Chapter

Noninvasive Biomarkers for the Diagnosis of Liver Fibrosis and Cirrhosis

Dorothy Rosique-Oramas, Moises Martinez-Castillo, Carolina Guzman, José Luis Pérez Hernández, Jacqueline Cordova-Gallardo, Luis Very-Pineda, Fatima Higuera-De La Tijera, Daniel Santana-Vargas, Eduardo Montalvo-Jave, Francisco Sanchez-Avila, Paula Cordero Perez, Linda Muñoz-Espinosa, David Kershenobich and Gabriela Gutiérrez-Reyes

Abstract

The clinical importance of monitoring liver fibrosis lies in the morbidity and mortality of the chronic liver diseases in relation to the stage and progression of fibrosis. Whether the fibrosis stabilizes or regresses depends on the specific treatment. Liver biopsy, the current standard for the diagnosis, has implicit limitations due to sampling heterogeneity. There are noninvasive imaging methods, such as transient elastography that measures the stiffness of the liver, but it has some limitations (feasibility and unreliability), particularly in obese patients. FibroTest is the most widely used noninvasive serological method worldwide which is efficacious in the extreme stages of fibrosis, but these methods cannot discern intermediate stages. Liver fibrosis is a dynamic response that involves multiple cellular and molecular events with an excessive deposit of extracellular matrix. Even though there is much information on the pathophysiology of fibrosis, that knowledge is still incomplete, greatly hindering the development of both an accurate treatment and a noninvasive diagnostic method with adequate sensitivity for all the stages of fibrosis. It is known that IGFBP participates in liver homeostasis, and thus these proteins can be used as serum biomarkers during the progression of liver fibrosis in chronic hepatitis C.

Keywords: liver fibrosis, inflammation, fibrolysis, noninvasive diagnosis

1. Introduction

The etiologic factors that mainly induce liver fibrosis are alcoholic liver disease (ALD), chronic hepatitis C (CHC), and nonalcoholic fatty liver disease (NAFLD). Fibrogenic response can be organized in four different phases according to damage evolution: activation, production of extracellular matrix proteins (ECM), and

deposition and degradation of ECM by hepatic stellate cells (HSCs) [1]. The inefficient resorption and control of ECM components promote the establishment and progression of fibrosis inducing the distortion in the architecture of the hepatic parenchyma.

Moreover, the regulation of wounding response is orchestrated by complex activities within different cells that include HSC, macrophages, myofibroblasts, cells derived from bone marrow, and fibrocytes [1–3]. The main type of ECM proteins produced by HSC comprises collagens, fibronectin, laminin, hyaluronan, proteoglycan, and other elements [3, 4]. It has been extensively reported that liver fibrosis progresses to cirrhosis in 20% of patients; furthermore, if the damage is not controlled, approximately 10% of people can progress to carcinoma hepatocellular, which usually causes death in all the cases [5].

The clinical importance of monitoring liver fibrosis progression is correlated with the reduction of morbidity and mortality of the chronic liver diseases [6]. Whether the fibrosis stabilizes or regresses depends on the specific treatment of the underlying disease, and the grade of fibrosis is a treatment indicator [4]. The clinical evidence documenting the fate of HSC during fibrosis regression in humans is limited, compared to the extensive evidence in animal models [4, 6, 7]. Promising studies showed strong evidence that the opportune and precise identification of the etiology and degree of liver fibrosis could be crucial for decision-making in the management and treatment.

Liver biopsy has been considered the keystone for the diagnosis of fibrosis and inflammation, necrosis, and iron deposition [8]; however, this invasive procedure has implicit important limitations due to sampling heterogeneity and possible surgical complications, variability in the interpretation by pathologists, elevated costs, and the difficulty of tracking the evolution of the disease [9, 10]. Furthermore, biopsy is not recommended in patients with ascites, coagulopathy, diabetes, metabolic syndrome, and ALD [11–13]. For these reasons, in the last years, the medical and research groups have evaluated novel noninvasive strategies to discriminate the liver pathologies.

2. Diagnosis of liver fibrosis

Noninvasive measures of liver fibrosis have streamlined the management of patients with CHC [6]. Imageology methods, such as transient elastography (FibroScan™), which measures the stiffness of the liver, have received great acceptance by clinicians and patients. Clinical trials showed that FibroScan results, which are expressed in kPa, reported similar results to that of METAVIR score during biopsy interpretation of liver fibrosis in patients with CHC [10]. Moreover, this procedure has also been validated in chronic hepatitis B, NAFLD, alcoholic liver disease, primary biliary cirrhosis, and primary sclerosing cholangitis [14, 15]. However, this methodology also has some limitations such as feasibility and unreliability, particularly in obese patients or under the circumstances of limited operator experience [16]. Furthermore, it is important to consider that this strategy is contraindicated during pregnancy, ascites, and implanted cardiac pacemaker patients [10].

A variety of “direct” serum markers reflecting ECM turnover (fibrogenesis and fibrolysis) and/or fibrogenic cell changes have been developed and used clinically [17]. In this sense, the multiple analyses of proteins and clinical trials provide valuable information of liver stage. FibroTest index is within the most accepted worldwide noninvasive serological method in the diagnosis of liver fibrosis by CHC, CHB, and NAFLD [17]. For this analysis are computed five surrogate parameters:

total bilirubin, haptoglobin, gamma-glutamyltranspeptidase (GGT), alpha2-macroglobulin, and apolipoprotein-A. It is important to mention that the validation of any surrogate parameter needs to be validated by the calculation of the area under the receiver operating characteristic curve (AUROC) using liver biopsy as reference [8]. Systematic analysis of several studies revealed that FibroTest displayed an excellent discrimination to identify cirrhosis with AUROC = 0.90 but showed a lesser ability to identify ≥F2 fibrosis stages (AUROC = 0.81). The authors conclude that this index is not ready to substitute liver biopsy in the intermediate stages [18]. Additionally, some limitations such as cost and external validation are documented.

Even though there is much information on the pathophysiology of fibrosis, that knowledge is still incomplete, greatly hindering the development of both an accurate treatment and a noninvasive diagnostic method with adequate sensitivity for all the stages of fibrosis. Such a method must also be able to be performed with the necessary frequency to establish disease progression or regression, as well as the changes that occur in the processes, such as chronic inflammation, fibrogenesis, and fibrolysis. Based on our knowledge of fibrosis pathogenesis, attention is now directed towards strategies for antifibrotic therapies and regulatory challenges for conducting clinical trials with these agents. New therapies are attempting to: (1) control or cure the primary disease or reduce tissue injury; (2) target receptor-ligand interactions and intracellular signaling; (3) inhibit fibrogenesis; and (4) promote resolution of fibrosis. Progress is urgently needed in validating noninvasive markers of fibrosis progression and regression that can supplant biopsy and shorten the duration of clinical trials. Both scientific and clinical challenges remain, however, in the past three decades of steady progress in knowledge liver fibrosis.

This entails analyzing the molecules involved in these processes along with the participation of proteins such as insulin-like growth factor binding proteins (IGFBPs). Recently, IGFBP-1 and insulin-like growth factor (IGF) have been proposed as markers for advanced fibrosis in NAFLD [19]. However, the completed evaluation of IGFBPs in liver diseases is not fully understood.

3. Insulin-like growth factor (IGF) complex

The IGF is a family of proteins with high sequence homology to insulin. The IGF system functions as an endocrine, paracrine, and autocrine regulatory axis for cell proliferation, survival, and apoptosis in different types of cells [20]. In general, the IGF system consists of two surface receptors (IGF1R and IGF2R), two ligands (IGF-1 and IGF-2), and a family of IGFBPs [20, 21].

Liver is the main source of IGFs but its highest concentrations are found in the blood. Both IGF-1 and -2 forms can be detected in small amounts in the kidney and other tissues of different species, for example, in the rat, IGF-I has been detected in serum, milk, amniotic fluid, and bile; it has also been detected in human adult bile [22]. In some experiments, it has been found that IGF-1concentrations are higher in bile of neonatal rats than in the adult rats. Thus, it is believed that IGF-I in bile should have an important role in the development of gastrointestinal tract. However, the precise role and the presence or absence of IGFs and IGFBPs in bile have not yet been clearly defined. The biosynthesis of IGFs depends mainly on the levels of growth hormone (GH), insulin, prolactin, and an adequate nutrition stimulus. In contrast, estrogens and cortisol can antagonize their formation [23].

The production of IGF-1 is stimulated by GH, which is secreted by somatotropic cells in the adenohypophysis. The hepatocytes present GH receptors that are stimulated by this hormone and in a consequence an increase in the transcription of

the IGF-1 gene is triggered. IGF-1 inhibits GH secretion either directly acting on the pituitary or indirectly by stimulating the hypothalamic secretion of somatostatin which, in turn, inhibits the release of GH. In this way, a negative feedback loop GH-IGF-1 is established [21, 23].

IGFBPs play important roles in the bioavailability of circulating IGF-I, and their synthesis is under metabolic and hormonal control. Their functions can be summarized as follows: (1) they act as protein carriers in serum and control the influx of IGF-I from the vascular space to the tissues. (2) They prolong the half-life of IGF-I and regulate its metabolism. (3) They provide temporal localization of IGF-1 with the aim to be available under specific requirements. (4) They modulate the interaction of IGF-I with its receptor, thus acting as indirect control of the biological actions of IGF-I.

At present, different types of IGFBPs have been described that are mainly produced by hepatocytes and secreted into the blood serum. They can be a high affinity for binding IGFs (IGFBP-1 to IGFBP-6) or a low affinity (IGFBP-7 among others). Normal serum IGF-I levels are approximately 40 nmol/L, and 99% of circulating IGF-Iis was estimated to be associated with the different IGFBPs, mainly IGFBP-3 [23–25].

In recent years, these proteins have gained great attention due the association as biomarkers in different pathologies [25].

4. IGFBPs and liver fibrosis

An increase in IGFBP-1 levels has been observed during nonalcoholic liver disease and cirrhosis of the liver. At the same time, serum IGFBP-3 concentrations are low, correlating with the severity of liver dysfunction, and signifying poor prognosis in hepatocellular carcinoma (HCC) [26]. In *in vitro* studies, IGFBP-7 expression has been found to hepatocyte apoptosis and HSC activation [27, 28]. In relation to the participation of IGFBP-2, -5 and -6, there is little evidence of their concentrations in serum and the possible association with liver diseases. Nevertheless, those proteins can inhibit angiogenesis (IGFBP-4) [29], regulate the role of TNF-α, tumor growth, and an increased expression in pulmonary and liver fibrosis (IGFBP-5) [30, 31] and promote prostate cancer cell migration (IGFBP-6) [22, 32]. The available information about the role, tissue production, and dependent and independent functions of IGFBPS as well as their regulation in related liver pathologies is resumed in **Table 1**.

In the present work, we include recently obtained data in our laboratory of a prospective comparative multicenter study to evaluate the production of IGFBPs according to liver fibrosis grade in patients with CHC. We provide a valuable and innovative approach to the analysis of IGFBPs as a group of proteins with important potential for improving diagnosis and maybe soon can be used as novel noninvasive biomarkers for liver fibrosis.

Even advanced stages of liver fibrosis have been described as reversible, stimulating considerable research to identify molecules for the development of antifibrotic therapies [35, 36].

IGFBPs are produced in the liver, but there is little evidence of their participation in the process of liver damage in humans. Their study can further improve the knowledge of liver fibrosis pathophysiology and enable the identification of therapeutic targets.

The aim of the present study was to measure the serum concentrations of the different IGFBPs in patients with CHC and analyze them according to the grade of fibrosis grade.

Protein	Production	IGF-dependent functions	IGF-independent functions	Participation in liver	Ref.
IGFBP-1	Liver Kidney Endometrium Amniotic fluid Fetal plasma	Modulates cell growth, differentiation, and metabolism	Proliferation, migration, and apoptosis	↑ Hepatic cirrhosis ↑ F3-F4 in NAFLD	[19]
IGFBP-2	CNS Liver Heart Kidney Prostate Adipocytes	Promotes the bioavailability of IGF to its ligands	Proliferation It binds with TGF-β	Unknown	[33]
IGFBP-3	Liver	Form a ternary complex with IGF and acid-labile subunit (90%)	Survival Activation of MAPK in pulmonary fibrosis Interaction with TGF-β	↓ Cirrhosis, and HCC	[26]
IGFBP-4	Liver Heart Bone Ovary Prostate Kidney	Inhibits angiogenesis Regulates bone formation	Tumor processes and in reproduction biology	Possible role in experimental liver regeneration	[29, 34]
IGFBP-5	Liver Bone Lung Testicle Ovary Uterus Placenta	Form a ternary complex with IGF and acid-labile subunit Inhibits and promotes tumor growth	Inhibits the actions of TNF-α Inhibits and promotes tumor growth proliferation	↑Experimental Fibrosis	[30, 31]
IGFBP-6	Liver Lung Intestine CSN	Inhibits the actions of the IGF-II	Promotes the migration of cancer cell lines Inhibition of angiogenesis	Unknown	[22, 32]
IGFBP-7	Liver	Cell adhesion in cancer cells	Cell proliferation, differentiation, adhesion, senescence, apoptosis, and angiogenesis. Tumoral suppressor Mutual regulation with TGF-β	↓HCC ↑ Experimental and clinical fibrosis	[27, 28]

CNS: Central nervous system, HCC: Hepatocellular carcinoma, F3-F4: advanced stage of fibrosis, ↑ up-regulation and ↓ down-regulation.

Table 1.
Proteins of the IGF system, IGFBPS production, functions, and their regulation in related liver pathologies.

5. Methods and patients for the study of IGFBPs in chronic hepatitis C

A prospective, cross-sectional, observational study was conducted. It included patients seen at the General Hospital of Mexico, University Hospital of Autonomous University of Nuevo Leon, and the National Institute of Medical Sciences and Nutrition, within the time frame of January 2011 to December 2015. The patients were treatment-naïve or untreated, and their grades of fibrosis were evaluated through

the FibroTest and FibroScan methods. According to fibrosis grade, the patients were divided into four groups: F0, F1-F2, F3, and F4. Given the small number of patients with stages F1 and F2, they were combined as a single group (n = 25). Patients with at-risk alcohol consumption (AUDIT < 8) and whose fibrosis grade was not determined using the same diagnostic methods stated above were excluded. The control group was made up of healthy subjects, defined as persons that were not at-risk drinkers (AUDIT < 8) and had negative serology for hepatitis A, B, and C viruses (n = 160).

All participants signed statements of informed consent and the study protocol followed the ethics guidelines of the 1975 Declaration of Helsinki. Blood samples (10 mL) were taken from all participants. The serum was separated and stored at −80°C until its use. The anthropometric variables obtained for each study subject were: sex, age, height (measured in centimeters with a stadiometer), weight (measured in kilograms with a manual scale), and body mass index (BMI) (kilograms/meters^2; weight/height^2 formula). The following biochemical tests were performed on all the study subjects: total bilirubin, direct bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). A detailed clinical history was carried out for each patient, through which the presence of clinical data of liver damage was intentionally evaluated. IGFBP determination was carried out through multiple suspension array technology (Millipore®). The use of antibodies made it possible to study the concentration of a variable number of proteins in a single serum sample. Thus, seven proteins were analyzed in a single assay, reducing intra-assay and inter-assay error, %CV of <10 and of <15 for intra- and inter-assay, respectively, without cross-reactivity. The HIGFBMAG-53 K07 kit was employed. The data were acquired utilizing Luminex200 MAGPX® Systems equipment (series number 10294005), following the supplier's specifications. The sensitivity of the minimum and maximum detection values for each protein was obtained using Luminex XPONENT software. The continuous variables were described as mean ± standard deviation and the qualitative variables as absolute and relative frequencies (percentages). The qualitative variables were analyzed using the chi-square test and ANOVA test, orthogonal analyses, and Spearman correlation was used for the quantitative variables. Statistical significance was set at $p < 0.05$. Logistic regression analysis with advanced fibrosis stage as the dependent variable was applied. Receiver operating curves (ROCs) were made. Statistical analysis was carried out using the SPSS version 22 program.

6. Clinical determinations

Demographic analysis showed the predominance of women in the group of CHC; furthermore, the age mean was 50 years in CHC in comparison with the mean of 37 years in CT. Nevertheless, body mass index (BMI) did not show differences in both groups of study. Liver function tests that include bilirubin and transaminases (AST and ALT) showed evident and significant increment of values that reflect the liver dysfunction in CHC patients (**Table 1**).

A total of 120 patients diagnosed with chronic hepatitis C (CHC) were included in the study. Many of the patients were women. All the patients were untreated and did not drink alcohol. The control population was made up of 165 healthy subjects with negative viral panels and no at-risk alcohol consumption (**Table 2**).

6.1 Serum determination of IGFBP proteins

As was previously mentioned, the multiple suspension arrangement (Luminex) was used to quantify the levels IGFBP-1 to IGFBP-7 proteins, both in serum of

patients with chronic liver disease and in CT. As we expected, a differential regulation of IGFBPs through the different stages of fibrosis in CHC was observed.

6.1.1 IGFBP-1 to IGFBP-7 quantification

IGFBP-1 concentration (ng/mL) was higher in patients compared with controls (1.35 ± 0.26 and 0.65 ± 0.12, respectively, p = 0.02), as were the IGFBP-2 values (16.26 ± 3.81 vs. 3.91 ± 0.35, p = 0.002). IGFBP-3 had the highest concentrations of all the IGFBPs, with a tendency to be lower in patients (778 ± 36) than in controls (878 ± 40, p = 0.066). IGFBP-4 concentrations were higher in patients than in

	CHC (120)	**CT (165)**	**P**
Gender n (%)			
Men	25 (29)	138 (89)	<0.001
Women	95 (71)	27 (11)	
Age (years)	51 ± 10	37 ± 9	<0.001
BMI (Kg/m^2)	27 ± 4	28 ± 4	0.464
Total Bilirubin (mg/dl)	1.37 ± 0.22	0.78 ± 0.03	<0.001
Direct Bilirubin (mg/dl)	1.21 ± 0.16	0.68 ± 0.03	<0.001
AST (UI/I)	84 ± 7	30 ± 1	<0.001
ALT (UI/I)	90 ± 6	28 ± 2	<0.001

AST, aspartate amine transferase; ALT, alanine anime transferase. Data are expressed as mean ± standard deviation.

Table 2.
Demographic data of study groups.

IGFBP (ng/mL)	**F0 (35)**	**F1–F2 (11–14)**	**F3 (21)**	**F4 (39)**	**CT (165)**	**p**
1	0.9 ± 0.5	1.5 ± 0.5	1 ± 0.5	1.4 ± 0.5	0.65 ± 0.12	NS
2	8.8 ± 8.4	10 ± 5	26 ± 9	18 ± 7	3.9 ± 3.5	F0vs.CT* F1-F2vs.CT* F3vs.CT† F4vs.CT†
3	695 ± 202	620 ± 350	844 ± 304	756 ± 391	878 ± 406	NS
4	25 ± 17	88 ± 76	37 ± 30	77 ± 29	21 ± 19	F1-F2vs.CT* F3vs.CT* F4vs.CT†
5	97 ± 71	237 ± 186	107 ± 36	324 ± 292	241 ± 118	F4vs.CT* F0vs.F4† F1-F2vs.F3†
6	136 ± 53	112 ± 68	168 ± 81	126 ± 59	122 ± 42	NS
7	20 ± 10	42 ± 30	91 ± 23	60 ± 42	33 ± 31	F3vs.CT† F4vs.CT† F0vs.F3† F0vs.F4* F1-F2vs.F3* F3vs.F4*

*Data are expressed as mean ± standard error (SE). *p < 0.05; † p < 0.005.*

Table 3.
Concentration of IGFBP 1 to 7 and fibrosis stages in patients and control groups.

controls (59 ± 14 vs. 21 ± 1.9, p = 0.008). IGFBP-5 values were similar between patients and controls (251 ± 26 vs. 241 ± 21, p = 0.786), as were IGFBP-6 concentrations (131 ± 6.6 vs. 122 ± 4.2, p = 0.244). IGFBP-7 concentrations were higher in patients (57 ± 4.4), compared with controls (33 ± 3.1) (p < 0.001).

6.1.2 Fibrosis stage and IGFBP analyses

Patients were classified according to 2 noninvasive methods for staging fibrosis: FibroTest and FibroScan. The 120 patients were divided into the following groups: F0 (n = 35), F1–F2 (n = 11–n = 14), F3 (n = 21), and F4 (n = 39). Significant differences were found for IGFBP-2, IGFBP-4, IGFBP-5, and IGFBP-7 (**Table 3**). The differences were mainly between fibrosis grade and the control group for IGFBP-2 and IGFBP 4. IGFBP-2, IGFBP-4, IGFBP-5, and IGFBP-7 concentrations correlated with the grade of fibrosis. There was an association between IGFBP-2 and fibrosis grade (**Figure 1A**), with an r of 0.263 (p = 0.001). IGFBP-4 was increased in F1–F2, F3, and F4, about the control subjects (**Figure 1B**). The correlation of IGFBP-4 with fibrosis grade produced an r of 0.228, (p = 0.003). According to fibrosis grade, there were significant differences between F0 vs. F4 (p < 0.001) and F1-F2 vs. F3 (p < 0.001) in the IGFBP-5 results (**Figure 2A**). We observed an oscillating pattern, given that stages F1–F2 and F4 had the highest concentrations.

There was a 2-fold greater increase in IGFBP-7 concentrations in patients, compared with controls. Upon fibrosis grade evaluation, we found a gradual increase in the concentration of that protein (**Figure 2B**), obtaining significant differences between F0 vs. F3 (p < 0.001), F0 vs. F4 (p < 0.001), F1–F2 vs. F3 (p = 0.002), and F3 vs. F4 (p = 0.005) (**Figure 2B**). The correlation of IGFBP-7 with fibrosis grade

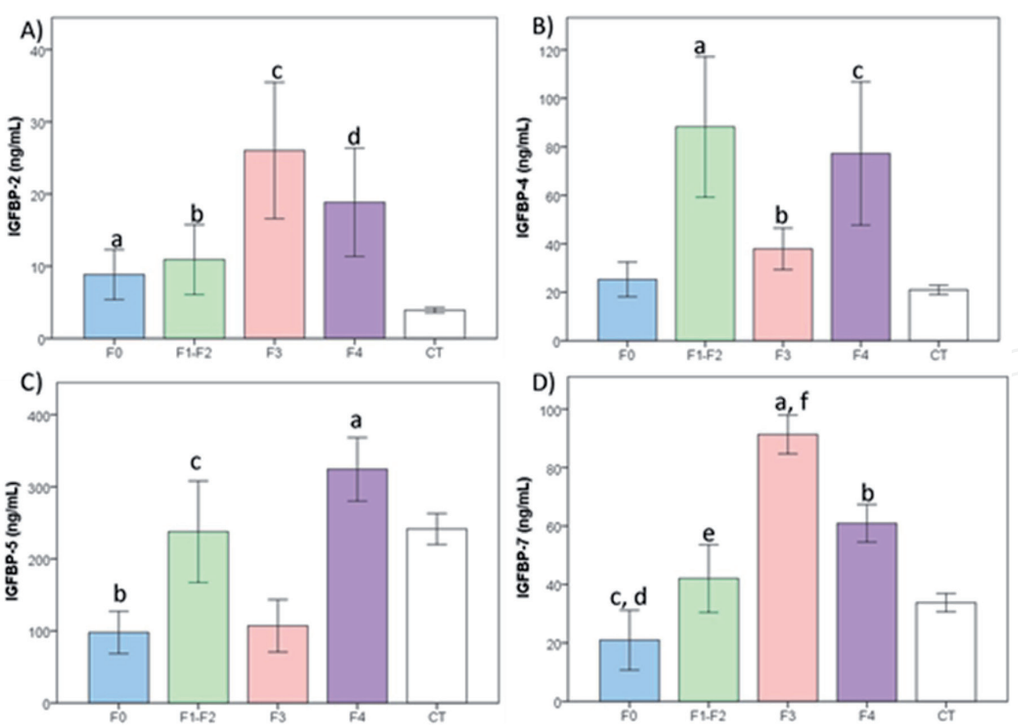

Figure 1.
IGFBP production in different fibrosis stages of CHC compared with healthy individuals. The concentration of IGFBPs (ng/mL) was determined at initial (F0), middle (F1–F2), and severe (F3 and F4) fibrosis stages in CHC and healthy individuals. (A) IGFBP-2 differences: a = F0vs CT (p = 0.002), b = F1–F2 vs. CT (p = 0.01), c = F3 vs. CT (p = 0.001), d = F4 vs. CT (p = 0.001). (B) IGFBP-4 differences: concentrations a = F1–F2 vs. CT (p = 0.002), b = F3 vs. CT (p = 0.008) and c = F4 vs. CT (p = 0.001). (C) IGFBP-5 differences: concentrations a = F4 vs. CT (p = 0.007), b = F0 vs. F4 (p < 0.001), c = F1–F2 vs. F3 (p < 0.001). (D) IGFBP-7 differences: concentrations a = F3 vs. CT (p = 0.001), b = F4 vs. CT (p < 0.001), c = F0 vs. F3 (p < 0.001), d = F0 vs. F4 (p = 0.008), e = F1–F2 vs. F3 (p = 0.002) and f = F3 vs. F4 (p = 0.005). Data are expressed as mean ± standard error (SE).

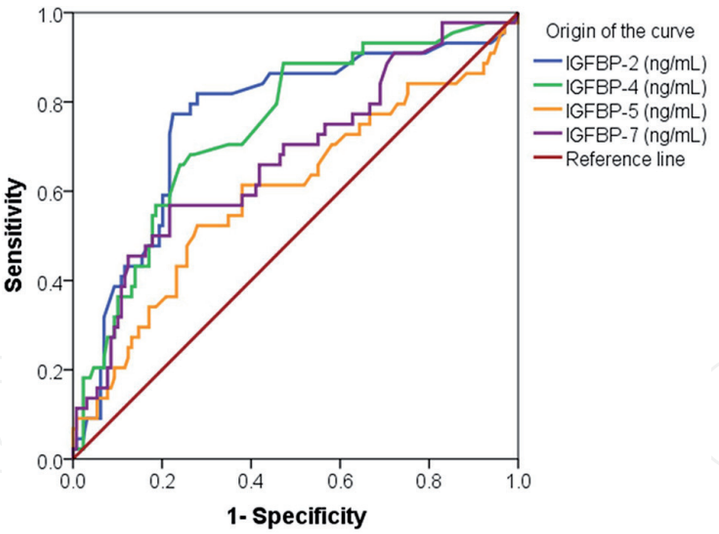

Figure 2.
ROC curve of the concentration of IGFBP-2, -4, -5, and -7 in patients with F4.

produced a statistically significant r of 0.384 (p = 0.001), indicating that IGFBP-7 was associated with the grade of fibrosis, signifying that it could be a serologic biomarker for liver fibrosis.

Moreover, our study provides for first time completed screening of seven IGFBPs and their serum concentration in the initial, middle, and severe fibrosis stages. For IGFBP-1, slight differences in middle and severe stages and CT were found, whereas nonsignificant differences were observed for de IGFBP-3, and -6 among F0, F1–F2, F3, F4, and CT classifications. However, evident variations were observed for IGFBP-2 and -4 according to the fibrosis degrees and CT group. (**Table 3**). The most evident concentration changes were observed in IGFBP-7, where the statistical analysis revealed a correlation in the fibrosis degrees and their production (**Table 3**).

6.1.3 Sensitivity and specificity of IGFBPs in severe fibrosis stage

Additionally, we performed calculation of the area under the receiver operating characteristic curve (AUROC) using the severe stage as a reference parameter with the aim to determine the sensitivity and specificity of IGFBP-2, -4, -5, and -7 with F4 stage (**Figure 2** and **Table 4**). The results showed that the value of area under the curve for IGFBP-2 was 0.760 (p < 0.001; 95% CI, 0.672–0.847); whereas in IGFBP-4 was 0.744 (p < 0.001; 95% CI, 0.659–0.828), IGFBP-5 was 0.600 (p = 0.049; 95% CI, 0.496–0.703), and for IGFBP-7 was 0.674 (p = 0.001; 95% CI, 0.579–0.770) (**Table 4**). Based on the AUROC interpretation, where the

Protein	Area	95%CI		p
		Lower limit	Upper limit	
IGFBP-2 (ng/mL)	0.760	0.672	0.847	0.000
IGFBP-4 (ng/mL)	0.744	0.659	0.828	0.000
IGFBP-5 (ng/mL)	0.600	0.496	0.703	0.049
IGFBP-7 (ng/mL)	0.674	0.579	0.770	0.001

Table 4.
Evaluation of the area under the ROC curve of IGFBP-2, -4, -5, and -7 in patients with severe fibrosis degree (F4).

values take more significance when they are close to the unit and with p value, we observed that the degree of predictive significance indicator of these proteins is: IGFBP2 < IGFBP-4 < IGFBP-7 < IGFBP-5.

7. Discussion

Our study is the first to quantify seven IGFBPs in patients with hepatitis C virus (HCV) and associate those protein levels with fibrosis grade. Different studies consider IGFBP-1 as an insulin-sensitive protein that participates in the development of metabolic diseases, such as insulin-resistance or metabolic syndrome, in patients with or without liver disease. Some authors have reported elevated levels of IGFBP-1 expression [37], whereas others have found low levels [38, 39]. Because of findings of its increased expression, IGFBP-1 has been identified as a possible biomarker for alcoholic liver disease (ALD). Controversial results have been reported in relation to NAFLD. A decrease in serum concentrations due to interaction with insulin was described [40, 41], but Hagström et al. reported elevated values, with higher concentrations in patients with advanced fibrosis [19]. High concentrations have also been associated with hepatocellular carcinoma, albeit the role of that protein is still contradictory [37, 42]. In our study, IGFBP-1 concentrations were higher in subjects with CHC, but no differences were found in relation to fibrosis grade. However, studies on patients with liver cirrhosis of different etiologies (hepatitis C, hepatitis B, NAFLD, ALD, and autoimmune hepatitis) have shown that IGFBP-1 increases [19, 43] and is higher in advanced fibrosis (F3 and F4) [19].

IGFBP-2 has been suggested as a biomarker for metabolic diseases, such as diabetes and insulin resistance [33]. In our study, IGFBP-2 concentrations were until 6-fold higher in patients, compared with controls. In accordance with fibrosis grade, there was also a gradual increase in IGFBP-2 concentration, but with no statistically significant differences between fibrosis stages, suggesting that IGFBP-2 participates in liver damage and the development of fibrosis caused by HCV. Our results concur with those reported for idiopathic pulmonary fibrosis, in which high levels of IGFBP-2 were determined [44].

Additionally, in this work, we can determine for first time the evaluation of the specificity and sensibility of the seven IGFBPs. Interestingly, IGFBP-2 showed higher area values (AUROC) in F4 and thus can be considered as the best predictive IGFBP protein indicator in severe fibrosis stage followed by -4, -7 and -5.

IGFBP-3 had higher concentration levels than those of the other six IGFBPs. Nevertheless, the concentrations showed a tendency to be lower in patients than in controls. IGFBP-3 has been the most widely studied protein because of its high affinity for IGF-I. It has been described as a biomarker for liver dysfunction classified with the Child-Pugh scale, with a lower concentration in patients with Child-Pugh class C [45]. Since 1995, many studies have been conducted on IGFBPs in patients with liver cirrhosis due to different causes. Results have shown a decrease in IGFBP-3 concentrations and an increase in IGF-I [46, 47]. IGFBPs are also thought to be associated with a high risk for liver cancer [48] and with poor prognosis [26]. Aleem et al. concluded that IGFBP-3 is superior to IGF-I and IGF-II for predicting the development of hepatocellular carcinoma in patients with cirrhosis caused by HCV [49], due to viral protein interaction that alters the IGF axis and the subsequent progression to liver cancer [50].

Miller et al. described the serum proteome of NAFLD, reporting increased IGFBP-3, compared with control subjects. They then analyzed fibrosis grade and severity and found that IGFBP-3 was able to distinguish between different disease stages [51]. In 2017, Chishima et al. studied the GH/IGF-I/IGFBP-3 axis

in patients with NAFLD and CHC and the relation to the histologic severity of NAFLD. IGFBP-3 levels were lower in patients with cirrhosis caused by NAFLD, whereas the levels did not decrease according to fibrosis grade in patients with HCV-induced chronic liver disease [46]. Our results concurred with those of that study.

Cirrhosis alters IGF-I production and suppresses protein metabolism. In studies on children with end-stage liver disease (ESLD) who underwent liver transplantation, the authors concluded that their results partially explained the failure to growth and the reduced number of functioning hepatocytes in patients with ESLD [52].

IGFBP-4 has been associated with the progression of lung cancer, finding high expression of that protein in lung tissue, and showing a decrease in survival [53]. In our study, IGFBP-4 concentration was 2-fold higher in patients, compared with controls, and behavior fluctuated in relation to fibrosis grade, with no statistically significant differences. In a study on patients with ESLD, no differences in IGFBP-4 were found upon comparison before and after liver transplantation [52]. In another study on cirrhotic patients and controls, there were no differences in IGFBP-4 when measured by the Western ligand blot technique [54]. Experimental studies have shown a regulation of the increase of that protein, along with IGFBP-1 and IGF-I, by AMPc, IL-6, IL-1β, and TNF-α [55, 56].

IGFBP-5 has been studied in animal models of progressive intrahepatic cholestasis, suggesting that it plays a possible role in the pathogenesis of chronic cholangiopathy. The same authors reported that IGFBP-5, in human stellate cells (LX-2), increased pro-fibrotic marker expression, and concluded that IGFBP-5 participates in liver fibrosis progression [30]. In our study, IGFBP-5 concentrations were similar between patients and controls. However, upon classifying them by fibrosis grade, we found differences in F0 vs. F4 and F1–F2 vs. F3. These findings concur with the results reported by Colak et al. who showed that IGFBP-5 played an important role in many pathophysiologic stages of liver fibrosis [57]. One of the functions of IGFBP-5 was the trans-differentiation of HSCs into myofibroblasts, improving the survival of those cells through anti-apoptotic effects on the activated HSCs, increasing collagen Iα1, TIMP-1, and MMP-1profibrotic gene expression.

It is known that IGFBP-6 can induce chemotaxis in T cells and monocytes, but not in B cells. It also increases oxidative stress and may be a late amplifier of neutrophil activation [58]. However, there have been few studies conducted on IGFBP-6 in liver diseases. In our study, IGFBP-6 concentrations were the same in patients and controls, and we found a tendency for concentrations to increase with the increase in fibrosis grade. IGFBP-6 has been described to be affected by HCV proteins and to participate in the progression to liver cancer [50, 59].

Finally, different studies demonstrate that IGFBP-7 (IGFBPrP1) contributes to liver fibrogenesis [27, 60, 61]. In our study, we found a significant increase in patients with liver disease. Likewise, we observed a gradual increase according to fibrosis grade. It was higher in F3, indicating that it could be a serum biomarker for liver fibrosis. IGFBP-7 has been widely studied in experimental models of liver fibrosis for identifying the mechanisms involved in the activation of HSCs and the signaling pathways, the result of which induces fibrosis. IGFBP-7 was inhibited in rat HSCs, inducing apoptosis in activated HSCs, and as a result, ameliorating liver fibrogenesis [61]. IGFBP-7 has also been shown to attenuate liver fibrosis through the regulation of MMPs/TIMPs in mice [62]. IGFBPrP1 contributed to the development of liver fibrosis in fibrotic and cirrhotic tissue biopsy samples and may be a novel molecule involved in the progression of liver fibrogenesis [28]. Studies conducted in vitro found that IGFBPrP1 induced liver fibrosis by means of HSC activation and hepatocyte apoptosis through the Smad 2/3 signaling pathway [27]. In addition, it acted as an initiator of liver fibrosis by inducing inflammation, HSC activation, and ECM protein deposit through the ERK1/2 pathway [63]. IGFBPrP1

has also been shown to promote fibrosis, by enhancing the TGF-β1 expression that it triggered, and the Egr1, PTEN, Hhip, MAP2K2 (MEK2), and MAPK3 (ERK1) genes were identified as candidates for the hepatic fibrosis-related pathway induced by IGFBPrP1 [60]. Mutual IGFBPrP1 and TGF-β1 regulation has been found that probably accelerates liver fibrosis progression [64]. IGFBPrP1 inhibition attenuates fibrosis by reestablishing the MMP2/TIMP2 and MMP9/TIMP1 balance concomitantly with the inhibition of HSC activation, low TGF-β1 expression, and ECM degradation [62]. Previous results, together with ours, show that IGFBP-7 is a molecule that can be diagnostically useful and a possible therapeutic target for liver fibrosis.

Currently, there are available treatment regiments against hepatitis C; in general, these drugs have as specific targets viral proteins (e. g. NS3/4A, NS5A, and NS5B) inhibiting their replication. The ratio of efficacy of different combinations of drugs can reach until 95% of efficiency [65, 66]. Regarding the action of these drugs and liver fibrosis, it has been reported that peginterferon and/or ribavirin, daclatasvir, sofosbuvir, and simeprevir can cause regression of liver fibrosis [67]. The specific molecular events induced by the administration of these drugs are not very well understood. However, it has been observed that the liver parenchymal injury and hepatocyte death are associated with the host's inflammatory response and reactive oxygen species promoted by virus proteins [68, 69], whereby the elimination of viral load abrogates the subsequent liver damage. Nevertheless, the molecular mechanisms triggered by the pharmacological therapy in the IGF system are not evaluated until now; it is possible that tissue fibrosis reversion can be orchestrated by IGF elements such as the IGFBPs. Thus, the study of these proteins could have beneficial implication for diagnosis and as well as complementary target to improve the liver regeneration.

8. Conclusion

We found higher serum concentrations of IGFBP-2, IGFBP-4, IGFBP-5, and IGFBP-7 in patients with CHC, and in accordance with fibrosis grade, IGFBP-2, IGFBP-4, and IGFBP-7 are associated with severe fibrosis. Based on our study, we strongly suggest the possibility that IGFBPs participate in ECM protein modulation and reuptake and regulate the progression of chronic liver disease and development of liver fibrosis. Therefore, we believe that IGF binding proteins play an important role in chronic hepatitis C and can be serum marker candidates for liver fibrosis.

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Conflict of interest

The authors have no conflict of interest or financial conflict with any organization or entity.

Author details

Dorothy Rosique-Oramas[1], Moisés Martínez-Castillo[1], Carolina Guzman[1], José Luis Pérez Hernández[3], Jacqueline Cordova-Gallardo[5], Luis Very-Pineda[1], Fatima Higuera-De La Tijera[3], Daniel Santana-Vargas[3], Eduardo Montalvo-Jave[3], Francisco Sanchez-Avila[2], Paula Cordero-Perez[4], Linda Muñoz-Espinosa[4], David Kershenobich[1,2] and Gabriela Gutiérrez-Reyes[1*]

1 HIPAM Laboratory, Experimental Medicine Unit, School of Medicine, National Autonomous University of Mexico, Mexico City, México

2 National Institute of Medical Sciences and Nutrition "Salvador Zubirán", Mexico City, México

3 General Hospital of Mexico "Dr. Eduardo Liceaga", Mexico City, México

4 University Hospital "Dr. José Eleuterio González", Autonomous University of Nuevo Leon, Monterrey, México

5 General Hospital "Dr. Manuel Gea González", Mexico City, México

*Address all correspondence to: gabgurey@yahoo.com.mx

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