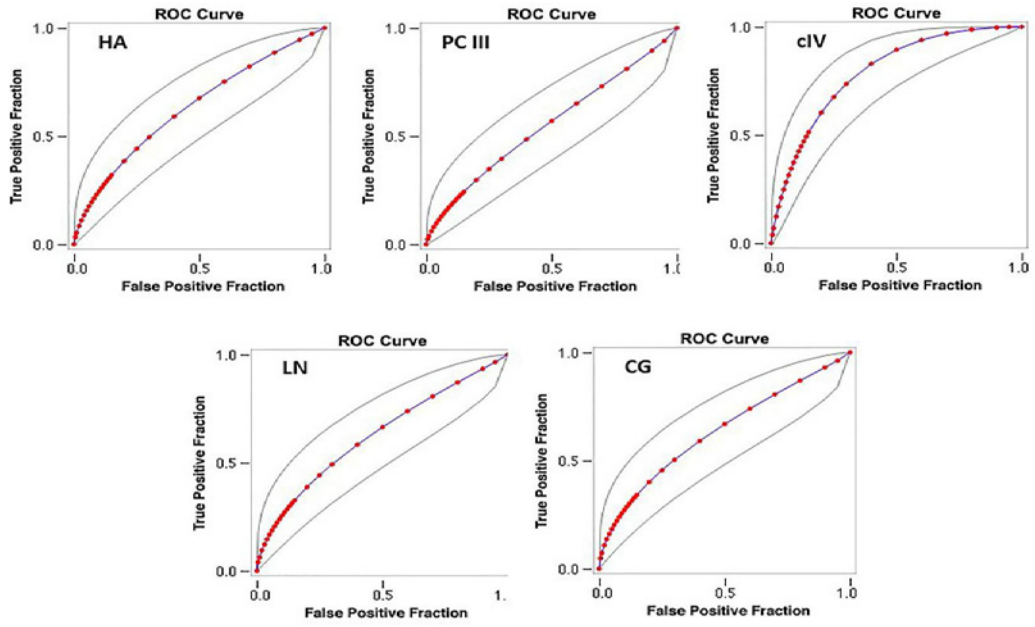

Figure 2: ROC curve for the detection of advanced fibrosis (F3-F4).
HA: Hyaluronic acid, cIV: Collagen type IV, PC III: Procollagen type III, LN: Laminin, CG: Cholylglycine.

after liver biopsy.

Serum biomarker analysis

Five direct serum biomarkers that reflect the Extracellular Matrix (ECM) for the determination of liver fibrosis were assessed. Chemiluminescence immunoassays were used to detect the following five serum biomarkers: Hyaluronic Acid (HA), collagen type IV (cIV), procollagen type III (PC III), Laminin (LN) and Cholylglycine (CG). Fasting blood samples were taken by vein puncture. The serum was separated by centrifugation and stored at -20ºC until it was assayed.

The five biomarkers of liver fibrosis were tested using a chemiluminescence immunoassay from SNIBE (China) and measured with a Maglumi 2000 fully automatic chemiluminescence immunoassay analyzer. Fasting blood samples were taken by vein puncture. The serum was separated by centrifugation and stored at -20ºC until it was assayed.

Results

Clinical and laboratory

A total of 102 patients with NAFLD were evaluated (24 patients were excluded due to incomplete medical record data). Among the included patients, the majority of patients were female (61.61%), with a mean age of 55.7±9.13 years. The mean Body Mass Index (BMI) was 32.1±5.9. The prevalence of Type II Diabetes Mellitus (T2DM), dyslipidemia, arterial hypertension and MtSwas 68.75%, 82.29%, 63,54% and 81.05%, respectively (Table 1).

Table 2 presents a logistic regression model to evaluate the relationship of markers (considered positive when they were above the suggested cut off point) with the presence of significant fibrosis (F2-F4). Patients with cIV abov e 30 ng/mL had a 5.57-fold chance of having fibrosis F2-F4 when compared with the chance of fibrosis of patients with cIV below 30 ng/mL, adjusting for the other markers.

Table 1: Demographic and clinical features of NAFLD Patients.

Feature	n=102
Age [years (Mean±SD)]	55.7±9.13
Female	61.61%
BMI [kg/m^2(Mean±SD)]	32.1±5.9
Type II Diabetes	68.75%
Dyslipidemia	82.29%
Arterial Hypertension	63.54%
Metabolic Syndrome	81.05%

The 95% confidence interval of this value was between 1.86 and 16.69. On the other hand, the markers HA, PIIIP, LN and CG were not statistically related to the presence of F2-F4 fibrosis after adjustment.

A logistic regression model was also made for the presence of advanced fibrosis (F3-F4) and is presented in (Table 3). The relationship of the markers (considered positive when they were above the suggested cutoff point) with the presence of fibrosis 3-4 was evaluated. Similarly, in individuals with significant fibrosis, those with cIV above 30 ng/mL had 7.61-times the chance of having F3-F4 fibrosis when compared with the chance of fibrosis F3-F4 of patients with cIV values below 30ng/mL, adjusting for the other markers. The 95% confidence interval of this value is between 2.27 and 25.54. The markers HA, PIIIP, LN and CG were not statistically related to the presence of F3-F4 fibrosis after adjustment.

Although the LN marker (above 50 ng/mL) did not reach statistical significance at an assumed level of 0.05, it had a positive relationship (patients with LN above 50 ng/mL had 2.83-times the chance of having advanced fibrosis (3-4) with a P value of less than 0.10). Note again that as with F2-F4 fibrosis, the upper limit for cIV is large, and the small sample size may have reduced the ability to obtain

Table 2: Logistic regression model of serum biomarkers in NAFLD patients according to significant fibrosis (F2-F4).

	Odds Ratio	CI 95% Inferior Limit	CI 95% Superior Limit	p value
Hyaluronicacid>100 ng/mL	2.95	0.41	21.07	0.28
Procollagentype III >30 ng/mL	0.98	0.27	3.56	0.981
Collagentype IV >30 ng/mL	**5.57**	**1.86**	**16.69**	**0.002**
Laminin>50 ng/mL	1.49	0.57	3.88	0.42
Cholylglycine>2.7 ug/mL	1.1	0.08	15.23	0.946

95% CI: 95% confidence interval.

Table 3: Logistic regression model of serum biomarkers in NAFLD patients according to advanced fibrosis (3-4).

	Odds Ratio	CI 95% Inferior Limit	CI 95% Superior Limit	p value
Hyaluronicacid>100 ng/mL	1.65	0.18	15.3	0.659
Procollagentype III >30 ng/mL	1.18	0.21	6.56	0.848
Collagentype IV >30 ng/mL	**7.61**	**2.27**	**25.54**	**0.001**
Laminin>50 ng/mL	2.83	0.87	9.29	0.085
Cholylglycine>2.7 ug/mL	1.19	0.11	13.37	0.889

95% CI: 95% confidence interval.

more accurate estimates.

The AUROC for the detection of significant fibrosis was better for cIV (0.718) than the otherserum biomarkers (fitted ROC area: HA=0.57, PIIIP=0.576, LN=0.567, CG=0.594) (Figure 1). In addition, the AUROC for the detection of advanced fibrosis was better for cIV (0.791) than the otherserum biomarkers (fitted ROC area: HA=0.633, PIIIP=0.553, LN=0.627, CG=0.632) (Figure 2).

Discussion

In the present study, we identified a simple serum biomarker type 4 collagen (cIV) that could predict the presence of significant fibrosis (F2-F4) in NAFLD patients and that could be a useful tool in routine clinical practice.

The estimated overall prevalence of NAFLD is 25-30% and may reach 40% in some areas of the United States [18-20]. This prevalence tends to increase, making NAFLD a very large worldwide public health problem in the future, with an increase in the number of liver transplantations secondary to NASH. In addition, patients with NAFLD are at an increased risk of overall mortality, cardiovascular disease, infectious disease, cirrhosis, and HCC [21]. Most of these complications related to NAFLD are associated directly with fibrosis [22,23]. Therefore, predicting the degree of fibrosis in patients using low-cost tools may be important, as there is a great need for better biomarkers to predict significant (F2) and advanced (F3-4) fibrosis in the general population. Currently, noninvasive tests are suboptimal in regard to helping with fibrosis diagnosis and risk stratification and being able to assist in the determination of liver biopsy indication [24]. However, a problem in evaluating the performance of noninvasive tests is that the gold standard for diagnosis, liver biopsy, is also imperfect and may lead to a bias in the analysis [25].

Therefore, in this study, we evaluated the efficacy of low-cost serum fibrosis markers in the prediction of not only advanced fibrosis but also significant fibrosis, including F2 fibrosis. For this, we studied a series of patients with NAFLD who underwent liver biopsy and evaluated these serum fibrosis markers, which could be done in the routine laboratory setting and could help in the diagnosis of liver fibrosis and the indication of liver biopsy in different populations worldwide.

According to biopsy results, cIV has been shown to be a good biomarker for the diagnosis of significant fibrosis (F2). Patients with cIV levels above 30ng/mL had a 5.57-fold higher chance of having fibrosis 2-4 than patients with cIV below 30ng/mL, after adjusting for the other markers, while the HA, PIIP, LN and CG biomarkers were not significantly related to the presence of fibrosis 2-4 after adjustment. These findings are very important because the most noninvasive clinical and laboratory scores divide individuals according to the absence or presence of advanced fibrosis into two large groups. Intermediate grades such as stage II fibrosis are in the gray zone, and cIV was able to discriminate the stages of significant fibrosis. In addition, cIV was also able to detect advanced fibrosis, which was similar to other noninvasive markers, such as FIB-4 and NAFLD fibrosis score. Recently, a Japanese study demonstrated, similar to our study, that cIV also had an AUROC of 0.803 for discriminating NAFLD patients with stage 2-4 fibrosis and 0.830 for discriminating patients with stage 3 and 4 fibrosis [26]. Another interesting Japanese study by Okanoue et al. Identified the combination of type IV collagen 7S and AST as a predictor for both NASH and related fibrosis. With this score, the authors revealed an AUROC 0.857/0.769 for NASH and 0.918/0.842 for NASH-related fibrosis [27]. The former was higher than those of the NAFIC score [26], BARD score, FIB-4 index and NAFLD fibrosis score.

On the other hand, some studies have demonstrated good performance of PIIIP [28,29]. In our study, these biomarkers were not significantly related to the presence of significant or advanced fibrosis. Recently, our group participated in a multicentric study that assessed the performance of PIIIP as a NASH-fibrosis biomarker as a diagnostic tool and determined its performance in comparison to established clinical scores and previously reported biomarker panels [24].

HA has long been described as a good marker of fibrosis [30,31]. On the other hand, some studies have illustrated that serum HA alone has limited accuracy in predicting the severity of liver fibrosis [32,33]. Because of this, some scores using HA with other clinical and laboratory variables have been validated, such as Enhanced

Liver Fibrosis Score (ELF) [34,35]. Recently, an Argentine group conducted a small study with biopsy-proven NAFLD patients in which the diagnostic accuracy of HA showed good performance for significant fibrosis with an AUROC of 0.92836. However, in the present study, we did not observe good accuracy of HA. Another interesting biomarker, LN, although it did not reach statistical significance at an assumed level of 0.05, had a positive relationship (patients with levels above 50 ng/mL had 2.83-times the chance of having advanced fibrosis (3-4) with a P value less than 0.10). In 2019, Srivastava et al. Demonstrated that noninvasive methods such as FIB-4 followed by ELF in indeterminate cases reduced the cost of primary care for NAFLD patients [37].Thus, using cIV and LN cut offs above 30 and 50ng/mL, respectively, can increase the chance of identifying more advanced fibrosis in NAFLD patients for a low cost. Our study demonstrated that type 4 collagen, a simple serum biomarker, could predict the presence of significant and advanced fibrosis in NAFLD patients and it would be a useful tool in routine clinical practice.

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