

Activated Hepatic Stellate Cells (Ito Cells) - Marker of Advanced Fibrosis in Chronic Viral Hepatitis C: A Pilot Study

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ABSTRACT

Background & Aims: The aim of this study is to determine whether activated hepatic stellate cells (HSCs) may represent a prognostic marker of progressive liver fibrosis in chronic viral hepatitis C (VHC) before antiviral therapy. The possible correlation between HSCs immunohistochemical features, histopathological aspects and clinical data before therapy were also studied.

Methods: This retrospective pilot study was conducted on 27 liver biopsies from VHC patients before antiviral therapy. HSCs's immunohistochemical analysis used the antibodies alpha-smooth muscle actin (α -SMA), glial fibrillary acidic protein (GFAP) and vinculin. We correlated immunopositive HSCs with HCV load, liver stiffness (LS), fibrosis stage and necro-inflammatory degree before treatment. Also, we assessed the association between liver fibrosis after therapy, the sustained virological response at 12 weeks after therapy (SVR 12) and the type of therapy.

Results: HSCs were increased in VHC patients compared to controls, mainly in the intermediate and periportal lobular regions. α -SMA and vinculin HSCs correlated positively with fibrosis stage ($p=0.044$), ($p=0.028$). Furthermore, α -SMA and vinculin HSCs were associated with LS ($p=0.027$), ($p=0.002$) and viral load ($p=0.021$), ($p=0.006$), but not with necro-inflammation degree. GFAP HSCs inversely correlated with fibrosis stage ($r=-0.475$), LS ($r=-0.422$) and HCV load ($r=-0.517$), but positively with necro-inflammation degree ($p=0.038$). Liver fibrosis post therapy correlated positively with SVR12 ($p<0.001$) and the type of therapy ($p=0.006$) and SVR12 correlated positively with treatment's type ($p=0.002$).

Conclusions: Activated HSCs may represent a marker of increased liver fibrosis in VHC. Different immunohistochemical markers can detect various HSCs subpopulations involved in the evolution of VHC and liver fibrosis.

Key words: hepatic stellate cells – α -smooth muscle actin – glial fibrillary acidic protein – vinculin – chronic hepatitis C – fibrosis.

Abbreviations: ECM: extracellular matrix components; GFAP: glial fibrillary acidic protein; HBV: hepatitis B virus; HCV: hepatitis C virus; HSC: hepatic stellate cell; IHC: immunohistochemical; LS: liver stiffness; S PegIFN + RBV: Peginterferon and ribavirin; SVR12: sustained virological response at 12 weeks post treatment; VHC: viral hepatitis C; α -SMA: α -smooth muscle actin.

INTRODUCTION

Chronic hepatitis represents the key factor in hepatic fibrogenesis and cirrhosis [1]. The frequent causes for chronic hepatitis are represented by hepatotropic viruses such as hepatitis B virus (HBV), hepatitis C virus (HCV), high alcohol intake, metabolic and autoimmune liver diseases [1].

Viral hepatitis represents a huge worldwide society health issue affecting hundreds of millions of people and leads to important morbidity and mortality [2]. Viral hepatitis B and hepatitis C (VHC) are responsible for a high number of chronic infections and represent the main cause of mortality from viral hepatitis [2].

Viral hepatitis C represents an international health issue, and was approximated that 71 million people are chronically infected with HCV [3]. The worldwide prevalence of HCV was 23,7 cases per 100,000 population in 2015, with an approximated 1,75 million recent HCV infections diagnosed in 2015 [3]. Worldwide, HCV genotypes 1 (44% of cases), 3

(25% of cases), and 4 (15% of cases) are responsible for the majority of the infections [3]. About 10-20% of people who have chronic VHC will have complications such as cirrhosis, liver failure, and hepatocellular carcinoma after 20-30 years [3].

Hepatic stellate cells (HSCs) represent the main element involved in liver fibrosis and cirrhosis [4–10]. In healthy liver they reside in the space of Disse in a quiescent state, storing the majority of vitamin A of the body and producing extracellular matrix (ECM) components like collagen III, IV and low amounts of collagen I [8–11].

Hepatitis C virus has the ability to activate HSCs directly through its core proteins, viral DNA or RNA or indirectly by apoptotic bodies and TGF- β 1 liberated by HCV infected hepatocytes, leading to liver fibrogenesis [12–14].

Once activated, HSCs lose their retinoids and become myofibroblasts with contractile and fibrotic properties resulting in a high amount of ECM accumulation [4, 6, 10, 14].

The current and effective therapy for VHC includes direct-acting antivirals (DAAs) which assure a sustained virologic response (SVR), with HCV eradication and hepatic fibrosis regression in the majority of patients [15–18].

Several evidence have shown that after HCV eradication, the number of activated HSCs diminishes through one of these mechanisms: inactivation, cellular senescence and elimination by apoptosis or autophagy [19–23]. In addition, hepatic fibrosis regression is associated with one of these HSCs fates [17–22]. Therefore, HSCs can return to a dormant, inactive state, but they retain the ability to reactivate faster than original quiescent cells [18–20]. This process is induced by many transcription factors such as transcription factor 21 (Tcf21), GATA Binding Protein 4/6 (GATA 4/6), LIM homeobox protein 2 (Lhx2), retinoic acid receptor beta (RAR β), interferon regulatory factor 1/2 (IRF 1/2), peroxisome proliferator-activated receptor gamma (PPAR γ), E26 transcription-specific transcription factors 1/2 (ETS 1/2), and Neurofibromin 1 (NF1) [24–26].

Another mechanism involved in activated HSCs reduction is senescence, which ends their proliferation, promoting fibrosis regression [22, 27, 28]. Recent data has revealed that activated HSCs which expressed a senescence marker, urokinase plasminogen activated receptor (uPAR), were eradicated, stimulating fibrosis decrease [29]. Ultimately, the number of activated Ito cells can be diminished by destruction via apoptosis or autophagy [17, 18, 21, 23]. Apoptosis of activated Ito cells has been described in vivo and in culture cells, being controlled by mediators of ECM degradation like matrix-metalloproteinase-2 (MMP2) [30, 31]. Activated HSCs are eliminated by autophagy through autophagosomes regulated by autophagy effector proteins [18, 23]. Some authors reported a decreased activation of Ito cells after hepatic damage, leading to reduced fibrosis in HSC-specific deletion of autophagy-related protein 7 (Atg7) mice in vivo [32].

Another research study conducted by Orrc et al. [16], using liver biopsies collected from VHC patients before and after DAAs therapy, examined the intrahepatic cellular modifications induced by DAAs therapy and their correlations with the treatment response. They didn't find any significant changes regarding activated HSCs during therapy or a correlation with the therapy outcome [16].

All these data support the influence of HSCs reduction in the reversal of liver fibrosis, post HCV elimination.

Activated HSCs are immunoreactive for α -smooth muscle actin (α -SMA), which represents the hallmark of their activation and for many antibodies like glial fibrillary acidic protein (GFAP), vinculin and others [6, 7, 10, 14, 33–36].

The purpose of this study was to determine if HSCs may represent a marker of progressive fibrosis in chronic VHC. Thus, we proposed to find an association between HSCs activity within the 3 zones of the hepatic lobule, the viral load and liver stiffness prior to the antiviral therapy and the histopathological features (liver fibrosis and grade of necro-inflammation) in VHC patients. In addition, we attempt to assess the immunoexpression of α -SMA, GFAP and vinculin in HSCs within the periportal zone, intermediate zone and periportal zone of the hepatic lobule.

METHODS

This retrospective observational, pilot study was carried out on 27 liver biopsy specimens from VHC patients, prior to antiviral therapy. We included liver biopsies from the archive of the Pathology Department of the Prof. Octavian Fodor Regional Institute of Gastroenterology and Hepatology. Cluj-Napoca, Romania, biopsied during 2011–2020. The control group consisted of 4 liver biopsy samples obtained from patients with benign liver tumors (hepatocellular adenoma and cavernous hemangioma) on a normal liver background.

The study was approved by the Ethical Committee of the Iuliu Hațieganu University of Medicine and Pharmacy Cluj-Napoca (approval nr. 71/11.03.2019) and respected the ethical guidelines of the 1975 Declaration of Helsinki.

The inclusion criteria were the same as those used for antiviral therapy: virological criteria – active virus replication (presence of HCV RNA), presence of anti-HCV antibodies; biochemical criteria – increased alanine aminotransferase (ALT) levels for more than six months; morphological criteria – chronic hepatitis – Metavir score necro-inflammatory activity (A) ≥ 1 and fibrosis (F) ≥ 1 . Also, for all VHC patients we included the following data: demographic, time interval since VHC diagnosis, blood HCV-RNA levels before treatment, type of treatment received, Metavir score of hepatitis, fibrosis stage and necro-inflammatory grade and evolution of fibrosis after therapy. In addition, we documented if patients presented sustained virological response at 12 weeks post treatment (SVR 12). SVR 12 refers that HCV RNA is undetectable (or under the inferior limit of assessment) at 12 weeks post-treatment end [37].

The exclusion criteria included patients co-infected with HBV, with human immunodeficiency virus, and patients with any other liver diseases.

Fibrosis prior antiviral therapy was evaluated by transient elastography (Fibroscan) and histologically through liver biopsy. Fibrosis after treatment was measured by Fibroscan. Transient elastography represents a non-invasive technique which measures the liver stiffness (LS) in order to assess the liver fibrosis [38–40].

We determined the correlation between the following variables: immunoreactive HSCs, viral load, LS, fibrosis stage

and necro-inflammatory activity prior antiviral therapy. Moreover, we assessed the relationship between post therapy LS, SVR 12 and the type of treatment.

Histopathological Assessment

The liver biopsies were fixed in 10% formaldehyde for 24 hours at a temperature of 4 °C. After embedding, fine 4 µm sections were cut from the paraffin blocks, which were later stained with Hematoxylin & Eosin (H&E). Furthermore, for a better assessment of fibrosis, special stains such as Masson's trichrome (TM) and Gomori were used.

We evaluated the stage of fibrosis on a scale from 0 to 4, applying the Metavir scoring system: stage 0 – absent; stage 1- portal fibrosis without septa (mild fibrosis); stage 2 - portal fibrosis with rare septa (moderate fibrosis); stage 3- numerous septa with porto-portal or porto-central bridging fibrosis, without cirrhosis (severe fibrosis); stage 4- cirrhosis. The necro-inflammatory activity was graded on a scale from 0 to 3: A0- no activity; A1- light activity; A2- moderate activity; A3- severe activity.

According to the severity of fibrosis and necro-inflammatory activity, we considered as “low Metavir score” stages 0, 1 and 2 of fibrosis and grade 0 and 1 of necro-inflammatory activity and a “high Metavir score” stages 3 and 4 of fibrosis and necro-inflammatory activity grade 2 and 3.

The presence of steatosis was assessed as the following: 0: absent, 1: mild (1-30% of hepatocytes), 2: moderate (31–60% of hepatocytes), and 3: severe (more than 60% of hepatocytes).

Immunohistochemical Assessment

For identification and characterization of hepatic stellate cells (number, distribution and morphology) immunohistochemical (IHC) analysis was performed. We used a Leica automatic Bond-Max system for slide processing. For α-SMA immunohistochemical staining, epitope retrieval was not recommended. Liquid mouse monoclonal antibody α-SMA dilution 1:50 (Novocastra clone asm-1) was applied during 30 minutes at 25° C.

Before IHC staining with GFAP and vinculin, heat mediated antigen retrieval with Tris/EDTA buffer pH 9.0 was performed. After this procedure, liver sections were incubated with mouse monoclonal anti-Vinculin antibody (Abcam, clone VCL/2575) diluted 1:100 and ready-to-use mouse monoclonal anti-GFAP antibody (Bond, clone GA5). For IHC reaction, diaminobenzidine was used as a chromogen substrate, followed by counterstaining with Hematoxylin.

Assessment of the immunopositive cells was evaluated using a semiquantitative technique by determining the number of positive cells on a x100 magnification (Leica Microscope 2500) in the 3 specific areas: 1 – periportal, 2 – intermediate (perisinusoidal) and 3 – central (perivenular), of three randomly chosen hepatic lobules. Counting was performed for each marker α-SMA, GFAP and vinculin.

The number of immunoreactive cells was classified as following: none: 0 positive cells, I: 1-5 positive cells, II: 6-15 positive cells, III: more than 15 positive cells.

According to the intensity of IHC staining of each marker, α-SMA, GFAP and vinculin, analysed HSCs presented weak, moderate and strong cytoplasmic immunoreactivity.

Statistical Analysis

Data analysis was performed in R 4.1.1, assuming p-values <0.05 as significant. Cramer's V, Chi², odds ratio (OR) and phi coefficient were used as measures of association between qualitative variables and Spearman's rho (R) was used to measure correlation between ordinal data. Numerical data was presented as mean ± standard deviation; qualitative data was presented as counts (%).

RESULTS

Out of the 31 patients, 27 (87%) had VHC and 4 (13%) were controls. Clinical and biological characteristics are shown in Tables I and II. Control patients had a normal histology of liver, without necro-inflammatory activity, fibrosis, or steatosis.

Liver stiffness measured by Fibroscan was positively associated with the stage of fibrosis determined histologically (R=0.696, p<0.001). We didn't find any correlation between LS and the necro-inflammatory activity (R=0.052, p=0.796). The HCV-RNA level prior treatment correlated positively with fibrosis assessed by Fibroscan (R=0.732, p<0.001) and histologically by liver biopsy (R=0.681, p<0.001). There was no association between viral load and the degree of necro-inflammation (R=-0.145, p=0.469).

Out of 27 VHC patients 14 were treated with DAAs and 13 with Peginterferon plus Ribavirin (PegIFN + RBV). Most of the patients (20) achieved SVR12 and fibrosis regression after treatment, LS decreased from 7.6 (3.3-13.8) kPa to 6.1 (2.8- 9.8) kPa (Table I). The evolution of fibrosis post therapy correlated positively with the SVR12 (p<0.001) and the type of therapy (p=0.006) (Table II). We observed a significant positive association between the SVR 12 and the type of treatment (p=0.002) (Table III).

Histopathological Features

According to Metavir scoring system, in our study 5 patients presented a low score and 22 a high score (Table I). The most common necro-inflammatory grade in VHC patients was 2 (66.7%) (Table I). The most frequent stage of fibrosis was 1 (48.1%) (Table I).

In patients with a low Metavir score, the necro-inflammatory activity was characterized by a mild portal lymphoplasmacytic inflammatory infiltrate, minimal interface hepatitis (mononuclear infiltrate at interface of portal tract and lobule) and mild lobular hepatitis (inflammation of the hepatic lobule), with hepatocyte ballooning degeneration, rare spotty necrosis and confluent necrosis. In this group fibrosis was mild and moderate represented by enlargement of portal tracts with stellate periportal fibrous extension and rare portal - portal or portal - central fibrous bridging.

Within the patients with a high Metavir score, 18 presented moderate and 2 severe necro-inflammatory activity with moderate to severe portal mononuclear inflammation with lymphoid follicles, moderate to severe interface hepatitis (Fig. 1 and 2B) and severe lobular hepatitis, with bridging and extensive necrosis. The stage of fibrosis ranged from moderate (8 patients) characterized by many fibrous septa forming bridges between the central veins and adjacent portal spaces or

Table I. Clinical characteristics of included VHC patients

Gender, n (%)	Female	21 (77.8)
Age (years)	M (min:max)	52 (31:73)
	$\mu \pm SD$	51.30 $\pm$ 11.2
Necro-inflammatory grade, n (%)	0	0
	1	7 (25.9)
	2	18 (66.7)
	3	2 (7.4)
Fibrosis stage, n (%)	0	0
	1	13 (48.1)
	2	5 (18.5)
	3	8 (29.6)
	4	1 (3.7)
Metavir, n (%)	0	0
	A1F1	2 (7.7)
	A1F2	3 (11.5)
	A2F1	10 (38.5)
	A1F3	2 (7.7)
	A2F2	2 (7.7)
	A2F3	5 (19.2)
	A3F2	0
	A2F4	1 (3.8)
	A3F3	1 (3.8)
Steatosis, n (%)	0	14 (51.9)
	1	11 (40.7)
	2	2 (7.4)
Interval time since VHC diagnosis (years)	M (min:max)	9 (5:18)
	$\mu \pm SD$	10.26 $\pm$ 3.54
HCV-RNA level prior to treatment (UI/ml)	M (min:max)	733231 (3556: 19832490)
	$\mu \pm SD$	2400000 $\pm$ 4110000
SVR12, n (%)	absent	7 (25.9)
	present	20 (74.1)
LS by Fibroscan (kPa) prior to treatment	M (min:max)	7.6 (3.3:13.8)
	$\mu \pm SD$	7.99 $\pm$ 2.9
LS by Fibroscan (kPa) after treatment	M (min:max)	6.1 (2.8:9.8)
	$\mu \pm SD$	6.29 $\pm$ 1.86
Type of treatment, n (%)	DAA's	14 (51.9)
	PegIFN + RBV	13 (48.1)
Evolution of fibrosis after treatment, n (%)	increase	1 (3.7)
	stationary	6 (22.2)
	decrease	20 (74.1)

$\mu \pm SD$: mean $\pm$ standard deviation; M (min:max): median (min:max); MW: Mann-Whitney test; OR: odds-ratio [95% CI] and p value from Fisher test; V: Cramér V (p value from Chi² test); A: necro-inflammatory activity; F: fibrosis; VHC: viral hepatitis C; HCV: hepatitis C virus; SVR12: sustained virological response at 12 weeks post treatment; LS: liver stiffness.

Table II. The evolution of fibrosis after treatment in function of SVR 12 and type of treatment.

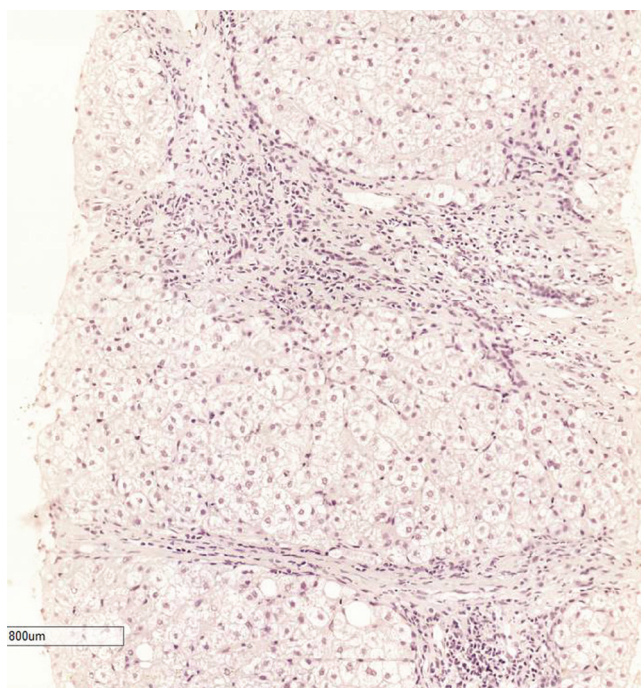
Evolution of fibrosis after treatment				
SVR 12	increase	stationary	decrease	(total)
absent	1 (100% / 14.3%)	5 (83.3% / 71.4%)	1 (5.0% / 14.3%)	7 (25.9%)
present	0	1 (16.7% / 5.0%)	19 (95.0% / 95.0%)	20 (74.1%)
(total)	1 (3.7%)	6 (22.2%)	20 (74.1%)	27 (100%)
V=0.81 (p<0.001); V = Cramér V p value from Chi ² test				
Type of treatment				
DAA's	0	0	14 (70.0% / 100%)	14 (51.9%)
PegIFN + RBV	1 (100% / 7.7%)	6 (100% / 46.2%)	6 (30.0% / 46.2%)	13 (48.1%)
(total)	1 (3.7%)	6 (22.2%)	20 (74.1%)	27 (100%)
V=0.61 (p=0.006); V = Cramér V p value from Chi ² test				

For abbreviations see Table I

Table III. The SVR 12 and type of treatment

Type of treatment			
SVR 12	DAA's	PegIFN + RBV	(total)
absent	0	7 (100% / 53.8%)	7 (25.9%)
present	14 (70.0% / 100%)	6 (30.0% / 46.2%)	20 (74.1%)
(total)	14 (51.9%)	13 (48.1%)	27 (100%)

OR=0.03 [0.00, 0.61], phi=0.61 (p=0.002); OR- odds ratios, phi coefficient. For the rest of abbreviations see Table I

**Fig. 1.** Regenerative hepatic nodules, separated by thick fibrous septa, severe portal inflammation and interface hepatitis in a high Metavir score (A3F4), (H&E).

portal veins to severe (1 patient) with architectural distortion of the liver parenchyma by fibrous thick collagen septa, separating

regenerative nodules with ductular reaction and cholestasis, leading to cirrhosis (Figs. 1, 2A, 2B).

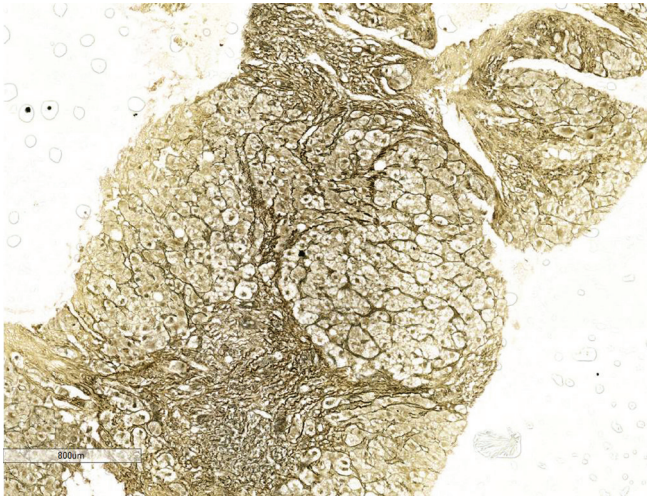


Fig. 2A. Alteration and replacement of the normal liver architecture by severe fibrosis and regenerative nodules, (Gomori stain).

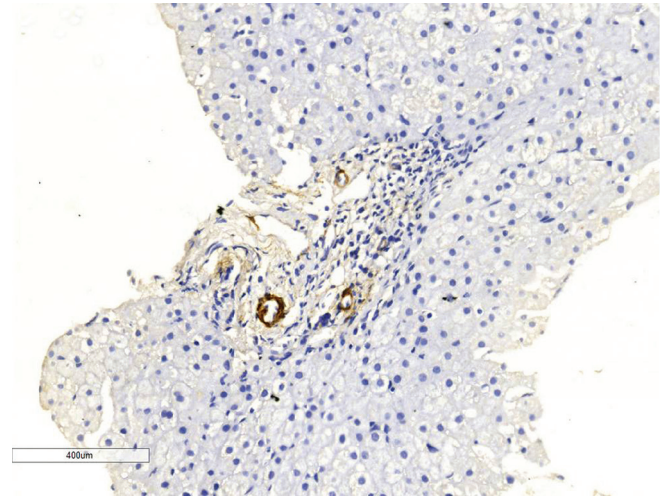


Fig. 3. Rare periportal α -smooth muscle actin (α -SMA) positive hepatic stellate cells in the control group (α -SMA, IHC).

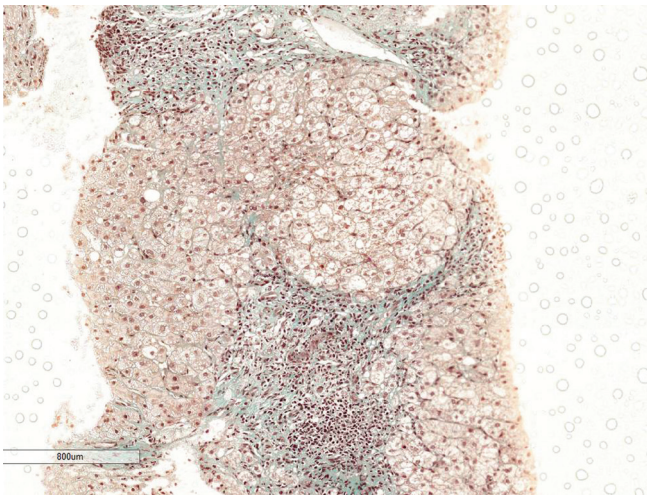


Fig. 2B. Alteration and replacement of the normal liver architecture by severe fibrosis and regenerative nodules, (Masson's trichrome stain).

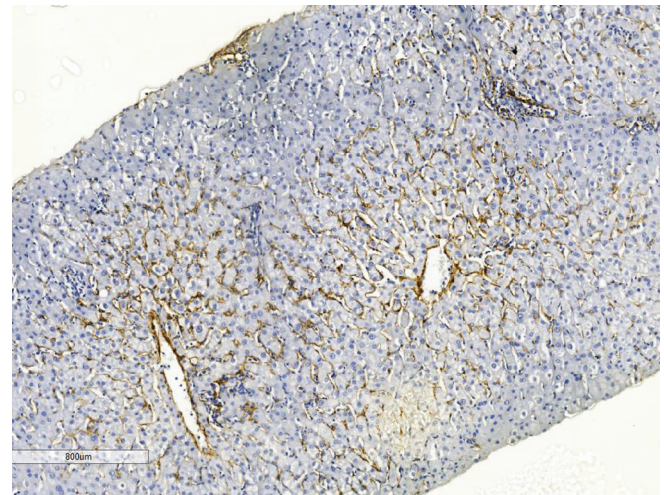


Fig. 4. Many periportal, perisinusoidal and perivenular α -smooth muscle actin (α -SMA) positive hepatic stellate cells, showing moderate immunorexpression in a high Metavir score VHC (A2F3), (α -SMA, IHC).

In the control group steatosis was absent, while in VHC group 13 patients presented microvesicular and macrovesicular steatosis (48%). Among them, 11 (40.7%) presented mild steatosis and 2 (7.4%) exhibit moderate steatosis (Table I).

Immunohistochemical Features

The number of HSCs immunostained with α -SMA, GFAP and vinculin in the 2 groups was semi quantitatively determined in the 3 characteristic regions of the hepatic lobules. HSCs appeared as star shaped cells, with thin, elongated cytoplasmic processes along the endothelial lining of the sinusoids (Figs. 3-8).

α -SMA Immunorexpression in HSCs

α -SMA-positive HSCs were statistically more frequent in VHC patients (Fig. 4) compared to controls (Fig. 3) only in zone 1 ($V=0.75$, $p<0.001$) and failed to reach significant differences in zones 2 ($V=0.40$, $p=0.179$) and 3 ($V=0.35$, $p=0.052$) (Supplementary file). The same relations are true for the immunorexpression intensity in zone 1: $V=0.81$ ($p<0.001$),

zone 2: $V=0.38$ ($p=0.221$) and zone 3: $V=0.22$ ($p=0.460$) (Supplementary file). In the VHC group, the α -SMA positive HSCs predominated in the periportal area and showed mainly moderate immunorexpression (Fig. 4), (Supplementary file). α -SMA positive HSCs were identified in all stages of fibrosis and all grades of necro-inflammatory activity (Supplementary file). The number of α -SMA-positive cells was significantly correlated with the stage of fibrosis ($R=0.391$, $p=0.044$) only in zone 3 (Fig. 4), (Supplementary file). Also, α -SMA HSCs of zone 1 correlated with LS ($R=0.425$, $p=0.027$) and viral load ($R=0.442$, $p=0.021$) (Table IV, Supplementary file). There was no correlation between the grade of necro-inflammatory activity and the number of α -SMA positive cells ($p=0.733$, $p=0.370$, $p=0.934$) in any zone of the hepatic lobule (Supplementary file). Also, α -SMA expression intensity of HSCs correlated with the necro-inflammatory activity only in zone 1 ($R=0.53$) (Supplementary file).

GFAP Immunorexpression in HSCs

The number of GFAP-positive HSCs was slightly higher in VHC patients (Fig. 6) compared to controls (Fig. 5), but

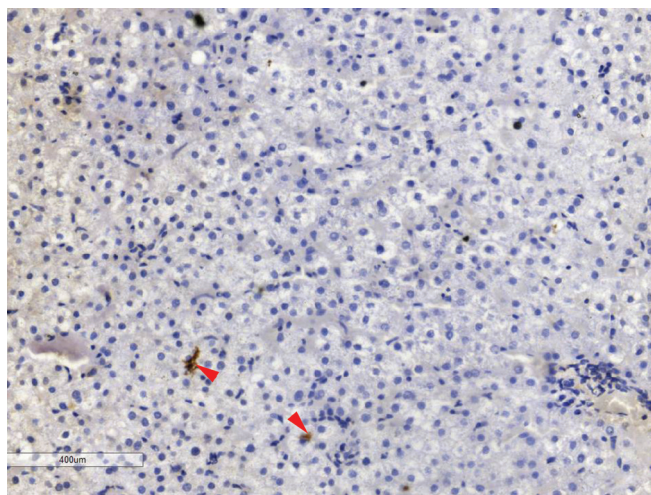


Fig. 5. Rare perisinusoidal glial fibrillary acidic protein (GFAP) immunoreactive hepatic stellate cells (arrows) in the control group (GFAP, IHC).

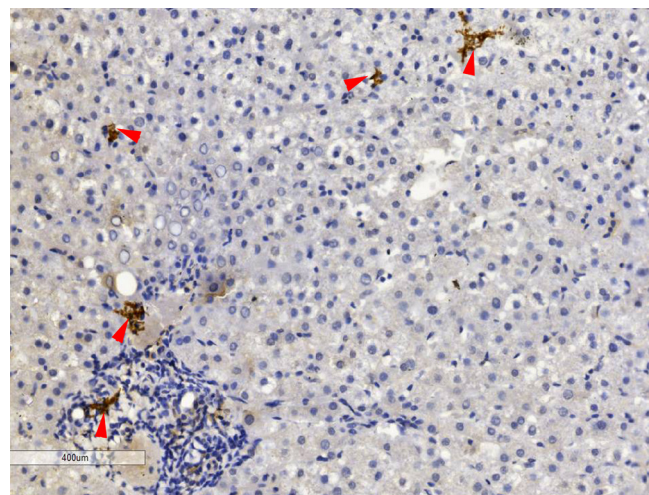


Fig. 6. Scattered periportal and perisinusoidal glial fibrillary acidic protein (GFAP) immunoreactive hepatic stellate cells (arrows) in a low Metavir score VHC (A1F1), (GFAP, IHC).

without statistical significance (Supplementary file). Note that only zones 1 and 2 had few GFAP-positive cells (Supplementary file). The number of GFAP-positive cells in zone 2 were negatively correlated with fibrosis stage ($R=-0.475$, $p=0.012$) and LS ($R=-0.422$, $p=0.029$) (Fig. 6), (Supplementary file). The same inverse correlation was found between positive HSCs and viral load ($R=-0.517$, $p=0.006$) (Supplementary file). Besides, the number of GFAP-positive cells in zone 2 was significantly positively correlated with the necro-inflammatory grade ($V=0.40$, $p=0.038$) (Supplementary file). We did not find any association between the expression of α -SMA and the expression of GFAP in HSCs (Table IV).

Vinculin Immunorepression in HSCs

Vinculin-positive HSCs were statistically more frequent in VHC patients (Fig. 8) compared to controls (Fig. 7) in zone 1 ($V=0.79$, $p<0.001$) and zone 2 ($V=0.44$, $p=0.049$) and failed to reach significant differences in zone 3 ($V=0.36$, $p=0.131$) (Supplementary file). However, the immunorepression intensity was significantly higher in zone 3 compared to control ($V=0.47$, $p=0.031$) while zones 1 ($V=0.29$, $p=0.106$) and 2 ($V=0.20$, $p=0.267$) did not show any significant difference (Supplementary file). The expression intensity was not significantly correlated with the necro-inflammatory grade

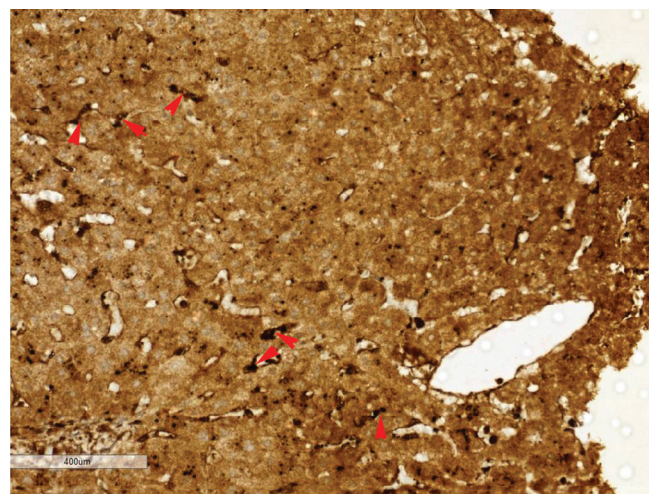


Fig. 7. Scattered perisinusoidal vinculin positive hepatic stellate cells (arrows) in the control group (vinculin, IHC).

($R=0.14$, $R=0.05$, $R=-0.08$) or fibrosis stage ($R=0.05$, $R=0.11$, $R=0.12$) in any zone (Supplementary file). The number of vinculin HSCs positive cells was significantly correlated with the fibrosis stage ($R=0.423$, $p=0.028$) and LS ($R=0.564$, $p=0.002$) only in zone 1 ($R=0.423$, $p=0.028$), (Fig. 8), (Supplementary

Table IV. Immunoreactive HSCs correlation with LS by Fibroscan (kPa) and HCV-RNA level prior treatment.

Immunoreactive HSCs	LS by Fibroscan (kPa) prior treatment	HCV-RNA level prior treatment (UI/ml)
Zone 1 SMA+ HSCs	$R=0.425$ ($p=0.027$)	$R=0.442$ ($p=0.021$)
Zone 2 SMA+ HSCs	$R=0.094$ ($p=0.640$)	$R=0.079$ ($p=0.695$)
Zone 3 SMA+ HSCs	$R=0.233$ ($p=0.242$)	$R=0.086$ ($p=0.668$)
Zone 1 GFAP+ HSCs	$R=0.134$ ($p=0.505$)	$R=-0.280$ ($p=0.157$)
Zone 2 GFAP+ HSCs	$R=-0.422$ ($p=0.029$)	$R=-0.517$ ($p=0.006$)
Zone 1 Vinculin+ HSCs	$R=0.564$ ($p=0.002$)	$R=0.522$ ($p=0.006$)
Zone 2 Vinculin+ HSCs	$R=0.242$ ($p=0.224$)	$R=0.284$ ($p=0.151$)
Zone 3 Vinculin+ HSCs	$R=-0.252$ ($p=0.205$)	$R=-0.252$ ($p=0.204$)

R = Spearman's rho correlation coefficient. HSC: hepatic stellate cell; LS: liver stiffness; HCV: hepatitis C virus; SMA: smooth muscle actin; GFAP: glial fibrillary acidic protein.

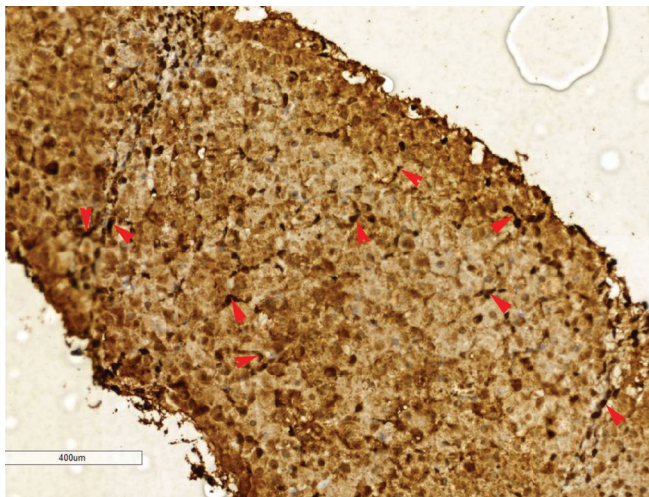


Fig. 8. Numerous periportal and perisinusoidal vinculin positive HSCs (arrows) showing strong immunoreexpression in a high Metavir score VHC (A3F3), (vinculin, IHC).

file). In addition, vinculin positive stellate cells were associated with viral load ($R=0.522$, $p=0.006$) (Supplementary file).

We found no correlation between vinculin-positive HSCs and the necro-inflammatory grade ($p=0.283$, $p=0.536$, $p=0.802$) (Supplementary file). Also, there was no association between the expression of vinculin, α -SMA expression and GFAP expression in HSCs (Supplementary file).

DISCUSSION

Viral hepatitis C, the main infectious disease responsible for cirrhosis and liver failure, exhibits histological hallmarks represented by different degrees of liver fibrosis and necro-inflammation, features which we tried to highlight in the present study [2, 3].

Liver fibrosis represents an active, continuous and excessive accumulation of ECM in the space of Disse, in response to chronic injury, affecting the circulation between hepatocytes and blood [4, 7, 41-43].

Meanwhile, fibrosis is accompanied by inflammation and angiogenetic alterations, leading to the destruction of the normal liver architecture [4, 7, 10, 42, 44].

In the present study we found a significant positive correlation between the stage of fibrosis determined on liver biopsy and LS measured by Fibroscan. This finding is in agreement with results reported by other authors [45-49].

We did not find any association between LS values and the necro-inflammatory activity. Cabibi et al. [48] found similar results in a study that included 111 patients with CHC [48], while others had different findings, revealing a significant correlation between LS and the necro-inflammatory activity [47, 49, 50].

Our study detected a positive association between the stage of fibrosis (evaluated by LS and histologically) and the viral load. These findings can be explained by the fact that active virus replication activates HSCs, which in turn secrete large amounts of collagen, leading to fibrosis. Our findings are in concordance with other researches [51-53] conducted on a higher number of VHC patients, showing a significant correlation between serum HCV RNA levels and fibrosis stage

and in contrast with others [54-56], which did not find any association between these two variables.

We did not reveal any relationship between blood RNA HCV levels and the necro-inflammatory grade. Some authors also didn't observe any correlation between serum HCV RNA titres and necro-inflammation [51, 54, 56], while other authors, who conducted a study of 1220 patients with chronic HCV infection, reported a strong association between the viral load and the necro-inflammatory activity ($p < 0.001$) [55].

A significant correlation between the SVR 12, evolution of fibrosis and the type of antiviral treatment, was detected in our work, reflecting the therapy efficiency. This observation is supported by several studies [17, 18, 57-64], which highlighted a significant decrease of liver fibrosis and serum HCV RNA levels, with SVR 12 in VHC patients after antiviral therapy, mainly with DAAs.

Hepatic stellate cells represent the pivotal element of fibrogenesis [4-7, 10, 44]. In response to persistent liver injury, they undergo activation, lose vitamin A lipid droplets and transform into contractile and proliferating myofibroblast-like cells, producing high amount of ECM [4, 6-8, 10, 13, 65].

Hepatic stellate cells activation is controlled by many cytokines and growth factors secreted by Kupffer cells, hepatocytes, platelets, leukocytes and sinusoidal endothelial cells [6, 7, 10, 13, 44, 66-69]. The principal mediators responsible for the activation of HSCs are represented by platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β), cell surface death receptor (Fas), reactive oxygen species (ROS), lipid peroxides, apoptotic bodies, extracellular vesicles (EVs) and LS [4, 7, 44, 67, 70-73].

Hepatitis C virus can activate HSCs directly or indirectly, initiating fibrogenesis [7, 13, 14].

Previous studies have shown that E2 protein of HCV, carboxyl and amino terminal of HCV core protein, as well as other HCV core proteins, have the ability to directly activate Ito cells [7, 12-14]. Moreover, HSCs can be indirectly activated by HCV-infected hepatocytes via TGF- β 1, ubiquitin carboxy-terminal hydrolase L1 (UCHL1) and various profibrogenic mediators [7, 13, 44, 74, 75].

A hallmark of their activation is their increase expression of α -SMA [6, 7, 10, 68, 75-77]. α -SMA, a cytoskeletal protein, represents a mesenchymal and a unique marker for smooth muscle cell differentiation [6, 7, 75, 76, 78, 79]. Numerous studies revealed that α -SMA is an authenticational marker of hepatic fibrosis [6, 7, 75-77, 80].

In healthy liver, scattered α -SMA positive HSCs are located perisinusoidally, perivenular, at the periphery of the hepatic lobule, in the periportal region, surrounding the hepatic artery branches and the terminal venules [75, 78, 80-83].

In our study, a small number of α -SMA HSCs were found in the control group, mainly in the perisinusoidal and in the periportal areas, in the vicinity of the hepatic artery branches and the terminal venules, presenting a weak α -SMA immunoreexpression. Similar results were reported by several prior studies [6, 74, 76, 78, 80-83].

In VHC patients the number of α -SMA positive cells was higher than in healthy subjects, especially in the periportal zone, displaying mainly a moderate and intense immunoreaction. α -SMA reactive HSCs were found in all stages and grades of

VHC. These findings are similar with those published by other authors [75, 78, 80, 84-86]. However, other studies revealed that α -SMA reactive HSCs were predominantly distributed in the intermediate zone of the hepatic lobule [76, 77, 79, 81]. These different findings regarding the location of activated α -SMA HSCs in VHC patients can be explained by different laboratory IHC methods, different IHC antibody clones and interpretation of IHC results.

The association between the number of α -SMA active HSCs, the stage of fibrosis and necro-inflammatory grade is still a questionable subject [75, 76, 78, 80]. Most of the existing reports highlighted a positive correlation between the stage of fibrosis and the number of α -SMA positive HSCs [75, 80, 84, 88-92].

In the current study we also identified a statistically significant positive correlation between the stage of fibrosis and the number of α -SMA positive HSCs in the pericentral zone of the lobule. Tomanovic et al. [81] found a positive correlation between the number of α -SMA HSCs and the stage of fibrosis in the portal spaces and fibrous septa. Moreover, Ionescu et al. [76] reported that α -SMA active HSCs were significantly correlated to the stage of fibrosis in all liver areas.

On the contrary, other researchers revealed a negative relationship between the number of α -SMA HSCs and fibrosis, assuming that HSCs were previously activated by HCV, even in the absence of increase fibrosis, or that activated HSCs were diminished by apoptosis, eventually returning to their inactive state, during the progression of VHC [33, 76, 78, 89, 90, 93, 94].

Furthermore, the number of α -SMA HSCs was associated with LS. This observation is supported by previous studies which highlighted that LS is involved in HSCs activation, leading to their proliferation [95-98].

Regarding the association between the number of α -SMA HSCs and the grade of necro-inflammation, our study did not find any statistically significant correlation between these two variables, which is in accordance to results reported by other published papers [33, 76, 78, 80, 93, 94]. This aspect could be explained by the fact that HSCs were already activated by HCV, even in the absence of severe necro-inflammatory activity [75, 80, 99].

On the other hand, some other authors suggested that the number of α -SMA HSCs correlated positively with the necro-inflammatory grade [66, 75, 84, 87, 91, 100].

The present study found a positive significant association between the number of α -SMA HSCs and the HCV viral load. Hepatitis C virus activates HSCs, promoting their proliferation and liver fibrosis [7, 11-14]. As far as we know, no such correlation has been described in the literature.

Glial fibrillary acidic protein represents a cytoplasmic intermediate filament, initially described and analyzed in astrocytes, preserving their conformation [7, 76, 78, 101, 102]. The literature review showed similarities between HSCs and astrocytes regarding their star shape, close contact to blood vessels and their reaction to tissue injury [7, 76, 78].

Over time, many studies have examined the expression of GFAP in HSCs in human liver, but the results remain controversial [33, 76, 78]. Some authors [78, 103] revealed few positive periportal GFAP HSCs in healthy human liver and increased number of GFAP HSCs in cirrhotic nodules, others [104] didn't find any positive GFAP HSCs in normal

liver samples, but detected scattered periportal GFAP HSCs in cirrhotic livers.

In our study, we found no significant difference between the number of GFAP positive HSCs in the VHC group compared to control group, perhaps because GFAP also stains inactive HSCs, supporting its double role as marker of quiescent and activated HSCs [7, 33, 105]. Recent research conducted by Lim et al. [106] via semi-quantitative RT-PCR and real-time PCR, discovered that inactive and culture-early activated HSCs expressed many types of GFAP slice, responsible for their activation.

Our study revealed that in the control group and in VHC group, GFAP positive HSCs were detected only in the perisinusoidal and periportal zones, lacking in the perivenular zone. Furthermore, GFAP HSCs were mainly distributed in the perisinusoidal region, suggesting their intimate contact to the sinusoid capillaries [76, 78]. This observation can be explained by the fact that the perisinusoidal and periportal zones are the most metabolically active and oxygenated zones, whereas the pericentral zone is the last metabolically and oxygenated zone. In the absence of oxygen, HSCs lose their affinity to GFAP. A number of previous studies reported similar findings [33, 76, 78].

Several reports concluded that GFAP represents an earlier and more specific marker of HSCs activation than α -SMA, indicating incipient fibrosis in VHC [33, 76, 78, 105]. This idea is supported by our study, which noticed an inverse correlation between the expression, number of GFAP positive HSCs, the stage of fibrosis and LS in the perisinusoidal zone. Also, our work showed that GFAP positive HSCs inversely correlated with HCV viral load. To our knowledge such correlation has not yet been analyzed. These findings lead to the idea that patients with low levels of GFAP expression in HSCs may represent a subgroup of VHC patients, which require a personalized antiviral therapy.

Regarding the association between GFAP positive stellate cells and the necro-inflammatory grade, we detected a positive correlation between these two variables. In contrast, the studies conducted by Hassan et al. [77] and Zakaria et al. [79] highlighted a negative correlation between GFAP positive stellate cells and the necro-inflammatory grade. These divergent results could be related to the small number of cases in our study, to the various staining procedures used or to a subpopulation of activated HSCs, which presents variable affinity for GFAP, depending to the phase of activation, during the necro-inflammatory process [33, 105, 106]. We didn't find any correlation between the expression of α -SMA and the expression of GFAP on HSCs. On the contrary, Hassan et al. [77] and Zakaria et al. [79] revealed a significant correlation between the expression of the two IHC markers. These differences could be explained by the diverse IHC techniques and antibody clones used, as well as because of our limited number of biopsies.

In the present study, the expression of α -SMA and GFAP on HSCs in some measure corresponded, several HSCs expressing both α -SMA and GFAP, while others being immunoreactive only for α -SMA or GFAP. These findings are in accordance with the results published by other researchers [33, 105].

There are few reports in the literature concerning the immunoreactivity of HSCs for vinculin [34, 107, 108].

We observed positive vinculin HSCs in both normal and VHC liver samples, mainly with a strong expression, being statistically more numerous in VHC patients, particularly in the periportal and perisinusoidal zones. Vinculin immunoreactive stellate cells appeared as tiny linear spots surrounding the sinusoidal wall. These findings are in concordance with other reports [34, 107], suggesting that vinculin is an immunohistochemical marker for both inactive and active HSCs.

To our knowledge, there is no data in the literature regarding the correlation between the number of positive vinculin HSCs, fibrosis stage, LS, the necro-inflammatory grade or viral load. In the present study the number of positive vinculin HSCs significantly correlated with the stage of fibrosis and LS in the periportal zone. These findings supports the hypothesis of Hijazi et al. [108] that vinculin, among other adhesion proteins, could have a key role in HSCs activation, promoting fibrogenesis. Moreover, positive vinculin stellate cells were positively associated with the viral load, suggesting that HCV activates HSCs, inducing their proliferation [7, 11–14].

No correlation between vinculin HSCs and the necro-inflammatory grade was detected in the current study, maybe because most of HSCs were quickly activated by HCV, whereas necro-inflammatory activity had just an additional implication.

There was no correlation between vinculin, α -SMA and GFAP immunoexpression in HSCs in our study. Although, the expression of vinculin, α -SMA and GFAP somewhat coincided, some HSCs expressed all the 3 markers, while others were positive for α -SMA, GFAP or vinculin. These findings may be due to existence of different subpopulations of HSCs, such as quiescent, early activated or late activated HSCs, which display different reactivity for these 3 markers [33, 78, 105, 106].

The limitations of our research consisted in the reduced number of subjects, the lack of a standard scoring system for counting Ito cells, the absence of an objective, accurate digital method for assessing the exact number of HSCs, the imbalance between the number of VHC biopsies and normal controls and the lack of hepatic biopsies after antiviral therapy, in order to highlight HSCs activity after viral eradication and fibrosis regression.

Our findings suggest that the number of activated HSCs could represent a helpful prognostic marker of advanced fibrosis in VHC patients. In order to confirm their potential prognostic value, further research is required, in large groups of patients, as well as analysis of liver biopsies post antiviral treatment and estimate the activated HSCs response to therapy.

As far as we know, this is the first study in Romania which analyses HSCs in VHC patients using 3 different immunohistochemical markers and their association with LS and viral load.

CONCLUSIONS

Activated HSCs, characterized by α -SMA, GFAP and vinculin immunoexpression were increased in number in VHC patients compared to healthy subjects. Positive α -SMA, GFAP and vinculin HSCs were predominantly located in the periportal and perisinusoidal zones of the hepatic lobules. α -SMA and vinculin reactive HSCs were statistically significantly correlated

with fibrosis stage and LS. GFAP positive HSCs correlated negatively with the stage of liver fibrosis, LS and viral load, being a more specific marker of initial activated HSCs than α -SMA, thus predicting early fibrosis.

Conflicts of interest: None to declare.

Authors' contributions: R.T.S. conceived, designed and wrote the study; C.S.M drafted and critically revised the manuscript; O.H.O. collected the clinical data and revised the method section of the study; T.Z. and B.A.G. performed the immunohistochemical analysis and interpreted the results; A.I. performed the statistical analysis; A.M.C. drafted the manuscript; C.M.M. critically revised and analyzed the final version of the manuscript. All authors approved the final version to be published and agree to be responsible for all aspects of the work.

Supplementary material: To access the supplementary material visit the online version of the *J Gastrointestin Liver Dis* at <http://dx.doi.org/10.15403/jgld-4726>

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