

Circulating Survivin and TIMP-1 in Hepatitis C

Virus Associated Liver Fibrosis

Abstract:

Aims: The present study was conducted to assess the clinical utility of survivin, an anti-apoptotic protein, and the profibrogenic tissue inhibitor metalloproteinase-1 (TIMP-1) as non-invasive biomarkers for the discrimination between stages of HCV-associated liver fibrosis that are largely asymptomatic.

Methodology: Circulating survivin and TIMP-1 levels as well as their corresponding proteins and genes expressions were assessed by ELISA, Western blot and RT-PCR in 100 HCV-infected patients at different stages of liver fibrosis in comparison to healthy controls.

Results: With the progression of hepatic fibrosis, each of circulating survivin and TIMP-1 as well as their ratio (survivin/TIMP-1) showed a stepwise increase and exhibited significant positive correlations with the fibrotic stage ($r=0.98$ & $p=0.002$, $r=0.95$ & $p=0.01$, and $r=0.93$ & $p=0.013$ respectively). A gradual increase in both survivin and TIMP-1 genes expression was also observed.

Conclusion: In conclusion, survivin, TIMP-1, and their ratio could represent promising novel biomarkers for prediction and discrimination of different stages of HCV-associated liver fibrosis. The higher sensitivity and specificity of survivin may provide new insights into its possible use as a target for the antifibrotic drugs. Further studies with validated testes on a large scale prospective clinical trial are required to ascertain these results.

Keywords: Survivin; Tissue inhibitor of metalloproteinase-1; Liver fibrosis; Hepatitis C virus

1. Introduction

Hepatitis C virus (HCV) infection is a universal health problem with an estimation of 130–170 million people infected throughout the world [1]. Globally, HCV infection shows a heterogeneous distribution with an average overall prevalence of 1–2% in most countries except in Egypt which is considered to have the highest worldwide prevalence (14.7% of the total population are seropositive for HCV, indicating that they had ever been exposed to the virus) [2-4]. More recently, in 2015 the Egypt Health Issues Survey (EHIS) reported that 6 % of the Egyptians individuals with age range of 1–59 years are seropositive for HCV. Of those individuals, 4 % were found to have an active HCV infection [5].

The course of chronic HCV infection comprises long-term multistage processes including fibrosis that represents a dynamic scarring process associated with accumulation of collagen and extracellular matrix (ECM) proteins. This in turn, could lead to the distortion of the hepatic architecture by forming ECM complexes and a fibrous scar with potential progression to cirrhosis [6-8]. It was estimated that, in the majority of HCV-infected patients, progression to cirrhosis can occur asymptotically after an interval of 15 to 20 years [6]. Therefore, there is an imperious medical need to regularly follow up the integrity of hepatic tissues in the susceptible patients.

Till recently, histological examination of the liver biopsy is considered as the gold-standard method for the assessment of fibrotic stage during the follow-up of patients [9, 10]. Unfortunately, besides being an invasive procedure, the concomitant complications and the heterogeneous variations in the sampling process are considered as actual challenges for the widespread applicability of liver biopsy for accurate prediction of the progression of liver

48 fibrosis [11]. Therefore, a reliable non-invasive method for accurate assessments of different
49 stages of liver fibrosis is still missing.

50 It was reported that hepatic stellate cells (HSCs) which account for 5% to 8% of total
51 liver cells are the major contributor of the fibrogenesis process [12]. In response to various
52 stimuli from parenchymal injury, the accompanied inflammatory reaction generates large
53 panels of profibrogenic signals that subsequently activate the quiescent HSCs to a
54 myofibroblast-like phenotype. This in turn , may lead to the induction of ECM synthesis and
55 deposition at the site of liver injury [13, 14]. The balance between the ECM degrading matrix
56 metalloproteinases (MMPs) and tissue inhibitors of the metalloproteinase family (TIMPs)
57 was reported to be strongly regulated by HSCs [12]. Tissue inhibitor of metalloproteinase-1
58 (TIMP-1) is a 28 kDa protein that prevents collagen degradation through inactivation of
59 MMPs, promoting liver fibrosis [15-17]. In order to play such a role during the liver fibrosis,
60 HSCs are required to exhibit an unusual high proliferation rate and enhanced survival via
61 resistance of apoptosis [18]. Survivin, a 16.5 kDa member of the apoptosis inhibitors protein
62 family, is up-regulated during HSCs activation, suggesting a potential role for survivin in
63 protecting HSCs from apoptosis and regulating cell division and proliferation during hepatic
64 fibrogenesis process [19, 20].

65 Accordingly, the aim of the present study was to evaluate the utility of both survivin
66 and TIMP-1 concentrations besides their ratio, genes and proteins expressions as reliable
67 non-invasive tools for the prediction and discrimination of the different stages of HCV-
68 associated liver fibrosis. Additionally, the current study aimed at investigating a possible
69 correlation of those serum biomarkers with the clinical status of the HCV infected Egyptian
70 patients.

71 **2. Patients and Methods**

72 **2.1. Study population**

73 The current study is a retrospective case-control study in which 100 chronic HCV-infected
74 Egyptian patients from both sexes with their age ranging from 35 to 66 years were enrolled.
75 They were randomly selected from a large cohort who were scheduled to receive antiviral
76 therapy at Assiut Virology Unit for control of HCV from April 2014 to December 2014.
77 Those patients were divided into five groups according to the histopathologically proven
78 fibrotic stages of the needle liver biopsies by the METAVIR score [21]. The first group (F0
79 stage) included 20 patients without any degree of fibrosis. The second group (F1 stage)
80 included 20 patients with portal fibrosis without septa. The third group (F2 stage) included 20
81 patients with portal fibrosis and few septa. The fourth group (F3 stage) included 20 patients
82 with septal fibrosis without cirrhosis. The fifth group (F4 stage) included 20 patients with
83 cirrhosis. F1, F2-F3, and F4 are considered as the mild, the moderate, and the advanced
84 fibrotic stages respectively. The study incorporated also 20 age and sex matched hepatitis
85 seronegative completely healthy subjects as a control group. All participants were subjected
86 to thorough clinical investigations with a complete record of their detailed history.

87 The present study was approved by the Ethical Committee of the Faculty of Medicine,
88 Assiut University, Egypt (IRB605/29/04/2014). An informed written consent was obtained
89 from each participant.

90 **2.2. Exclusion creiteria**

91 Patients were excluded if they had human immunodeficiency virus (HIV), hepatitis B virus
92 (HBV), decompensated liver cirrhosis, comorbidities, schistosomiasis, chronic inflammatory
93 liver diseases, hepatocellular carcinoma (HCC) associated cirrhosis or previously received
94 interferon or any other antiviral therapy before enrolement in this study.

95 **2.3. Blood sampling**

96 Eight ml venous fasting blood sample were collected from each subject at the time of
97 registration for the study before any therapeutic measures under strict aspeptic conditions and

were delivered into 2 tubes: 4 ml into an EDTA treated tube for subsequent RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) protocol and the other 4 ml were allowed to clot in another tube for serum isolation, which were kept frozen at -80 °C until used for laboratory estimations.

2.4. Biochemical estimations

The criteria for HCV diagnosis are based serologically on detecting positive anti-HCV IgG, using enzyme linked fluorescent assay in human serum (VIDAS® bioMérieux SA, France). Subsequent quantitative determination of HCV RNA as an indicator of the viral load (IU/ml) in the HCV-IgG seropositive patients was established using a direct RT-PCR assay kit (Abbott GmbH, Germany). Quantitative estimations of serum survivin and TIMP-1 levels were achieved using the corresponding enzyme-linked immunosorbent assay (ELISA) kits (Biospes Co., Ltd., China). Serum albumin, total bilirubin levels as well as serum alanine aminotransferase (sALT) and serum aspartate aminotransferase (sAST) activities were estimated using corresponding commercially available colorimetric or kinetic assay kits.

2.5. Western blotting assessments of survivin and TIMP-1 proteins

Immunoblotting of both survivin and TIMP-1 proteins in serum samples was carried out using Western blot technique after the removal of the high abundant plasma proteins (albumin and immunoglobulines) using albumin and IgG Depletion SpinTrap kits (GE Healthcare Life Sciences, Sweden). Proteins in each corresponding serum sample were denatured at 95 °C for 5 minutes in 2× Laemmli buffer followed by addition of 5% 2-mercaptoethanol. SDS–PAGE electrophoresis was achieved by loading 30 µg protein per lane at 50 volts through stacking gel followed by 125 volts through 18% and 15% resolving gels for survivin and TIMP-1 respectively, during approximately 2 hours and transferred to a polyvinylidene difluoride (PVDF) membrane using T-77 ECL semidry transfer unit (Amersham BioSciences, UK Ltd) for 2 hours. Immunoblotting was performed by incubating

the PVDF membrane in a Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% non-fat milk for one hour at 4 °C, followed by an overnight incubation at 4 °C with rabbit anti-survivin polyclonal antibody (Bioss Inc., USA) or rabbit anti-TIMP-1 polyclonal antibody (Biospes Co., Ltd., China) at dilutions of 1:1500 and 1:1200, respectively. After being washed three times with TBST buffer, each membrane was incubated for one hour at room temperature with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Novus Biologicals, USA) at a dilution of 1:5000. Subsequent to four times washing with TBST, the membrane bound antibodies were detected with a commercially available BCIP/NBT substrate detection Kit (Genemed Biotechnologies, Inc., USA). Equivalent protein loading for each lane was confirmed by stripping and re-blotting each membrane at 4 °C against mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Novus Biologicals, USA) at a dilution of 1:1000. Each analysis was repeated to assure reproducibility of results. Quantification of each corresponding analysis was further performed using Image J software and expressed as the relative band density to the GAPDH.

2.6. RT-PCR assessments of survivin and TIMP-1 mRNA levels

Total RNA was extracted from the whole blood using a total RNA purification Kit (Jena Bioscienc, Germany). The quality and quantity of the extracted RNA was controlled by a NanoDrop spectrophotometer (Biotech inc, USA). RNA quality was subsequently confirmed by gel electrophoresis. Then cDNA was synthesized from 2 µg total RNA using a RevertAidTM First strand cDNA synthesis kit (Thermo Fisher Scientific Inc, USA). Quantification of the resultant cDNA was determined using the NanoDrop spectrophotometer. Amplifications of mRNA were performed by Biometra cycler (Jena, Germany) using a ready-to-use MyTaqTM mix kit (Bioline Inc., USA) and specific custom-made PCR primers (Vivantis Technologies Sdn. Bhd., Malaysia) to amplify the human

survivin, TIMP-1 and β -actin-cDNA according to the NCBI reference sequences (Table 1). For survivin, samples were subjected to initial denaturation at 95 °C for 3 minutes then underwent 35 cycles (denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 40 seconds) followed by a filling extension at 72 °C for 5 minutes. For TIMP-1, the same procedures were used but with annealing temperature of 54 °C for 30 seconds. RT-PCR of β -actin was performed in parallel as an internal control with annealing temperature of 58 °C for 30 seconds. The RT-PCR products were analyzed by electrophoresis using 2% agarose gel (Roche Diagnostics, GmbH, Germany), stained with ethidium bromide and visualized by UV light using UV Transilluminator (UVT-20 M, Herolab, Germany). Each analysis was repeated to assure reproducibility of results. Quantification of each corresponding gel analysis was further performed using Image J software and expressed as the relative band density to the β -actin.

[Table 1]

2.7. Statistical analyses

Statistical analyses of the data were carried out using GraphPad prism (Graphpad Software, Inc., USA). Data comparisons were performed using analysis of variance (ANOVA) followed by Tukey's *t*-test. The levels of significance were accepted with $p < 0.05$ and all relevant results were displayed as mean $\pm$ SD. The correlations between the continuous variables were performed using the Pearson's correlation. Sensitivity, specificity, the best cut-off values and the diagnostic efficacy index of survivin, TIMP-1 and survivin/TIMP-1 were calculated from the corresponding Receiver Operating Characteristic (ROC) curves.

3. Results

3.1. Clinical, demographic and biochemical data

Table 2 shows the clinical, demographic and biochemical data of the patients and controls.

[Table 2]

3.2. Immunosorbent assays of circulating survivin and TIMP-1

As shown in Table 2, serum survivin levels were significantly increased in F2, F3, and F4 groups ($p < 0.001$ for each) in comparison to the healthy controls. In F2, F3, and F4 these increase was significant ($p < 0.00$ and $p < 0.001$ for each, respectively) in comparison to F0 and F1 fibrotic stages. The increase in serum survivin levels was significant in F3 ($p < 0.05$), and F4 ($p < 0.001$) as compared to F2 fibrotic stage.

It is noticed that serum survivin levels showed a significant positive correlation with the fibrotic stage ($r = 0.98$ & $p = 0.002$, Figure 1A, Table 3 and Table S1 in the supplementary data). Also, significant positive correlations were detected between serum survivin and each of sALT, sAST, total bilirubin and viral load ($r = 0.64, 0.71, 0.44$ and 0.49 , respectively & $p < 0.0001$ for each). While, a significant negative correlation was recorded between serum survivin and serum albumin level ($r = -0.25$ & $p = 0.004$, Table 3 and Figure S2 in the supplementary data).

On the other hand, serum TIMP-1 was significantly increased in the advanced stages of liver fibrosis ($p < 0.001$ for each of F3 and F4) in comparison to the healthy controls. In comparison to F0, F1, and F2 fibrotic stages, the increase in serum TIMP-1 was significant in each of F3 and F4 ($p < 0.001$ for each), as illustrated in Table 2.

Notably, serum TIMP-1 levels showed significant positive correlations with the fibrotic stage ($r = 0.95$ & $p = 0.01$), age ($r = 0.28$ & $p = 0.0015$), sALT ($r = 0.48$ & $p < 0.0001$), sAST ($r = 0.46$ & $p < 0.0001$), total bilirubin ($r = 0.33$ & $p = 0.0002$), and viral load ($r = 0.45$ & $p < 0.0001$) as illustrated in Figure 1B and Table 3 as well as Figure S3 and Table S1 in the supplementary data). Additionally, it exhibited an insignificant negative correlation with serum albumin (Table 3).

197 Interestingly, a strong positive correlation was detected between serum levels of
198 survivin and TIMP-1 ($r=0.51$ & $p<0.0001$, Table 3 and Figure S2 in the supplementary data).
199 Moreover, the serum survivin/TIMP-1 ratio was significantly increased in the moderate (F2 -
200 F3) and advanced (F4) fibrotic stages in comparison to the healthy controls, non fibrotic (F0)
201 and mild (F1) fibrotic stages ($p<0.001$ for each) as seen in Table 2.

202 The correlations between the fibrotic stage and the different parameters are
203 represented in Table S1 and Figure S1 in the supplementary data. Both age and sAST showed
204 significant positive correlations with the fibrotic stage ($r=0.98$ & $p=0.0016$) and ($r=0.95$ &
205 $p=0.013$) respectively. Also, sALT showed a positive correlation with the fibrotic stage but it
206 did not reach the statistical significance ($r=0.87$ & $p=0.051$). Furthermore, there was a
207 significant positive correlation between the fibrotic stage and survivin/TIMP-1 ratio ($r=0.93$
208 & $p=0.019$) as seen in Figure 1C and Table S1 in the supplementary data.

209 [Table 3]

210 3.3. ROC analyses in different disease stages

211 The overall diagnostic performance of each of serum survivin, TIMP-1 and their ratio in
212 discriminating HCV fibrotic patients was assessed by ROC curve analysis (Figure 1D-F). It is
213 evident from the results that the best cut-off value of serum survivin was 1125 ng/L with 81%
214 sensitivity and 95% specificity producing a diagnostic efficacy index of 0.90. In case of
215 serum TIMP-1, the best cut-off value was 102.5 $\mu\text{g/L}$ with 43% sensitivity and 95%
216 specificity, producing a diagnostic efficacy index of 0.77. Concerning the serum
217 survivin/TIMP-1 ratio, the best cut-off value was 19.1 with 66% sensitivity and 95%
218 specificity, producing a diagnostic efficacy index of 0.86.

[Figure 1]

3.4. Immunoblotting detection of survivin and TIMP-1 proteins

Western blot results showed over-expressions in both survivin (Figure 2A) and TIMP-1 (Figure 2B) proteins with the progression of hepatic fibrosis.

[Figure 2]

3.5. RT-PCR assessments of survivin and TIMP-1 mRNA levels

RT-PCR results revealed a gradual increase in the mRNA levels of both survivin (Figure 2C) and TIMP-1 (Figure 2D) with the progression of liver fibrosis.

4. Discussion:

Liver fibrosis, the scarring of the hepatic parenchymal tissues, is the common final pathway of almost every chronic inflammatory liver injury which may accumulate and ultimately lead to the formation of cirrhosis with serious complications such as portal hypertension, hepatic synthetic impairment and HCC [22, 23]. For patients with chronic liver diseases, including HCV, determination of the severity of hepatic fibrosis is considered as a routine procedure to dictate the need and timing of therapy, and may also define treatment response [22]. Since decades, this assessment of hepatic fibrosis has traditionally been achieved by histopathological examinations of a liver biopsy. However, the invasiveness of the liver biopsy procedures is considered as a serious drawback that increases the risk of potential complications including bleeding which ranges from 0.3% to 0.5%, and mortality for up to 0.1% [24, 25]. Additionally, since the biopsy core only represents 1/20000 to 1/50000 of the whole liver tissues, biopsy is prone to a sampling error and variability that could lead to unfaithful appreciations of the fibrosis staging [11, 26, 27]. For these reasons, alternative non-invasive methods for accurate evaluation of liver fibrogenesis are of the pressing medical needs.

Some research groups investigated the usefulness of serum activities of sALT and sAST as reproducible fibrotic biomarkers. Both sALT and sAST showed obvious variations in different stages of liver fibrosis and therefore they were considered as useful indicators of liver fibrosis [28-30]. These results are in harmony with our findings where sAST and sALT were correlated with the fibrosis score although the sALT correlation was not statistically significant which could be attributed to the small sample size of the present study.

Other researchers investigated the variability of serum albumin and total bilirubin in different stages of liver fibrosis and concluded that neither of them could be considered as a dependable marker for monitoring liver fibrosis [31]. These findings are also confirmed in our work where the recorded changes in both serum albumin and total bilirubin levels were not significantly correlated with the fibrotic stage. Additionally, no significant correlation was found in our results between the viral load and the fibrotic stages.

Survivin is a 16.5-kDa protein that inhibits apoptosis and regulates cell division and proliferation. The expression of survivin is found to be undetectable or at very low levels in normal tissues, whereas it is found at high levels in embryonic and fetal tissues, in transformed cells, during HSCs activation and in various malignancies, including liver cancer [19, 32-34].

In the current study, circulating levels of survivin showed a stepwise increase along the progression of hepatic fibrosis with a significant positive correlation with the fibrotic stage. Also, significant correlations were found between serum survivin and the other laboratory indices including sALT, sAST, total bilirubin, serum albumin, and the viral load as well as the patient's age.

Moreover, survivin protein and gene expressions confirmed the above described results and stepwise up-regulations of the survivin protein and mRNA fragment were recorded with the advancement of hepatic fibrosis from F1 to F4 compared to the controls.

The results of the current study are in accordance with many studies in which survivin up-regulation has been described in liver diseases that are accompanied by HSCs activation, suggesting a potential role for survivin in protecting HSCs from apoptosis during hepatic fibrogenesis [19, 33]. However, survivin exhibits a role in regulating the survival of the normal adult cells [35].

Similarly, the current study also evaluated TIMP-1 as a biochemical serum marker of liver fibrosis. TIMP-1 is a 28 kDa protein that can irreversibly bind to and inactivate MMPs which are associated with matrix degradation [15]. It was reported that TIMP-1 is induced during liver injury [36]; where it plays an important role in promoting liver fibrosis [17, 37] and inhibiting liver regeneration [16].

In the present study, circulating levels of TIMP-1 were significantly increased in the advanced stage of liver fibrosis in comparison to the healthy control group, whereas its increased levels in the early stages of liver fibrosis did not reach the statistical significance. However, TIMP-1 levels in those patients showed a positive correlation with the fibrotic stage. In respect to the other laboratory indices, serum TIMP-1 levels showed significant correlations with sALT, sAST, total bilirubin, and the viral load as well as the patient's age.

Additionally, the results of the current study revealed up-regulation of TIMP-1 protein and mRNA fragment with the promotion of liver fibrosis stage where they are moderately up-regulated during mild and moderate fibrotic stages followed by a marked up-regulation during the advanced stage. These observations are in agreement with the results of previous studies which reported TIMP-1 correlations with the histopathological fibrotic scores [38, 39]. These results clearly reflect the profibrogenic effects of TIMP-1 which may be mediated via preventing collagen degradation. This in-turn occurs through the inhibition of MMPs and protecting against activated HSCs death [40, 41]. Rationally, this could elucidate the cooperative role of survivin and TIMP-1 in the fibrotic process and explain the

significant positive correlation between serum survivin and serum TIMP-1 which has been recorded in the current study.

In contrast to the subtle increase in the early stages of liver fibrosis, the observed dramatic up-regulation of TIMP-1 during the advanced stages of liver fibrosis as shown in the present study can be attributed to the increased MMPs expressions by HSCs at the early stage of liver fibrosis, causing the liver ECM to degrade, while at the advanced stages, the fully activated HSCs over-express TIMPs especially TIMP-1 that inhibits MMPs activities [17, 42, 43]. This increased production of TIMPs relative to MMPs may be the important factor in the progression of liver fibrosis [44]. On the other hand, the values of survivin/TIMP-1 ratio were significantly higher in the moderate and advanced fibrotic stages than in the healthy controls, non fibrotic and mild fibrotic stages.

When assessing the diagnostic performance of circulating survivin, TIMP-1 and survivin/TIMP-1 ratio in discriminating liver fibrosis patients, survivin and survivin/TIMP-1 ratio showed better sensitivity over TIMP-1 with a superiority of survivin over survivin/TIMP-1 ratio. This indicates the predictive role of survivin in discriminating liver fibrosis stages and suggests its use as a potential non invasive marker for the early liver fibrosis. While mild liver fibrosis is largely asymptomatic, there is urgent need for measures that may induce regression of these fibrotic changes before its progression into cirrhosis and organ failure. These liver fibrotic changes in HCV patients are recently proved by some investigators to be reversible [45-47], if the underlying liver injury is successfully treated [48]. Thus, survivin could also act as a target for antifibrotic drugs for liver fibrosis and/or cirrhosis.

5. Conclusion

The findings of the present study indicated that survivin and TIMP-1 could be used as promising novel biomarkers for prediction and discrimination of HCV-associated liver

fibrosis patients, with a priority for survivin. Circulating survivin could be considered as a potential valuable tool for the detection of the early asymptomatic stage of liver fibrosis in HCV-infected patients with high sensitivity and specificity. Moreover, survivin could be used as a target for the antifibrotic drugs that may induce regression of liver fibrosis and/or cirrhosis that is recently proved to be reversible processes. In addition, survivin/TIMP-1 ratio can be used as a diagnostic marker for differentiating the early and advanced stages of liver fibrosis in HCV-infected patients. Further studies with validated tests on a large scale prospective clinical trial are required to ascertain these results.

Financial Disclosure: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Atoom A, Taylor N, Russell R. The elusive function of the hepatitis C virus p7 protein. *Virology*. 2014;462(1):377-387.
2. El-Zanaty F, Egypt demographic and health survey 2008. 2009, Cairo, Egypt: Ministry of Health and Population.
3. Esmat G. Hepatitis C in the Eastern Mediterranean Region. *Eastern Mediterranean health journal* 2013;19(7):587-8.
4. Fallahian F, Najafi A. Epidemiology of hepatitis C in the Middle East. *Saudi journal of kidney diseases and transplantation*. 2011;22(1):1-9.
5. El-Zanaty F, Egypt Health Issues Survey 2015. 2015, Cairo, Egypt and Rockville, Maryland, USA: Ministry of Health and Population and ICF International.
6. Bataller R, Brenner D. Liver fibrosis. *Journal of clinical investigation*. 2005;115(2):209-18.
7. Rockey D, Bissell D. Noninvasive measures of liver fibrosis. *Hepatology*. 2006;43(S1):S113-S120.
8. Wu YJ, Xu MY, Lu LG. Clinical Advances in Fibrosis Progression of Chronic Hepatitis B and C. *Journal of clinical and translational hepatology*. 2014;2(4):222-227.
9. Fallatah H. Noninvasive Biomarkers of Liver Fibrosis: An Overview. *Advances in Hepatology*. 2014;2014(2014):1-15.

- 352 10. Goodman Z. Grading and staging systems for inflammation and fibrosis in chronic
353 liver diseases. *Journal of hepatology*. 2007;47(4):598-607.
- 354 11. Bedossa P, Dargère D, Paradis V. Sampling variability of liver fibrosis in chronic
355 hepatitis C. *Hepatology*. 2003;38(6):1449-1457.
- 356 12. Tacke F, Weiskirchen R. Update on hepatic stellate cells: pathogenic role in liver
357 fibrosis and novel isolation techniques. *Expert Rev Gastroenterol Hepatol*.
358 2012;6(1):67-80.
- 359 13. Bachem M, Meyer D, Melchior R, Sell K, Gressner A. Activation of rat liver
360 perisinusoidal lipocytes by transforming growth factors derived from
361 myofibroblastlike cells. A potential mechanism of self perpetuation in liver
362 fibrogenesis. *Journal of Clinical Investigation*. 1992;89(1):19-27.
- 363 14. Mallat A, Lotersztajn S. Cellular mechanisms of tissue fibrosis. 5. Novel insights into
364 liver fibrosis. *American Journal of Physiology-Cell Physiology*. 2013;305(8):C789-
365 C799.
- 366 15. Friedman S. Cytokines and fibrogenesis. in *Seminars in liver disease*. 1998.
- 367 16. Mohammed FF, Pennington CJ, Kassiri Z, Rubin JS, Soloway PD, Ruther U,
368 Edwards DR, Khokha R. Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell
369 cycle via HGF activation in murine liver regeneration. *Hepatology (Baltimore, Md.)*.
370 2005;41(4):857-67.
- 371 17. Parsons CJ, Bradford BU, Pan CQ, Cheung E, Schauer M, Knorr A, Krebs B, Kraft S,
372 Zahn S, Brocks B, Feirt N, Mei B, Cho MS, Ramamoorthi R, Roldan G, et al.
373 Antifibrotic effects of a tissue inhibitor of metalloproteinase-1 antibody on
374 established liver fibrosis in rats. *Hepatology (Baltimore, Md.)*. 2004;40(5):1106-15.
- 375 18. El-Attar HA, Kandil MH, El-Kerm YM, El-Ghandour MK. Comparison of serum
376 survivin and alpha fetoprotein in Egyptian patients with hepatocellular carcinoma
377 associated with hepatitis C viral infection. *Asian Pac J Cancer Prev*. 2010;11(4):897-
378 903.
- 379 19. De Minicis S, Seki E, Uchinami H, Kluwe J, Zhang Y, Brenner D, Schwabe R. Gene
380 expression profiles during hepatic stellate cell activation in culture and in vivo.
381 *Gastroenterology*. 2007;132(5):1937-1946.
- 382 20. Ou D, Lee B, Lin L, Liou J, Liao S, Hsu C, Cheng A. Vertical blockade of the IGFR-
383 PI3K/Akt/mTOR pathway for the treatment of hepatocellular carcinoma: the role of
384 survivin. *Mol Cancer*. 2014;13(2):1-11.

- 385 21. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C.
386 Hepatology. 1996;24(2):289-293.
- 387 22. Adams LA. Biomarkers of liver fibrosis. Journal of gastroenterology and hepatology.
388 2011;26(5):802-809.
- 389 23. Iredale JP, Thompson A, Henderson NC. Extracellular matrix degradation in liver
390 fibrosis: Biochemistry and regulation. Biochim Biophys Acta. 2013;1832(7):876-83.
- 391 24. Cadranel J, Rufat P, Degos F. Practices of liver biopsy in France: results of a
392 prospective nationwide survey. Hepatology. 2000;32(3):477-481.
- 393 25. Mc Gill D, Rakota J, Zinsmeister A, Oh B. A 1-year experience with major
394 hemorrhage after percutaneous liver biopsy. Gastroenterol. 1990;99(5):1396-1400.
- 395 26. Regev A, Berho M, Jeffers L, Milikowski C, Molina E, Pyrsopoulos N, Feng Z,
396 Reddy K, Schiff E. Sampling error and intraobserver variation in liver biopsy in
397 patients with chronic HCV infection. The American journal of gastroenterology.
398 2002;97(10):2614-2618.
- 399 27. Bedossa P. Intraobserver and interobserver variations in liver biopsy interpretation in
400 patients with chronic hepatitis C. Hepatology. 1994;20(1):15-20.
- 401 28. Gordon SC, Fang JW, Silverman AL, McHutchison JG, Albrecht JK. The
402 significance of baseline serum alanine aminotransferase on pretreatment disease
403 characteristics and response to antiviral therapy in chronic hepatitis C. Hepatology.
404 2000;32(2):400-4.
- 405 29. Grigorescu M. Noninvasive biochemical markers of liver fibrosis. J Gastrointestin
406 Liver Dis. 2006;15(2):149-59.
- 407 30. Kruger FC, Daniels CR, Kidd M, Swart G, Brundyn K, van Rensburg C, Kotze M.
408 APRI: a simple bedside marker for advanced fibrosis that can avoid liver biopsy in
409 patients with NAFLD/NASH. S Afr Med J. 2011;101(7):477-80.
- 410 31. Hongbo L, Xiaohui L, Hong K, Wei W, Yong Z. Assessing routine and serum
411 markers of liver fibrosis in CHB patients using parallel and serial interpretation. Clin
412 Biochem. 2007;40(8):562-6.
- 413 32. Kim G, Lim S, Kim Y. Expression of HuR, COX-2, and survivin in lung cancers;
414 cytoplasmic HuR stabilizes cyclooxygenase-2 in squamous cell carcinomas. Modern
415 Pathology. 2011;24(10):1336-1347.

- 416 33. Zhu H, Chen X, Zhang W, Luo S, Zhang B. Expression and significance of new
417 inhibitor of apoptosis protein survivin in hepatocellular carcinoma. *World J*
418 *Gastroenterol.* 2005;11(25):3855-3859.
- 419 34. Kappler M, Kotzsch M, Bartel F, Füssel S, Lautenschläger C, Schmidt U, Würfl P,
420 Bache M, Schmidt H, Taubert H. Elevated expression level of survivin protein in soft-
421 tissue sarcomas is a strong independent predictor of survival. *Clinical cancer research.*
422 2003;9(3):1098-1104.
- 423 35. Fukuda S, Pelus L. Survivin, a cancer target with an emerging role in normal adult
424 tissues. *Molecular cancer therapeutics.* 2006;5(5):1087-1098.
- 425 36. Nobili V, Parkes J, Bottazzo G, Marcellini M, Cross R, Newman D, Vizzutti F,
426 Pinzani M, Rosenberg WM. Performance of ELF serum markers in predicting fibrosis
427 stage in pediatric non-alcoholic fatty liver disease. *Gastroenterology.*
428 2009;136(1):160-7.
- 429 37. Gieling RG, Burt AD, Mann DA. Fibrosis and cirrhosis reversibility - molecular
430 mechanisms. *Clinics in liver disease.* 2008;12(4):915-37.
- 431 38. Boeker K, Haberkorn C, Michels D, Flemming P, Manns M, Lichtinghagen R.
432 Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in
433 patients with chronic hepatitis C. *Clinica chimica acta.* 2002;316(1):71-81.
- 434 39. Walsh K, Timms P, Campbell S, Norman R, Macsween M, Morris A. Plasma levels
435 of matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases-1
436 and-2 (TIMP-1 and TIMP-2) as noninvasive markers of liver disease in chronic
437 hepatitis C comparison using ROC analysis. *Digestive diseases and sciences.*
438 1999;44(3):624-630.
- 439 40. Iredale JP. Tissue inhibitors of metalloproteinases in liver fibrosis. *The international*
440 *journal of biochemistry & cell biology.* 1997;29(1):43-54.
- 441 41. Murphy F, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur M, Benyon C, Iredale J.
442 Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of
443 metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition
444 implications for reversibility of liver fibrosis. *Journal of Biological Chemistry.*
445 2002;277(13):11069-11076.
- 446 42. Gieling RG, Burt AD, Mann DA. Fibrosis and cirrhosis reversibility - molecular
447 mechanisms. *Clin Liver Dis.* 2008;12(4):915-37.

- 448 43. Iredale J, Thompson A, Henderson N. Extracellular matrix degradation in liver
449 fibrosis: Biochemistry and regulation. *Biochimica et Biophysica Acta (BBA)-*
450 *Molecular Basis of Disease*. 2013;1832(7):876-883.
- 451 44. Iredale J, Murphy G, Hembry R, Friedman S, Arthur M. Human hepatic lipocytes
452 synthesize tissue inhibitor of metalloproteinases-1. Implications for regulation of
453 matrix degradation in liver. *Journal of Clinical Investigation*. 1992;90(1):282-7.
- 454 45. Friedman SL, Sheppard D, Duffield JS, Violette S. Therapy for fibrotic diseases:
455 nearing the starting line. *Sci Transl Med*. 2013;5(167):167sr1.
- 456 46. Schuppan D, Pinzani M. Anti-fibrotic therapy: lost in translation? *Journal of*
457 *hepatology*. 2012;56 Suppl 1(S66-74.
- 458 47. Tacke F, Trautwein C. Mechanisms of liver fibrosis resolution. *Journal of hepatology*.
459 2015;63(4):1038-9.
- 460 48. Ellis EL, Mann DA. Clinical evidence for the regression of liver fibrosis. *Journal of*
461 *hepatology*. 2012;56(5):1171-80.

462 Table 1: The primers used for amplification of survivin, TIMP-1 and β -actin in
463 RT-PCR detections

Gene	Primer sequences (5'-3')	Product size (bp)	Annealing temp. (°C)	Gen Bank Accession Number
Survivin	F: AGTCCCTGGCTCCTCTACTG R: CGTGTGGAGAACGTGACAGA	380	55	U75285.1
TIMP-1	F: ACTTCCACAGGTCCCACAAC R: GCAGTTTGCAGGGGATGGAT	204	54	NM_003254.2
β-actin	F: AGCGGGAAATCGTGCGTGAC R: ACATCTGCTGGAAGGTGGAC	453	58	XM_006715764.1

464 Table 2: Clinical, demographic and biochemical data of patients and controls

	Control	Group-I	Group-II	Group-III	Group-IV	Group-V
Fibrotic stage	Healthy	F0	F1	F2	F3	F4
Sex (M/F)	14/6	13/7	11/9	15/5	12/8	10/10
Age (year)	44.8 $\pm$ 7.4	47.4 $\pm$ 7.0	50.4 $\pm$ 7.1	52.5 $\pm$ 8.3*	53.8 $\pm$ 7.7**	57.7 $\pm$ 6.3***,†††,‡
Viral load (IU/ml) $\times 10^3$	Undetectable	208 $\pm$ 175	202 $\pm$ 156	468 $\pm$ 344	6792 $\pm$ 3754†††,‡‡‡,§§§	2236 $\pm$ 2058†,‡,§,•••
sALT (U/L)	12.6 $\pm$ 2.9	16.4 $\pm$ 2.9	39.1 $\pm$ 7.1***,†††	43.2 $\pm$ 12.5***,†††	44.8 $\pm$ 13.4***,†††	49.4 $\pm$ 15.9***,†††,‡
sAST (U/L)	13.9 $\pm$ 3.8	36.4 $\pm$ 7.3**	42.1 $\pm$ 16.5***	64.7 $\pm$ 21.3***,†††,‡‡	81.0 $\pm$ 38.7***,†††,‡‡‡	78.6 $\pm$ 12.3***,†††,‡‡‡
Total bilirubin (μ mol/L)	11.3 $\pm$ 2.9	13.5 $\pm$ 3.4	13.7 $\pm$ 3.9	14.7 $\pm$ 3.2	15.0 $\pm$ 3.6*	19.5 $\pm$ 5.5***,†††,‡‡‡,§§,••
Serum albumin (g/L)	47.7 $\pm$ 4.0	39.9 $\pm$ 3.4**	40.2 $\pm$ 1.7**	38.7 $\pm$ 2.5***	40.0 $\pm$ 5.7***	40.6 $\pm$ 6.2***

Serum survivin (ng/L)	851±200	1135±329	1403±567	2870±775***,†††,‡‡‡	3764±1291***,†††,‡‡‡,\$	4350±1373***,†††,‡‡‡,\$\$\$
Serum TIMP-1 (µg/L)	74.1±18.6	78.2±14.9	85.4±13.6	90.7±20.6	117.4±13.3***,†††,‡‡‡,\$\$\$	120.1±25.1***,†††,‡‡‡,\$\$\$
Survivin/TIMP-1 Ratio	12.0±3.6	15.3±6.0	17.1±8.0	33.1±11.2***,†††,‡‡‡	32.9±13.4***,†††,‡‡‡	38.0±14.1***,†††,‡‡‡

465 Data are presented as mean ± SD (n = 20). *, †, ‡, § and ● indicate significant changes from
466 control, F0, F1, F2 and F3 groups respectively. *, †, ‡, § and ● indicate significant change at
467 $p < 0.05$; **, ††, ‡‡, §§ and ●● indicate significant change at $p < 0.01$; ***, †††, ‡‡‡, §§§ and
468 ●●● indicate significant change at $p < 0.001$. sALT, serum alanine aminotransferase; sAST,
469 serum aspartate aminotransferase; TIMP-1, tissue inhibitor of the metalloproteinase-1.

470 **Table 3: Pearson's correlations between different parameters and each of serum**
471 **survivin (ng/L) or TIMP-1 (µg/L)**

	Survivin		TIMP-1	
	<u>r value</u>	<u>p value</u>	<u>r value</u>	<u>p value</u>
sALT	0.64	***	0.48	***
sAST	0.71	***	0.46	***
Total bilirubin	0.44	***	0.33	***
Serum albumin	-0.25	**	-0.15	NS
Viral load	0.49	***	0.45	***
Fibrotic stage	0.98	**	0.95	*
Survivin	-	-	0.51	***

472 * indicates significant correlation at $p < 0.05$; ** indicate significant correlation at $p < 0.01$ and
473 *** indicate significant correlation at $p < 0.001$. NS indicates non significant correlation.
474 sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; TIMP-1,
475 tissue inhibitor of the metalloproteinase-1.
476

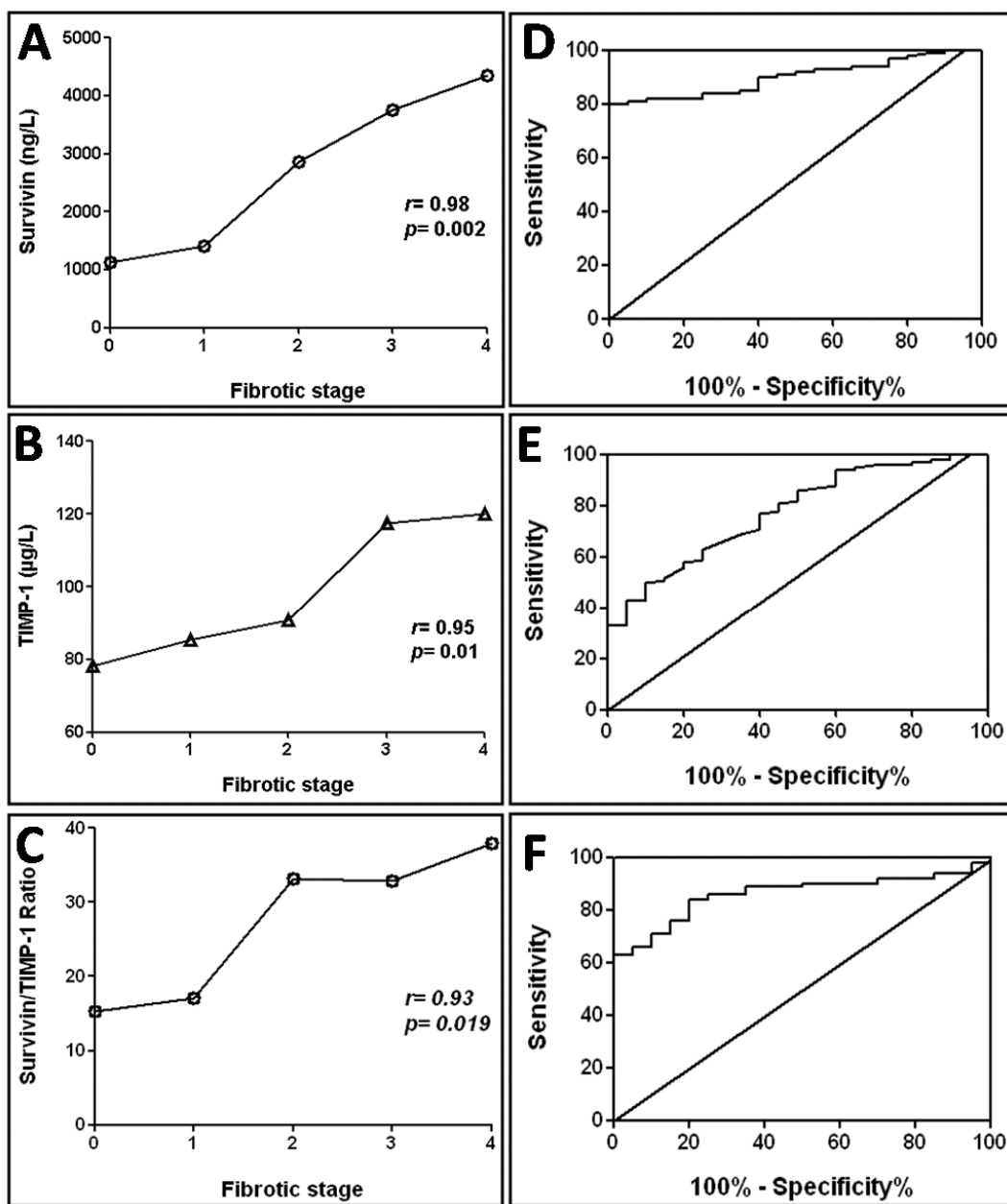


Figure 1: Correlations between the fibrotic stage and each of serum survivin (A), TIMP-1 (B), and survivin/TIMP-1 ratio (C) in the studied patients. D, E, and F are ROC curves for serum survivin, TIMP-1, and survivin/TIMP-1 ratio, respectively in liver fibrosis.

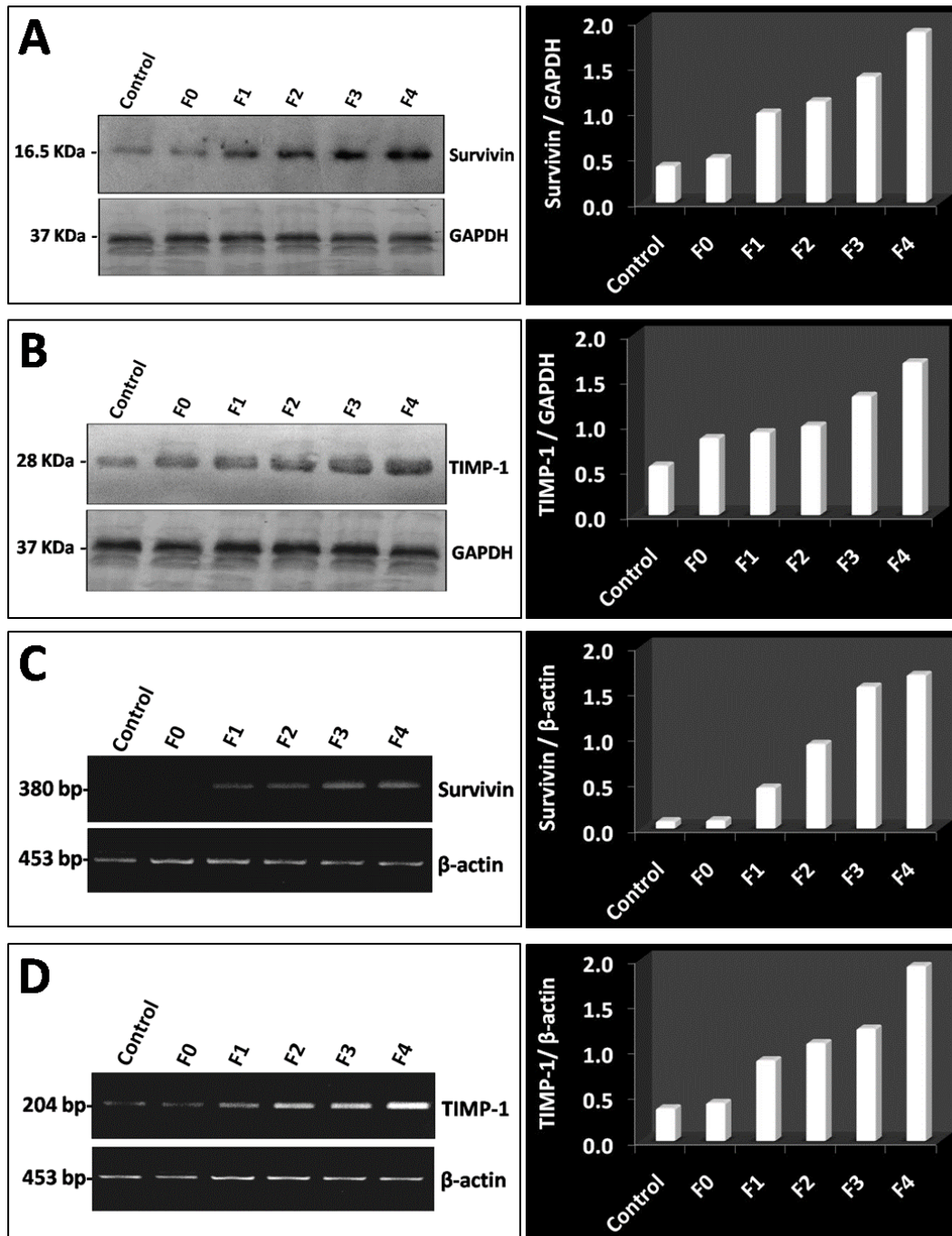


Figure 2: Illustration of the Western blot assessments of survivin (A) and TIMP-1 (B) as well as RT-PCR detection of mRNA fragments survivin (C) and TIMP-1 (D) in different stages of liver fibrosis. GAPDH and β -actin were used in parallel as internal controls. The right panels represent the corresponding quantification of each analysis measured by Image J software and expressed as the relative band density to the GAPDH or β -actin. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TIMP-1, Tissue inhibitor of the metalloproteinase-1

492 **Table S1: Pearson's correlations between different parameters and the fibrotic stages.**

	Fibrotic stage	
	<u>r value</u>	<u>p value</u>
sALT	0.87	NS
sAST	0.95	*
Total bilirubin	0.86	NS
Serum albumin	0.28	NS
Viral load	0.59	NS
Survivin	0.98	**
TIMP-1	0.95	*
Survivin/TIMP-1 Ratio	0.93	*

493 * indicates significant correlation at $p<0.05$; ** indicate significant correlation at $p<0.01$ and
 494 *** indicate significant correlation at $p<0.001$. NS indicates non significant correlation.
 495 sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; TIMP-1,
 496 tissue inhibitor of the metalloproteinase-1.
 497
 498

499

500

501

502

503

504

505

506

507

508

509

510

511

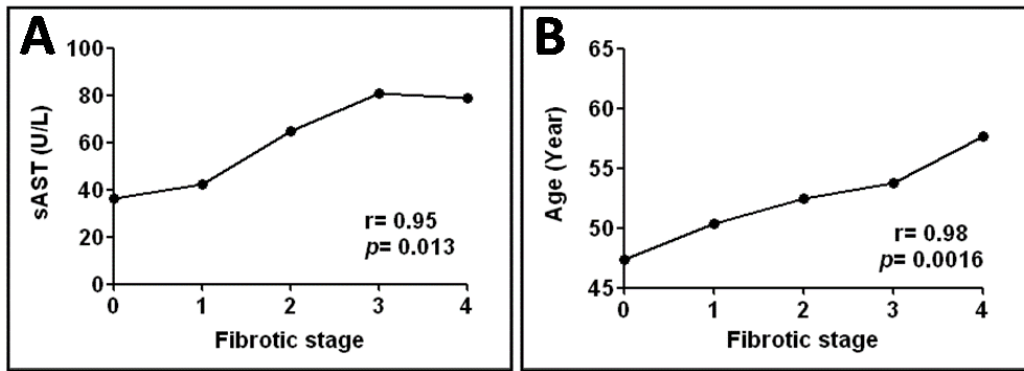


Figure S1: Significant correlations between sAST and age with the fibrotic stages in the studied patients. sAST, serum aspartate aminotransferase.

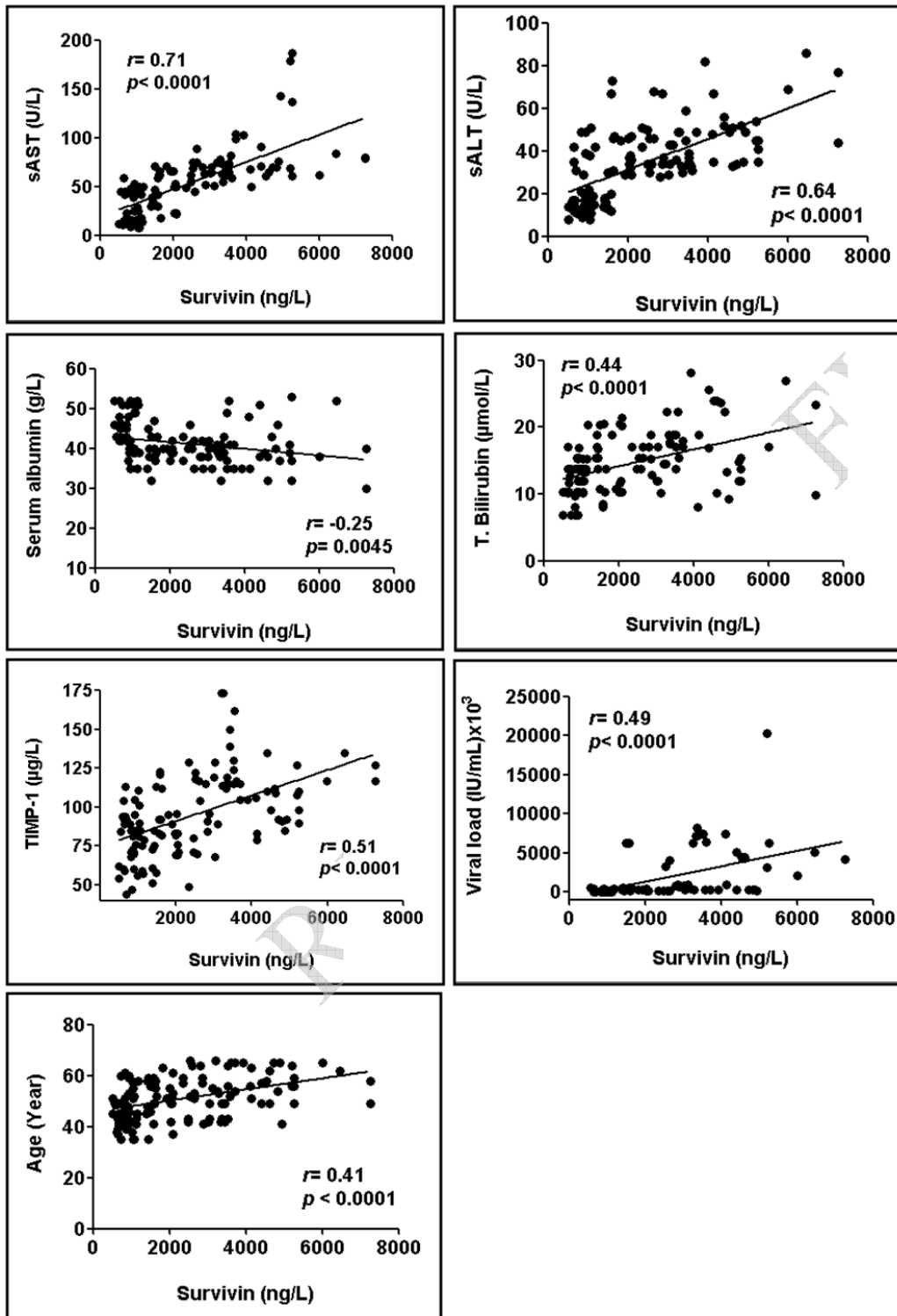


Figure S2: Significant correlations between different parameters and serum survivin in the studied patients. sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; TIMP-1, tissue inhibitor of the metalloproteinase-1; T. Bilirubin, total bilirubin.

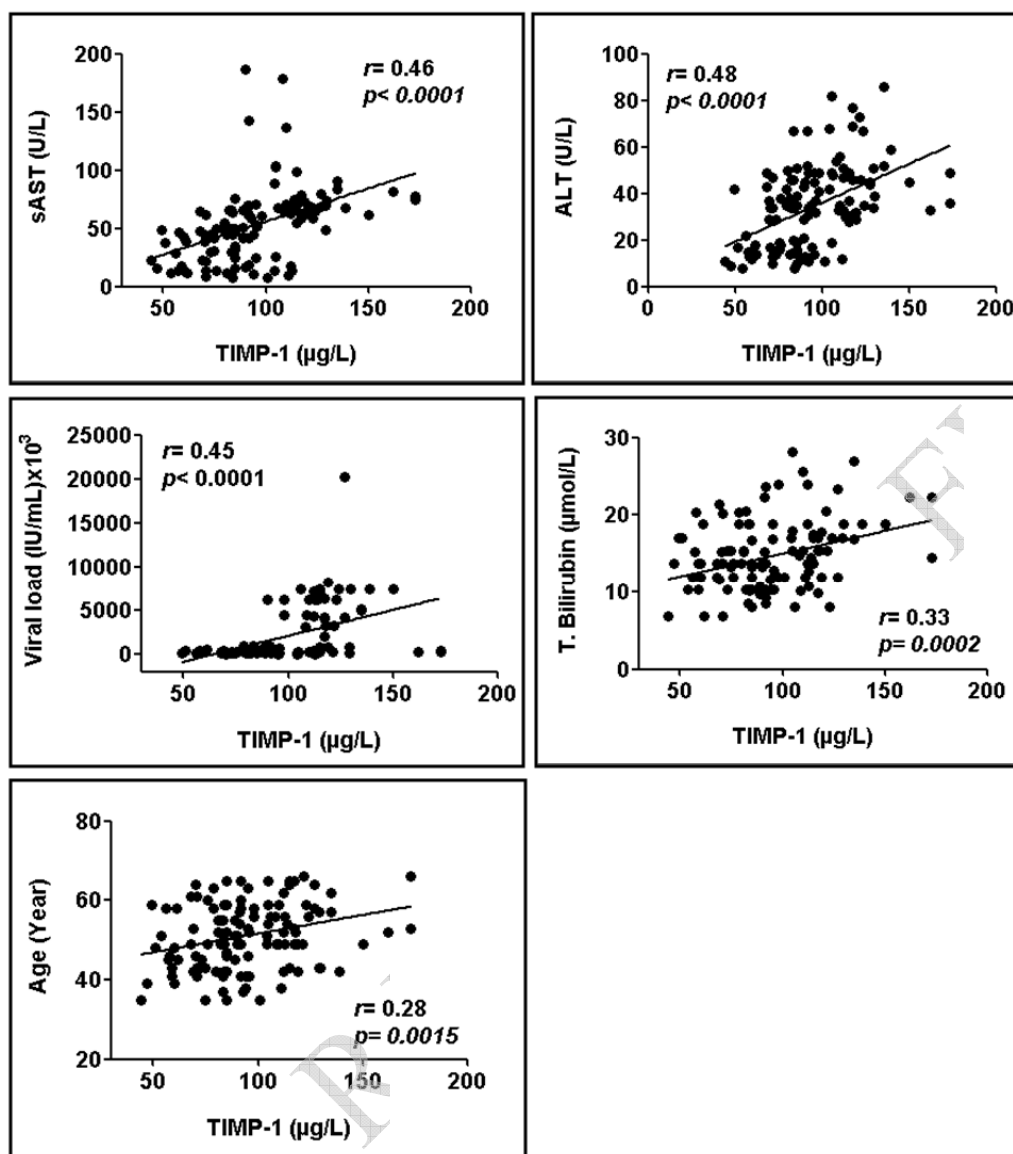


Figure S3: Significant correlations between different parameters and serum TIMP-1 in the studied patients. sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; T. Bilirubin, total bilirubin.