Fibrinogen α-Chain as a Serum Marker of Liver Disease

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Abstract

Liver fibrosis is the hepatic response to an insult characterized by an accumulation of extracellular matrix proteins. If the underlying cause is not treated or eliminated, the disease can progress and may lead to several clinical complications including hepatocellular carcinoma or even death. Thus, detection, staging, and follow-up of liver fibrosis are the main issues in the prognosis and treatment of patients with chronic liver disease. In recent years, new advances in mass spectrometry-based proteomics technology and protein fractionation techniques have improved protein identification as well as protein quantification in many different samples and diseases including liver fibrosis. In particular, the fibrinogen α chain and more specifically the serum levels of the 5.9 kDa fragment of fibrinogen α C-chain have shown to be altered in several hepatic etiologies. In fact, these results have been reproduced by different laboratories, and recently a marked downregulation of this protein fragment has also been described in the initial stages of liver fibrosis. In this chapter, we have described the potential role of fibrinogen α chain and particularly the 5.9 kDa fragment of fibrinogen α C-chain as a circulating marker of liver fibrosis.

Keywords

Biomarker • Liver • Early detection • Fibrinogen • Fibrinogen alpha chain • Fibrosis • Mass spectrometry • Proteomics

List of Abbreviations

AH	Autoimmune hepatitis
ALD	Alcoholic liver disease
CDT	Carbohydrate-deficient transferring
ECM	Extracellular matrix
ELF	Enhanced liver fibrosis
ESI	Electrospray ionization
GGT	Gamma-glutamyltransferase
HA	Hyaluronic acid
HBV	Hepatitis B virus
HCV	Hepatitis C virus
LT	Liver transplantation
m/z ratio	Mass-to-charge ratio
MALDI-TOF/TOF MS	Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry
MS	Mass spectrometry
NASH	Nonalcoholic steatohepatitis
PIIINP	Amino-terminal peptide of type III procollagen
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
SID-MS	Stable-isotope dilution mass spectrometry
TGF-β	Transforming growth factor beta

| TIMP-1 | Tissue inhibitor of matrix metalloproteinase-1 |
| TOF | Time of flight |

Key Facts of Liver Function and Structure

- The liver is the largest organ in the body and is located in the upper-right region of the abdominal cavity.
- It is comprised of four different lobes.
- It is the only organ that has a dual blood supply: the hepatic artery and the portal vein.
- While the hepatic artery provides the oxygen necessary to achieve the different vital functions, the portal vein, which comes from the intestines, is detoxified in the organ. Both blood flows converge and form the hepatic vein which finally drains into the inferior vena cava.
- Several cell types compose this organ, and they can be classified as parenchymal and non-parenchymal cells.
- Hepatic stellate cells (HSC), Kupffer cells, and hepatocytes are the most relevant cell types. The latter, however, are the principal cell types in the liver and occupy around 80% of its volume.
- The liver, but generally hepatocytes, executes many different functions including protein synthesis, bile synthesis and its secretion, toxin metabolism, etc.

Key Facts of Mass Spectrometry (MS)

- It was invented by J. J. Thomson.
- Its function is to separate, identify, and even quantify molecules based on their mass-to-charge ratio (m/z). However, molecules must be firstly converted into gaseous ions.
- A mass spectrometer consists of three basic elements:
  - Ion source: this is where the sample is ionized. Examples of this component are electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and surface-enhanced laser desorption/ionization (SELDI).
  - Mass analyzer: this is where molecules are separated according to their mass-to-charge ratio (m/z). Examples of this component are time of flight (TOF) and quadrupole ion trap, among others.
  - Ion detector: this is used to detect and record the relative abundance of each charged molecule.

Definitions of Words and Terms

| Alternative splicing | This is a biological process by which multiple mRNAs (and subsequently proteins) are generated from the same |

	gene. Basically, DNA is transcribed into a pre-mRNA, which contains both introns and exons. Subsequently, mature mRNA is formed by the total or partial retention of exons, creating a diverse panel of mRNA from a single pre-mRNA. This phenomenon enhances proteome diversity and the functional capacity of each gene.
Coagulation	This is a physiological process by which fibrinogen is converted to fibrin by the action of thrombin. This cleavage leads to the appearance of a fibrin clot which turns liquid blood into a thickened mass.
Fibrinolysis	This is the mechanism by which fibrin clots are broken down by the action of plasmin. Its physiological function is to prevent the growth of blood clots and reduce their potential side effects.
HA	Hyaluronic acid is one of the components of extracellular matrix (ECM). Its serum levels have proven to have a good correlation with the severity of liver disease. In fact, it is an excellent tool to identify late stages of liver fibrogenesis. However, it is not as useful for the detection of early fibrotic stages.
HCV	Hepatitis C virus is one of the main causes of liver fibrosis and cirrhosis worldwide and the main indication for liver transplantation. However, recent advances in drug development have allowed the obtaining of better and highly effective drugs that will predictably reduce all the clinical complications caused by this virus.
Liver biopsy	Until recently, liver biopsy has been the gold standard method to stage liver fibrosis. This invasive procedure involves the removal of a small sample of liver tissue to be further histologically examined to determine the underlying cause and the severity of the liver disease.
m/z ratio	The mass-to-charge ratio is the relation between the mass (m) of a molecule and its charge number (z). In this chapter, the molecules analyzed by MS are proteins. In addition, since many of the ions carry a charge of $+1$, the m/z ratio is often considered to be the molecular weight of the compound.
MALDI-TOF/TOF MS	This is a proteomic approach that allows the analysis, identification, and even quantification of several biomolecules including proteins. It can be used for biomarker discovery.
NASH	Nonalcoholic steatohepatitis is one of the most advanced consequences of nonalcoholic fatty liver diseases (NAFLD). Subsequently, NASH can lead to cirrhosis, liver failure, or even HCC. In terms of prevalence, this phenomenon has

increased in Western societies during the last decades due to sedentary habits, unhealthy diet, and the metabolic syndrome. Basically, it is characterized by fat accumulation in the liver which causes inflammation and damage.

Posttranslational modifications
These are biological mechanisms that enhance proteome diversity. In addition, they play a pivotal role in several cellular processes. Posttranslational modifications (PTM) include all the modifications generated during or after protein synthesis. Phosphorylation, methylation, and proteolysis are just some examples of the wide variety of these modifications.

Scheuer classification
This is one of the most commonly used classifications for the assessment of chronic hepatitis in liver biopsy specimens. Basically, liver fibrosis can be staged from zero to four. No fibrosis corresponds to fibrosis stage 0 (F0); minimal portal fibrosis would be staged as F1, periportal fibrosis (F2), fibrosis beyond the portal tract making septums (F3), and, finally, cirrhosis (F4).

SELDI-TOF-MS
It is a high-throughput proteomic approach similar to MALDI-TOF, which is also used for biomarker discovery. However, it incorporates solid-phase chromatography that reduces the complexity of the biological sample protein and allows better selectivity of the proteins of interest.

TGF-β
Transforming growth factor beta is mainly produced by Kupffer cells and is the most important cytokine that promotes wound healing and repair. TGF-β induces apoptosis of hepatocytes and activates HSC.

TIMP-1
Tissue inhibitor of metalloproteinase 1 is produced by several types of hepatic cells and inhibits the degradation of newly formed collagen fibrils by MMPs. It is also highly upregulated during fibrogenic processes.

Introduction

Liver fibrosis is a dynamic and reversible wound-healing process characterized by the accumulation of extracellular matrix proteins (ECM) in response to chronic injury (Bataller and Brenner 2005). Despite the remarkable regenerative capacity of the liver, if the underlying cause is not treated and eliminated, chronic liver injury can lead to cirrhosis, hepatocellular carcinoma, liver failure, or even death (Bataller and Brenner 2005; Lee and Friedman 2011; Forner et al. 2012; Schuppan and Kim 2013; Tsochatzis et al. 2014). Many different causes can contribute to liver fibrosis. However, the main etiologies in developed countries

are alcoholic liver disease (ALD), cholestasis, chronic hepatitis B virus (HBV) and C virus (HCV) infections, and, recently, nonalcoholic steatohepatitis (NASH) (Schuppan and Kim 2013; Friedman 2010). Knowledge of the extent of hepatic fibrosis is critical to define and establish its prognosis and progression as well as make treatment decisions. However, in clinical practice, early detection of liver fibrosis is, in many cases, relatively complex since the detection and clinical symptoms of hepatic decompensation can occur within weeks, months, or even years after the onset of injury (Hernandez-Gea and Friedman 2011). In fact, some studies have pointed out that about 40% of patients with liver disease are asymptomatic for 10–15 years before any clinical complication appears (Heidelbaugh and Bruderly 2006). Thus, prompt detection of hepatic fibrogenesis is of major importance in the prognosis and treatment of patients with chronic liver disease.

At present, liver biopsy remains the gold standard method to stage liver fibrosis (Fernandez-Varo and Jimenez 2011). Nevertheless, this procedure must be performed by a trained physician due to its invasiveness. In addition, it may involve several clinical complications including pain, hemorrhage, potential morbidity, or even death. Furthermore, the small amount of specimen obtained in the liver biopsy might not represent the fibrosis stage of the whole organ due to the heterogeneity of fibrosis. Moreover, interobserver variability should also be taken into account, and liver biopsy does not allow fibrosis progression/reversion follow-up (Castera and Pinzani 2010; Bedossa et al. 2003; Fernandez-Varo and Jimenez 2011). For these reasons, many efforts have been made during the last decade to find new noninvasive procedures as alternatives to liver biopsy. These efforts have essentially been focused on developing imaging techniques and identifying new circulating biomarkers (Carrión et al. 2010; Castera et al. 2008; Crespo et al. 2012). Despite both options having several important advantages including their noninvasiveness and easy sequential follow-up of the disease, the latter allows better accessibility (Alrawashdeh and Crnogorac-Jurcevic 2011).

Ideally, a marker of liver fibrosis should be specific and highly sensitive when discriminating the different stages of liver fibrosis of any etiology. It should also be easy to measure and economical as well as reproducible. In addition, it should not vary with inflammation or with any metabolic disorder and should be useful to monitor fibrosis progression/regression (Baranova et al. 2011). Finally, a biomarker should also be able to discriminate the underlying causes of liver disease. Nonetheless, there is currently no marker that fulfills all these criteria (Watkins 2009). However, the advances made in this field have allowed the identification of new potential and very promising biological markers. Additionally, the combination of several serum markers appears to be another interesting strategy in discriminating different stages of liver fibrosis (Sebastiani et al. 2006; Castera 2012; Crespo et al. 2012). In fact, one of the most well-known algorithms is enhanced liver fibrosis (ELF), which combines serum levels of three different direct biological markers: HA, PIIINP, and TIMP-1. This algorithm was developed in an international multicenter study including a cohort of 1,021 patients

with different hepatic etiologies (Rosenberg et al. 2004). Age was initially included in the algorithm but was finally excluded on validation of the ELF tool. In fact, ELF is highly accurate for detecting patients with significant fibrosis and cirrhosis (Martinez et al. 2011; Crespo et al. 2012). However, several studies have reported lower diagnostic capacity in early and mild stages of hepatic fibrosis in which the values obtained from fibrotic patients are similar to those of healthy subjects (Martinez et al. 2011; Lichtinghagen et al. 2013). In fact, this is a common trend in many other noninvasive markers and algorithms. Thus, discriminating early stages of liver fibrosis has remained a challenge in this disease.

In view of the fact that hepatocytes, the predominant cell type in the liver, are responsible for the synthesis of most intrahepatic and extrahepatic proteins including plasma coagulation proteins such as prothrombin or fibrinogen (Tennent et al. 2007), it is feasible to think that even subtle changes in liver architecture can incur abnormalities in protein synthesis and be further detected in the bloodstream. Thus, circulating proteins have been one of the most studied areas of research. Recent advances in mass spectrometry-based proteomics technology and protein fractionation techniques have improved protein identification as well as protein quantification in many different samples and diseases (Aebersold and Mann 2003; Camerini and Mauri 2015). In particular, thanks to these technical advances, not only changes in many abundant and non-abundant proteins can now be detected but also in protein fragments released into the circulation (Hortin 2006). In this context, some studies have demonstrated alterations in circulating serum fibrinogen fragments.

Fibrinogen Structure

Human fibrinogen is one of the most abundant proteins in blood and plays a pivotal role in the coagulation cascade (Mosesson et al. 2001; Davalos and Akassoglou 2012). Structurally, this 340 kDa acute phase glycoprotein synthesized in the liver is comprised of two symmetric sets of three different chains known as Aα, Bβ, and γ. The predominant Aα chain is the longest polypeptide chain, containing 610 residues and weighs 63.5 kDa. The Bβ (56 kDa) and γ (48 kDa) chains consist of 461 and 411 amino acids, respectively. However, all the fibrinogen chains are very heterogeneous as a result of alternative splicing, posttranslational modification, and even proteolytic degradation (Herrick et al. 1999). The N-terminus of each chain is bound by disulfide knots, forming its central E domain. In this region, the Aα and Bβ chains present a small peptide sequence known as fibrinopeptide A and B, respectively, which are crucial for preventing the conversion of fibrinogen to fibrin. At the opposite end, the C-terminus fragments of the Bβ and γ chains together with a portion of the Aα chain constitute both outer D domains. The end peptide sequence of the Aα chain is called the αC domain and forms "free-swimming appendages" (Doolittle 1973) which can interact not only with each other but with the central

region of the molecule or even with other αC domains (intermolecular interactions) in fibrin polymerization. In addition, this region is susceptible to being cleaved from fibrinogen by several proteases including plasmin and being released into the systemic circulation where it is further degraded (Cesarman-Maus and Hajjar 2005). However, some fragments from this region can be detected before their dissolution and can be used as potential biomarkers of many diseases including liver fibrosis.

Coagulation and Fibrinolysis

Under physiological conditions, both coagulation and fibrinolysis mechanisms are precisely regulated by many different elements (Cesarman-Maus and Hajjar 2005). Due to different stimuli, fibrinogen is converted to fibrin by thrombin. Afterward, the fibrin clot is broken down in order to prevent excessive clot formation and subsequently blood vessel blockage. Several factors are involved in this complex process, although plasmin is basically the enzyme that lyses the fibrin mesh at different locations, generating fibrinogen fragments of different sizes (Cesarman-Maus and Hajjar 2005). The resulting fragments are released into the circulation and are subsequently degraded by other proteases. However, this process is accelerated during liver injury (Takahashi et al. 1990). Furthermore, it has also been well established that these breakdown fragments can exert direct effects on tissue repair (Herrick et al. 1999). Therefore, it is possible that the soluble fragments released into the bloodstream or even the molecules related to coagulation or fibrinolysis processes could be differentially expressed in liver diseases and be used as biomarkers of hepatic fibrosis. Nevertheless, it seems that depending on the etiology, the mechanisms of degradation and even the cleavage sites could be different. In fact, some studies have reported that conformational changes occur in fibrinogen molecule after moderate alcohol consumption (Gorinstein et al. 2003). It is also well known that there is a lower concentration of serum fibrinogen levels in patients with moderate alcohol consumption (Sierksma et al. 2002). Additional investigations have suggested that moderate consumption entails no changes in fibrinolysis (van Golde et al. 2002), despite other studies describing the presence of clear changes in this mechanism (Dimmitt et al. 1998).

Fibrinogen as a Marker of Liver Cirrhosis

It is well established that serum fibrinogen levels remain unaltered in patients with stable liver diseases. However, this behavior differs in patients with more advanced hepatic fibrosis (de Maat et al. 1995). In fact, several studies have reported that fibrinogen levels are usually diminished in these patients, even though some cirrhotic patients may present fibrinogen serum concentrations within the normal range

(Pluta et al. 2010). It is of note that Child-Pugh C cirrhotic patients show the lowest serum levels, probably due to an overall reduction of protein synthesis in this stage. Furthermore, dysfibrinogenemia is also often observed in patients with acute liver failure (Mistry and Jain 2011). Therefore, serum fibrinogen alterations seem to mainly be found in advanced liver disease, and only in very specific cases such as acute liver failure are early alterations observed.

Early stages of liver fibrosis are associated with a clear inflammatory process. In this context, the analysis of fragmentome (also called peptidome) may play a pivotal role for its detection. The fragmentome approach is the analysis of the entire set of tiny peptide components most of which are the fruit of the degradation of the abundant proteins in serum or plasma (Hortin 2006). In fact, it has been reported that fragmentome modifications may be better indicators of inflammation and even organ injury than intact proteins. With regard to fibrinogen, several molecular fragments of the α, β, and γ chains are cleaved and released into the bloodstream due to several stimuli. A more in-depth discussion of β and γ chain fragments is outside the scope of this chapter. However, α chain fragments are particularly interesting due to their rapid alteration in early stages in liver fibrosis (Marfà et al. 2014). Indeed, recent studies using MS technology have identified altered fragments of the fibrinogen α chain with a 5.9 kDa C-terminal fragment of the fibrinogen α chain being one the most frequently described.

5.9 kDa Fibrinogen Alpha C-Chain Fragment as a Marker of ALD

At present, determination of blood ethanol levels is performed for recent alcohol intake. On the other hand, there are several biochemical markers for the diagnosis of ALD. Among them, carbohydrate-deficient transferrin (CDT) and gamma-glutamyltransferase (GGT) are the most commonly used (Anton et al. 2002). However, in some heavy drinkers, no alterations are observed in blood, with GGT serum concentrations being within the standard range (Matsuda et al. 1993).

The first study to identify and describe a clear alteration in the expression of this fibrinogen fragment in ALD was in 2004 (Nomura et al. 2004). In this study, the fibrinogen α C-chain fragment was detected by SELDI-TOF-MS. Serum levels of this circulating fragment were found to be downregulated at admission and significantly increased after 3 months of abstinence from alcohol (Fig. 1). Thus, this investigation demonstrated that this peptide allowed the monitoring of potential relapses as well as alcohol abstinence. In addition, it is of note that this circulating biomarker was able to detect all the heavy drinkers, including patients with normal GGT serum levels which were unaltered in 25% of cases, thereby establishing its diagnostic value in ALD (Sogawa et al. 2007) and highlighting its promising role as a potential biomarker of alcohol intake. However, the number of patients included in the study was low and the vast majority were men (94%). Moreover, all had consumed more than 100 g of alcohol per day for a minimum of 10 years, indicating that all of these subjects most likely had
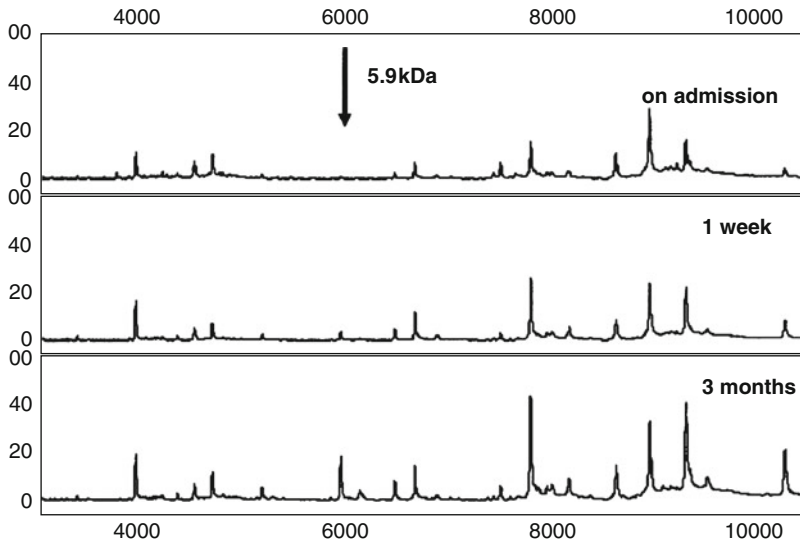
Fig. 1 Differential proteomic profile between ALD patients at admission and during alcohol abstinence. Representative portion of the SELDI-TOF-MS spectra comprised between 4,000 and 10,000 m/z of serum samples obtained from ALD patients at admission and at 1 week and 3 months of abstinence from alcohol. The upregulation of the 5.9 kDa fibrinogen α C-chain fragment is clearly associated with the time of alcohol abstinence (Figure from Nomura et al. (2004) with permission from the Publisher John Wiley and Sons, Inc)

advanced rather than early liver fibrosis. Nonetheless, these results were validated a few years later by the same group using the MALDI-TOF/TOF MS approach (Sogawa et al. 2009). In the same study, three more altered peptides were also detected by MALDI-TOF/TOF MS in serum samples from heavy drinkers and were identified as fragments of fibrinopeptide A (m/z 1466), phosphorylated fibrinopeptide A (m/z 1616), and the fibrinogen α C-chain (m/z 2660).

Later, Sogawa et al. (2011) performed another study in a group of healthy subjects and patients with different liver diseases, including ALD. Basically, the investigation was focused on the quantitative determination of the 5.9 kDa fragment of fibrinogen α chain by the use of the MALDI-TOF-MS method with a stable-isotope dilution mass spectrometry (SID-MS). This widely used MS approach, which allows the discovery and the quantitative determination of potential circulating disease bio-markers, was able to determine the 5.9 kDa α fibrinogen fragment more accurately than ordinary MS. Ultimately, Noda et al. (2011) developed a quantitative enzyme-linked immunosorbent assay (ELISA) which was able to perform a more accessible and affordable quantification analysis to determine 5.9 kDa fragment levels in serum samples from patients with ALD and healthy subjects. However, taking into account the complexity of working with specific peptide fragments and not the whole protein, this technique was characterized and validated using the SID-MS approach, showing a good correlation with the ELISA outcomes (Fig. 2).
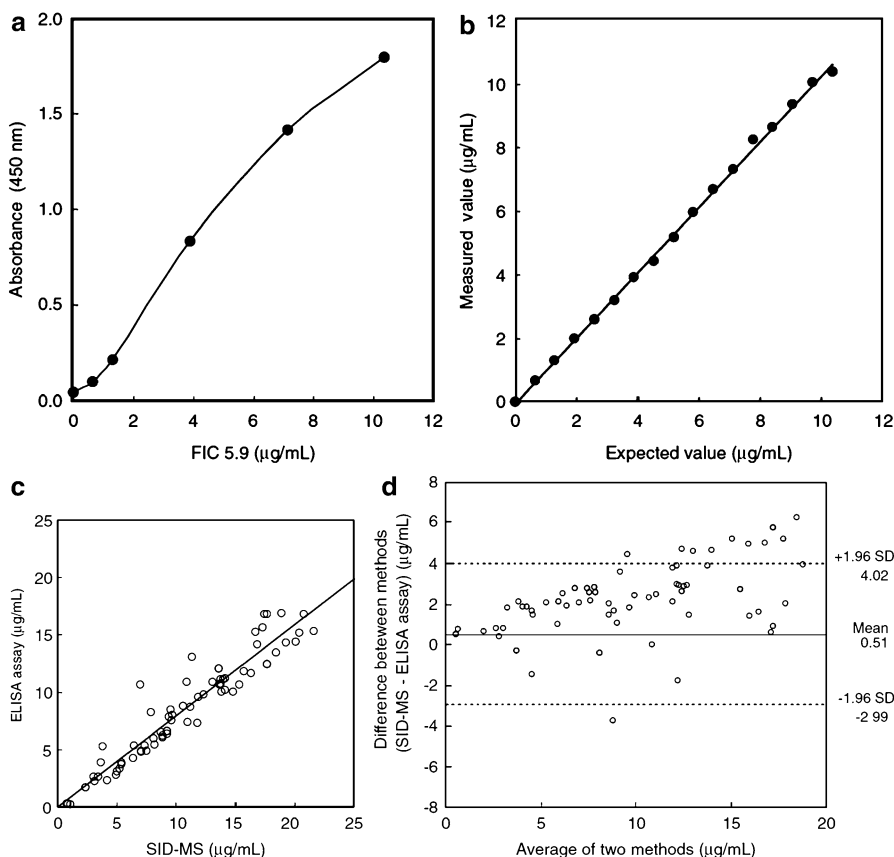
Fig. 2 Characterization of the ELISA assay and comparison to the SID-MS method. (**a**) Standard curve between the 5.9 kDa fibrinogen α C-chain fragment concentration (μg/ml) and colorimetric measures (450 nm). (**b**) ELISA validation test of linearity ($r^2 = 0.998$). (**c**) Correlation of the 5.9 kDa fibrinogen α C-chain fragment concentration with SID-MS results. (**d**) Examination of the agreement between SID-MS and ELISA procedures (Bland-Altman plot). Results are given as mean (*solid line*) ± 1.96 SD (*dotted lines*) (Modified figure from Noda et al. (2011) with permission from the Publisher John Wiley and Sons, Inc)

Early Serum 5.9 kDA Fibrinogen Alpha C-Chain Fragment Alterations in HCV Patients

In 2011, Sogawa et al. demonstrated for the first time a subtle downregulation in the 5.9 kDa fragment of fibrinogen α C-chain in serum samples from HCV patients compared to a control group. These differences were increased when the samples were analyzed by the SID-MS approach. However, this observation was very preliminary as only eight HCV patients were included in this investigation. Afterward, Sogawa et al. (2013) analyzed 88 serum samples from HCV patients and
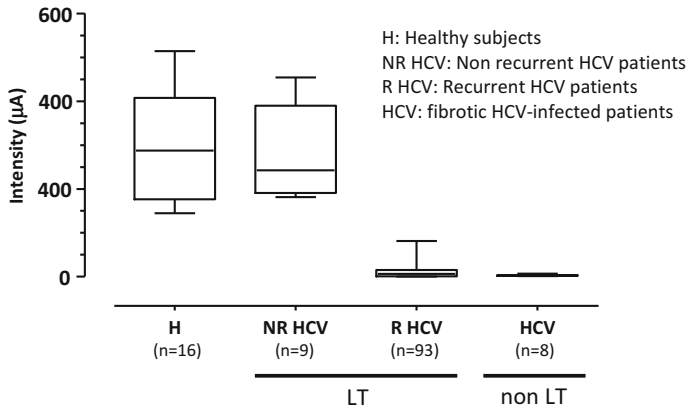
Fig. 3 Comparison of the 5.9 kDa fibrinogen α C-chain fragment levels between HCV patients and healthy subjects. SELDI-TOF-MS intensity values of the 5.9 kDa fibrinogen α C-chain fragment from different groups of HCV patients, nonrecurrent HCV patients, and healthy subjects. The intensity of the peptide released is markedly suppressed in all HCV patients under an active fibrogenic process regardless of liver transplantation (Modified figure and legend from Marfà et al. (2014) and used under the Creative Commons Attribution (CC BY) license. Lack of a 5.9 kDa Peptide C-Terminal Fragment of Fibrinogen a Chain Precedes Fibrosis Progression in Patients with Liver Disease. PLoS ONE 9(10): e109254. doi:10.1371/journal.pone.0109254.g003)

quantified the 5.9 kDa fragment with the use of a homemade ELISA (Noda et al. 2011), confirming previous reports and indicating a clear downregulation of this fragment in serum samples from HCV patients with different stages of fibrosis. Later, in a study focused on the identification of early circulating serum biomarkers of active fibrogenesis, Marfà et al. (2014) also identified the same 5.9 kDa fragment of the fibrinogen α C-chain by the SELDI-TOF-MS technique. In fact, this investigation took advantage of the faster recurrence of hepatic fibrosis that usually occurs in HCV patients submitted to liver transplantation (LT). A marked downregulation of this fragment was detected in serum samples from ten HCV-positive patients submitted 6 months after LT surgery compared to nine LT patients without HCV-RNA recurrence who also underwent antiviral treatment before surgery. It is of note that at 6 months after LT most of these patients showed no evidence of hepatic fibrosis. In addition, all patients underwent a liver biopsy 1 year after LT. Forty percent of the cohort had a fibrosis stage lower than two according to Scheuer classification (Scheuer 1995), indicating that the 5.9 kDa fragment of fibrinogen α C-chain seems to be altered long before any histological evidence is available, thereby enhancing its relevance as a biomarker of early fibrogenic processes. In the same study, 83 HCV LT patients were also analyzed in order to validate the results obtained, and the outcomes confirmed that this fragment behaved as an early serum biomarker of fibrosis. Furthermore, the 5.9 kDa peptide was also determined in serum samples from eight HCV non-LT patients with different degrees of fibrosis, and the results were identical (Fig. 3).

5.9 kDA Fibrinogen Alpha C-Chain Fragment Behavior in Other Hepatic Etiologies

The serum levels of the 5.9 kDa fragment of the fibrinogen α C-chain were also evaluated in samples from patients with etiologies other than HCV infection and ALD, such as NASH, HBV, autoimmune hepatitis (AH), and cryptogenic liver damage (Marfà et al. 2014) (Fig. 4). The results of this investigation were in line with those obtained in HCV-infected patients and in ALD patients, since the absence of the 5.9 kDa fragment was confirmed in all these subjects. Therefore, the lack of the 5.9 kDa peptide is a common finding in patients with an active fibrogenic process regardless of whether this is the consequence of altered lipid metabolism, alcohol intake, or viral infection (Fig. 5). In addition to this and in contrast to other previously described biomarkers of liver fibrosis, the 5.9 kDa fragment of the fibrinogen α C-chain could be useful to identify those subjects under an active fibrogenic process including those in an early stage of liver damage rather than to stage fibrotic patients.
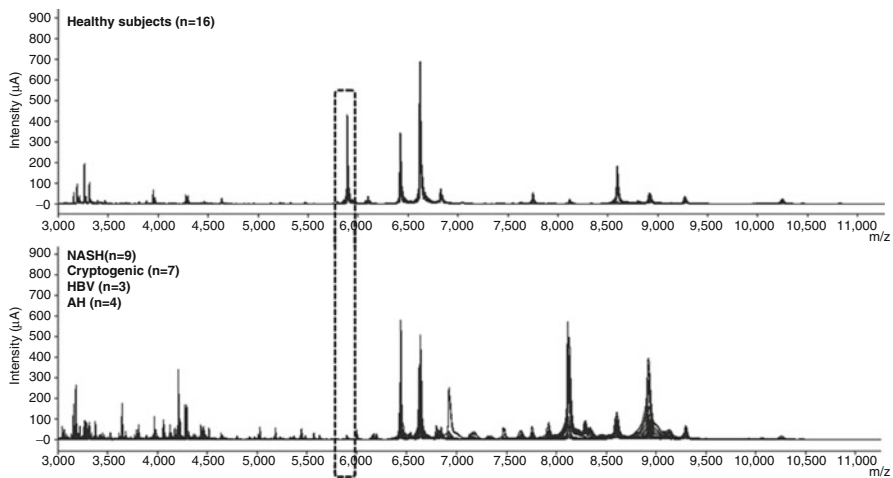

Fig. 4 Lack of the 5.9 kDa fibrinogen α C-chain fragment in fibrotic patients. Fragment of the SELDI-TOF-MS spectra ranging from 3,000 to 11,000 m/z of serum samples obtained from 16 healthy subjects and 23 patients with liver fibrosis of several etiologies including NASH, AH, HBV, and cryptogenic. The lack of the 5.9 kDa fibrinogen α C-chain fragment is clearly associated with fibrogenesis regardless of its etiology (Modified figure and legend from Marfà et al. (2014) and used under the Creative Commons Attribution (CC BY) license. Lack of a 5.9 kDa Peptide C-Terminal Fragment of Fibrinogen a Chain Precedes Fibrosis Progression in Patients with Liver Disease. PLoS ONE 9(10): e109254. doi:10.1371/journal.pone.0109254.g002)
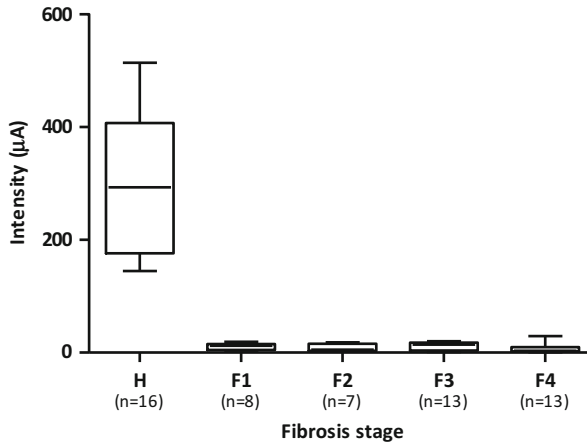
Fig. 5 Comparison of the 5.9 kDa fibrinogen α C-chain fragment levels among stages of liver fibrosis. SELDI-TOF-MS intensity values of the 5.9 kDa fibrinogen α C-chain fragment between patients with different stages of liver fibrosis and healthy subjects (H). The intensity of the released peptide was clearly suppressed in all fibrotic patients including subjects with an early fibrogenic process (F1) (Figure obtained from the data from Marfà et al. (2014) and used under the Creative Commons Attribution (CC BY) license. Lack of a 5.9 kDa Peptide C-Terminal Fragment of Fibrinogen a Chain Precedes Fibrosis Progression in Patients with Liver Disease. PLoS ONE 9(10): e109254. doi:10.1371/journal.pone.0109254)

Cell Regulation and Serum Stability of the 5.9 kDA Fibrinogen Alpha C-Chain Fragment

The mechanisms by which the 5.9 kDa fragment of the fibrinogen α C-chain is diminished in serum samples from patients with an active fibrogenic process are not fully understood. It is well established that IL-6 is a major regulator of fibrinogen gene expression (Fuller and Zhang 2001), suggesting that mediators involved in inflammatory response could be responsible for the altered serum content of the 5.9 kDa fragment in patients with liver fibrosis. To test this hypothesis, Marfà et al. (2014) recently exposed HepG2 cells, a human liver hepatocellular carcinoma cell line, to several well-known profibrogenic and proinflammatory agents including TNF-α, lipopolysaccharide, angiotensin II, endothelin-1, apelin, fibronectin, interleukin-1β, and TGF-β. Of note was that only TGF-β was able to reduce the amount of the 5.9 kDa fragment in the cell culture medium of these cells. This was associated with a downregulated mRNA expression of the fibrinogen α chain gene (FGA) but not of the transcripts corresponding to the β and γ chain genes (FGB and FGG, respectively) (Fig. 6). This was the first experimental data demonstrating that TGF-β is a specific regulator of the 5.9 kDa fragment.
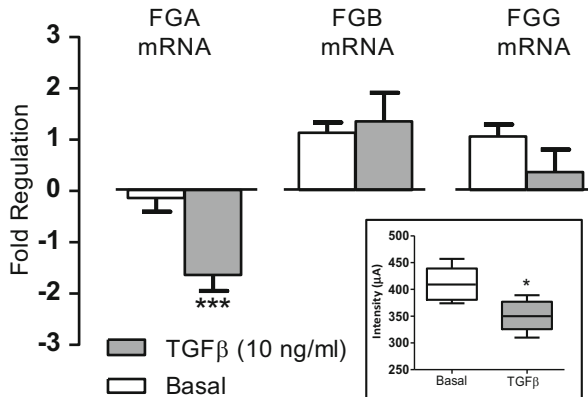
Fig. 6 TGF-β reduces fibrinogen α chain expression in HepG2 cells. Fold regulation in fibrinogen alpha chain (FGA), beta chain (FGB), and gamma chain (FGG) gene regulation in HepG2 cells after 6 h of treatment with TGF-β (10 ng/ml). Results are given as mean ± SE; ***$p < 0.001$ versus basal. Statistical analysis was calculated by the unpaired student's t test. The insert shows the intensity values of the 5.9 kDa peak detected in the cellular supernatant of HepG2 cells after 48 h of treatment with TGF-β (10 ng/ml). Results are given as mean ± SE; *$p < 0.05$ versus basal. Statistical analysis was calculated by the Mann-Whitney U test (Original figure and legend from Marfà et al. (2014) and used under the Creative Commons Attribution (CC BY) license. Lack of a 5.9 kDa Peptide C-Terminal Fragment of Fibrinogen a Chain Precedes Fibrosis Progression in Patients with Liver Disease. PLoS ONE 9(10): e109254. doi:10.1371/journal.pone.0109254.g005)

Demographic variables as well as preanalytical conditions are major issues for considering the diagnostic feasibility of any biomarker. In this regard Sogawa et al. (2013) demonstrated that gender and age did not affect the serum levels of the 5.9 KDa fragment (Fig. 7) but it is modified by factors such as the interval between venipuncture and serum separation. These results indicate that sample processing conditions should be accurately established in order to avoid analytical misinterpretations.

Potential Applications to Prognosis, Other Diseases, or Conditions

At present there is no study specifically addressed at estimating the strength of the 5.9 kDa fibrinogen α C-chain fragment as a prognostic or diagnostic tool in liver disease. On the other hand, taking this biomarker from the research lab to the daily routine in the clinical diagnostic lab depends not only on accurately defining the sensitivity, specificity, positive predictive value, and negative predictive value as indicators of early fibrogenic activity but also determining the availability of high-throughput methods, such as automatic immunoassays, to measure its concentration in biological fluids.
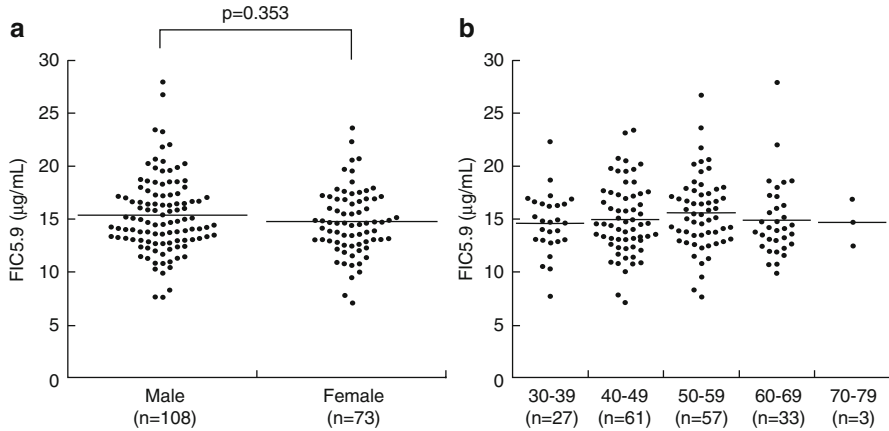
Fig. 7 Serum levels of the 5.9 kDa fibrinogen α C-chain fragment according to age and gender. 5.9 kDa fibrinogen α C-chain fragment concentration based on gender (**a**) and age (**b**). No differences were observed between groups (Figure from Sogawa et al. (2013) with permission from the Publisher John Wiley and Sons, Inc)

Finally, neither is it known whether the 5.9 kDa fragment is a specific biomarker for liver fibrosis. In this regard, a study performed by Pang et al. (2006) showed that the MS spectra of patients with severe acute respiratory syndrome (SARS) during the outbreak period displayed a diminution in the intensity of the 5.9 kDa fragment in comparison to non-SARS patients.

The SELDI-TOF-MS proteomic technique has also been widely used to investigate altered expression of the 5.9 kDa fragment in other pathologies including different types of neoplasia. Several experimental data have suggested that this fragment could be a biomarker in this condition. For instance, a diminution in the same m/z intensity of the fragment has been described in breast, ovarian, prostate, and non-small cell lung cancers (Engwegen et al. 2006; Belluco et al. 2007). In contrast, increased intensities were found in colorectal cancer and pancreatic adenocarcinoma (Koopmann et al. 2004; Engwegen et al. 2006). It should be noted that none of these investigations identified the amino acid sequence of the protein peak. However, it is presumably the same 5.9 kDa fragment of fibrinogen α C-chain since the sample processing and MS methods were very similar to those investigations in which the amino acid sequence identification was performed. Therefore, it is quite plausible that the 5.9 kDa fragment of fibrinogen α C-chain can also be useful as a biomarker in some types of neoplastic diseases.

Summary Points

- Until the last decade, invasive techniques were the only option to stage liver fibrosis.

- Recent technological advances have allowed the development of new imaging techniques and the identification of novel circulating protein markers to detect and monitor the evolution of liver diseases.
- Although many circulating biomarkers are appropriate for the identification and follow-up of the severe stages of liver fibrosis, their diagnostic capacity is lower in early and mild stages.
- The 5.9 kDa fragment of the fibrinogen α C-chain detected in serum is able to identify patients in early stages of liver fibrosis.
- No differences were observed between fibrotic patients in terms of age, gender, etiology, or fibrosis stage when the 5.9 kDa fragment of the fibrinogen α C-chain was evaluated.
- TGF-β seems to be one of the potential stimuli that affect the release of this 5.9 kDa fibrinogen peptide.

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