

## Correlation between Tgf-B1 and Fsp-1 Expression in Chronic Viral Hepatitis - an Immunohistochemical Study

DANIELA ELISE TACHE<sup>1</sup>, Ș.G. FIRU<sup>2</sup>, ANA MARINA ANDREI<sup>3</sup>,  
ANCA PREDESCU<sup>4</sup>, ȘTEFANA OANA PURCARU<sup>1</sup>,  
AMELIA MIHAELA DOBRESCU<sup>5</sup>, ANICA DRICU<sup>1</sup>,  
CĂTĂLINA GABRIELA PISOSCHI<sup>3</sup>, ELENA CAMELIA STĂNCIULESCU<sup>3</sup>

<sup>1</sup>Biochemistry Dept., Faculty of Medicine, University of Medicine and Pharmacy of Craiova

<sup>2</sup>PhD Student, Clinic of Nephrology, Emergency County Hospital No. 1 Craiova

<sup>3</sup>Biochemistry Dept., Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova

<sup>4</sup>Histology Dept., Faculty of Dentistry, University of Medicine and Pharmacy of Craiova

<sup>5</sup>Genetics Dept., Faculty of Medicine, University of Medicine and Pharmacy of Craiova

**ABSTRACT:** Infection with hepatitis C virus (HCV) is the most important stimulus for chronic hepatitis and subsequent progression to cirrhosis and hepatocellular carcinoma. Fibrosis that follows inflammation represents the main complication. One of the mechanisms that could be associated with development of liver fibrosis is epithelial-mesenchymal transition (EMT). Transforming Growth Factor  $\beta$ 1 (TGF- $\beta$ 1) is an important mediator of fibrosis and also able to trigger phenotypic changes in EMT. Fibroblast-specific protein 1 (FSP-1), a marker of fibroblasts in organs undergoing tissue remodeling, is used to identify cells that derive from EMT. In this study, we assessed the expression of TGF- $\beta$ 1 and FSP-1 in liver biopsies obtained from HCV-infected patients using immunohistochemistry and correlated them in order to evaluate the relation between fibrosis and EMT in liver disease progression. Staining of liver sections revealed increased amount of type III collagen and clusters of inflammatory cells invading portal spaces. The number of TGF- $\beta$ 1-positive cells was directly proportional to the incidence of liver injury. In cases of mild fibrosis, FSP-1 positive cells were observed in cells lining sinusoids. As fibrosis progressed, increased number of FSP-1 positive fibroblasts, isolated cholangiocytes and hepatocytes was observed. Even EMT via the activation of TGF- $\beta$  signaling pathway is recognized as a pathogenic mechanism of HCV-induced liver disease, FSP-1 alone couldn't be used as a valuable marker for cells that undergo EMT.

**KEYWORDS:** hepatitis C virus, fibrosis, epithelial-mesenchymal transition, TGF- $\beta$ 1, FSP-1

### Introduction

Infection with hepatitis C virus is the main cause of liver fibrosis and subsequent progression to cirrhosis and hepatocellular carcinoma, over 200 million people being estimated to be infected worldwide [1]. The prevalence rate of HCV infection in Romanian adult population was reported to be 3.23% [2].

Liver fibrosis is a complex tissue response resulting in the accumulation of extracellular matrix (ECM) components which leads to the disorganization of liver architecture, appearance of a scar at the site of injury and compromise liver functions. These changes are caused by the presence of inflammation accompanied by the release of proinflammatory cytokines and activation of myofibroblasts (MFs), cells involved in the aberrant synthesis of ECM. In this way fibrosis could be considered the result of the imbalance between ECM production and breakdown [3], an active process in which phases of progression and regression may

alternate, ECM suffering quantitative and qualitative changes [4].

Recent studies regarding liver fibrosis focuses on the MFs as key cells involved in fibrogenesis. They are proliferative cells that exhibit enhanced capacity to survive, migrate and accumulate at sites of liver injury in response to various stimuli [5]. They also possess properties and functions of both smooth muscle cells and fibroblasts, resulting in their contractile nature and increased capacity to produce ECM molecules [6].

Although the MFs origin in injured liver is still under debate, the majority of studies focus on hepatic stellate cells (HSCs) as the main source. Activated HSCs, transdifferentiated into MFs, can migrate and accumulate at the sites of tissue lesion, being able to secrete ECM components in higher quantities on one hand and, on the other hand, regulate ECM breakdown [7]. Beside HSCs, others cells (portal and bone-marrow derived fibroblasts) may become MFs [8].

Recently, epithelial to mesenchymal transition (EMT) has been proposed as a mechanism by which other types of adult liver cells may become MFs [9]. EMT is a complex biological process in which epithelial, polarized cells normally interacting with the basal membrane, undergo a variety of transformations to acquire a mesenchymal phenotype, increasing their migrating ability, invasiveness, resistance to apoptosis, and overproduction of ECM [1,10].

Several growth factors are related to EMT, among which isoform 1 of transforming growth factor  $\beta$  (TGF- $\beta$ 1), key mediator of tissue fibrosis, is considered an important promoter able to trigger phenotypic changes in this process [3].

Loss of epithelial markers, such as E-cadherin, and *de novo* expression of mesenchymal markers (vimentin,  $\alpha$ -smooth muscle actin, fibroblast specific protein 1) represent hallmarks of EMT [11].

Fibroblast specific protein 1 (FSP-1), also known as S100A4, belongs to the S100 superfamily of calcium-binding proteins. These proteins do not exhibit enzymatic activity and it is considered that they are involved in controlling various biological functions of other proteins [12]. Being used to label fibroblasts in organs undergoing tissue remodeling, FSP-1 could also identify cells that became able to synthesize increased amounts of collagen after EMT.

In this study, we analyzed the expression of TGF- $\beta$ 1 and FSP-1 in liver biopsies obtained from HCV-infected patients and correlated them in order to evaluate the relation between inflammation, fibrosis and EMT in liver disease progression.

## Material and Methods

This study was performed on 18 paraffin embedded liver biopsy samples obtained from HCV-infected patients and provided by the courtesy of Pathology Department, Emergency County Hospital, Craiova.

*Histological analysis.* Paraffin embedded liver specimens were cut at 4–5  $\mu$ m, and routinely stained with H&E, trichrome Masson and silver impregnation in order to assess the degree of fibrosis according to METAVIR scoring system and to evaluate the amount of reticulin fibers.

*Immunohistochemical detection of TGF- $\beta$ 1 and FSP-1.* Immunohistochemical reactions were performed on liver sections prepared as mentioned above using the following primary

antibodies: i) monoclonal mouse anti-human TGF- $\beta$ 1 (Santa Cruz Biotechnology Inc., sc 52893) and ii) polyclonal rabbit anti-human S100A4 (Dako, A5114), both diluted 1:200. After inhibition of the endogenous peroxidase with hydrogen peroxide in methanol and blocking of nonspecific binding with the appropriate normal serum, an overnight incubation with the primary antibodies at 4°C, in a humid chamber, was performed. The next day reactions were processed for EnVision-Dual Link System-HRP (Dako) or avidin-biotin-peroxidase (Vector Laboratories) techniques and developed with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Sigma-Aldrich Co.). Nuclear counterstaining was performed with Mayer's hematoxylin. Slides were observed and registered with a Nikon Eclipse microscope coupled to a digital camera. Images were finally processed using the Microsoft Office Picture Manager. For the negative controls, the primary antibodies were substituted with phosphate buffer saline, pH 7.4.

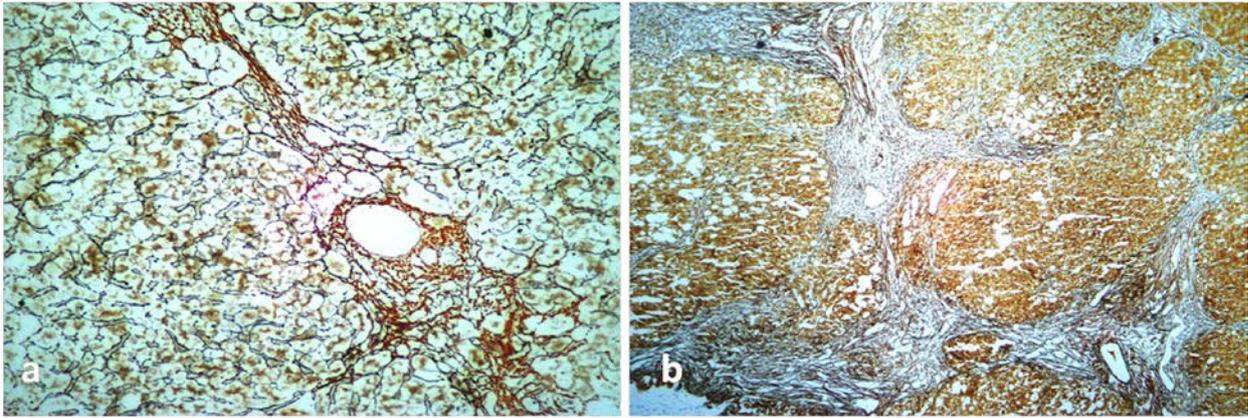
*Assessment of immunohistochemical results.* Evaluation of the immunohistochemical staining was performed by two different observers independently according to the following: positive reaction was indicated by the presence of brown deposits in the labeled structures while negative reaction was indicated by their absence, after the evaluation of the whole slide. A semiquantitative analysis was performed in order to score the number of positive cells in ten nonoverlapping random fields using a subjective grade system: 0 = no positive cells in the field; 1 = few positive cells (<10%); 2 = increased numbers of positive cells, especially immune cells (<30%); 3 = increased number of positive cells, especially in areas of fibrosis (>30%) and 4 = diffuse positive reaction in hepatocytes.

*Statistical analysis.* Score values were compared with t-test. Pearson correlation coefficients were calculated to assess the relationship between various histological findings.

## Results

Sections stained using H&E and Masson revealed fibrosis degrees from F2 to F4, according to the METAVIR scoring system: F2 – portal fibrosis and rare fibrous septa (44.45% of all cases included); F3 – septa without cirrhosis (22.27%); F4 – cirrhosis (33.34%).

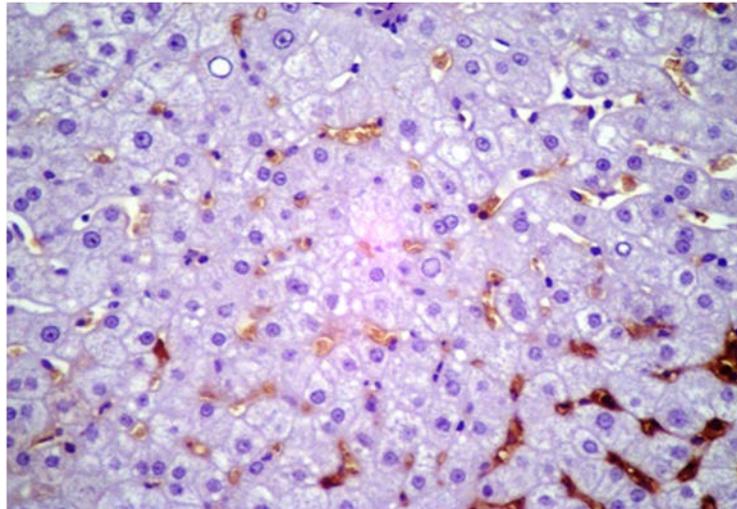
Silver impregnation revealed an increase of type III collagen amount from F2 to F4 liver samples (Fig.1).



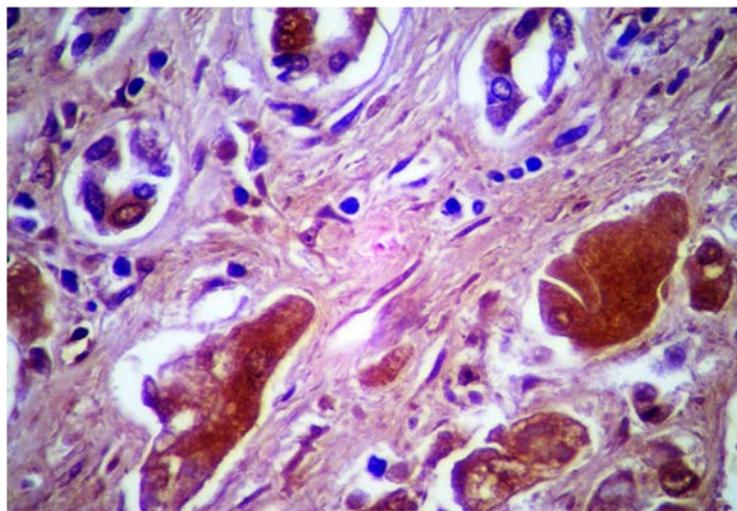
**Fig.1. Gomori silver impregnation – increased amount of type III collagen from F2 fibrosis (a, x100) to cirrhosis (b, x40)**

TGF- $\beta$ 1 positive reaction was observed in 94.45% of biopsies. The number of TGF- $\beta$ 1-positive cells was proportional to the severity of liver injury. We observed that liver sections with mild fibrosis revealed TGF- $\beta$ 1-positive cells only in endothelial cells of sinusoids and

occasionally in immune cells from the portal areas (Fig.2). With progression of liver damage, positive reaction for TGF- $\beta$ 1 expanded to disorganized hepatocytes located nearby collagen bundles and to cholangiocytes (Fig.3).



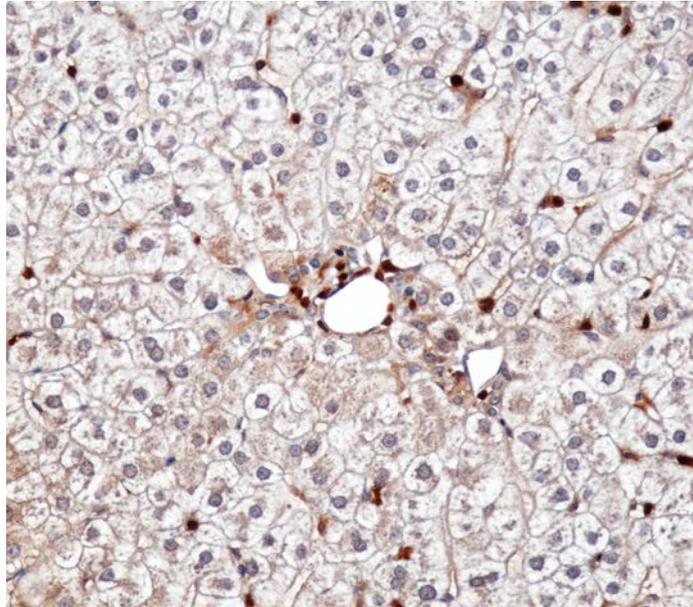
**Fig.2. TGF- $\beta$ 1 positive cells lining sinusoids (F2, x200)**



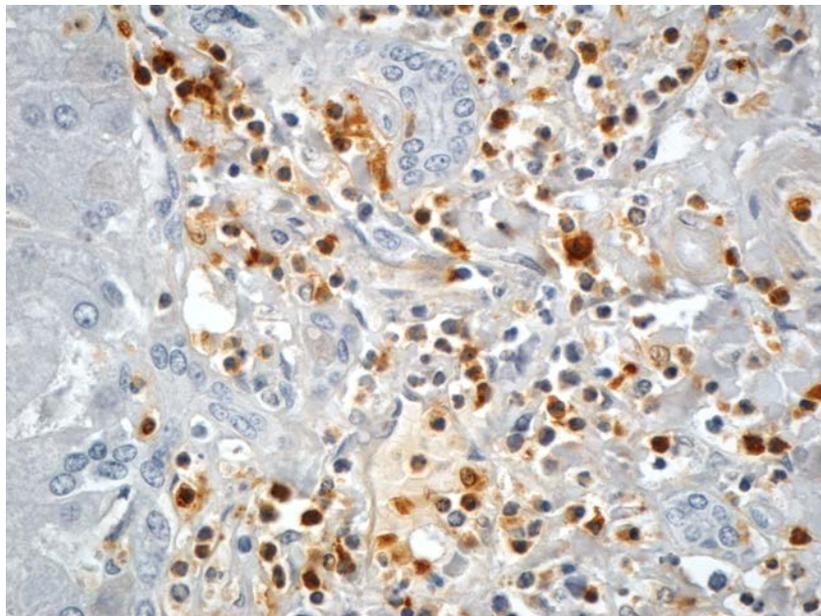
**Fig.3. Disorganized parenchyma, thick fibrotic septa with some TGF- $\beta$ 1 positive hepatocytes, macrophages and cells from the bile ducts (cirrhosis, x400)**

Analyzing the immunohistochemical expression of FSP-1, a heterogeneous distribution was observed among liver cells. Regardless the fibrosis degree, immune cells were constantly positive for FSP-1. In F2/F3 liver sections, FSP-1 positive cells were observed lining sinusoids (Fig.4), being probably Kupffer cells or endothelial cells. As

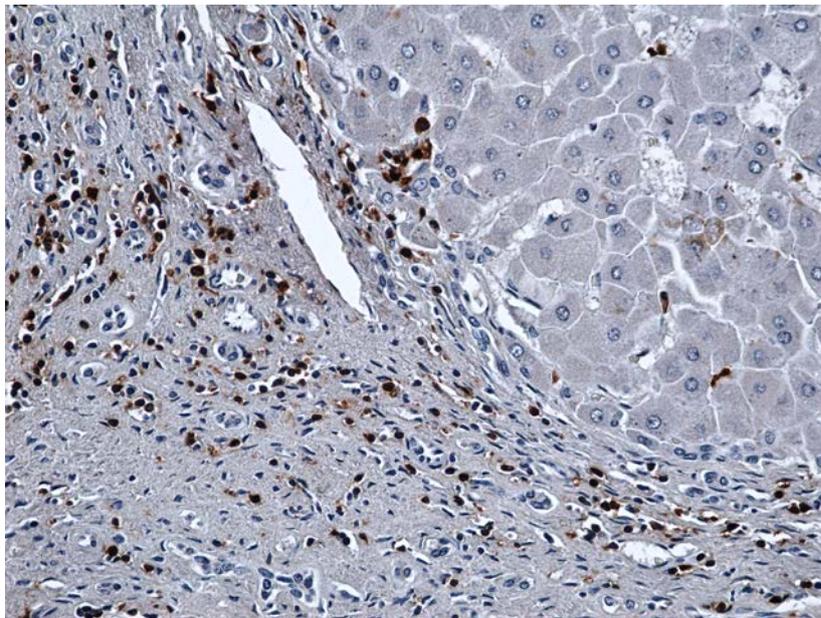
fibrosis progressed we observed an increase of FSP1-positive fibroblasts in F3/F4 liver sections (Fig.5) where FSP-1 positive cells could be noticed also in isolated cholangiocytes and in the vessel wall. Few hepatocytes also expressed FSP-1 protein (Fig.6). The number of FSP-1 positive immune cells was also increased, mainly in portal areas and along fibrotic septa.



**Fig.4. FSP-1 positive cells lining sinusoids, probably Kupffer cells or endothelial cells (F2, x200)**



**Fig.5. FSP1-positive fibroblasts, isolated cholangiocytes and cells from the vessel wall (F3, x400)**



**Fig.6. FSP-1 positive fibroblasts and immune cells invading fibrotic septa; few FSP-1 positive hepatocytes nearby fibrotic area (cirrhosis, x200)**

The quantified scores for TGF- $\beta$ 1 and FSP-1 expression corresponding to each stage of fibrosis are reported in Table 1. TGF- $\beta$ 1 was statistically highly significant only when comparing stages F2 to F4 while FSP-1 when comparing stages F3 to F4 ( $p < 0.001$ ). Pearson correlation between TGF- $\beta$ 1 and FSP-1 expression related to fibrosis stage revealed a strong positive correlation only for cirrhosis samples ( $r = 0.86$ ).

**Table 1. Scores for TGF- $\beta$ 1 and FSP-1 in various stages of HCV induced liver fibrosis**

Stage	No. of Cases	Score	
		TGF- $\beta$ 1*	FSP-1
2	8	1.63 $\pm$ 0.92	1.88 $\pm$ 0.83
3	4	2.53 $\pm$ 0.45	2.25 $\pm$ 0.5
4	6	3.34 $\pm$ 0.45	3.44 $\pm$ 0.48

\* Data are given as mean  $\pm$  SD

## Discussion

As we observed, progression of fibrosis and accumulation of type III collagen was associated with increased TGF- $\beta$ 1 expression and the presence of FSP-1 positive collagen producing cells.

The most important cytokine controlling collagen homeostasis in fibrotic diseases is TGF- $\beta$ , mainly its TGF- $\beta$ 1 isoform. The involvement of TGF- $\beta$ 1 in liver fibrosis is complex, this cytokine being implicated in the initiation or progression of fibrosis and thus

considered the best described inducer for EMT [13].

There is evidence that several types of adult liver cells may undergo EMT becoming MFs such as cholangiocytes, hepatocytes, HSCs [9, 14], portal fibroblasts and bone-marrow derived fibroblasts [8]. Cholangiocytes from small and medium sized bile-ducts transform into invasive fibroblasts during chronic liver disease as a response to local TGF- $\beta$  [15]. *In vivo* studies are still controversial showing either that hepatocytes cannot undergo EMT during liver fibrosis [16] or that TGF- $\beta$ 1 induced apoptosis and EMT of hepatocytes into fibroblasts or fibroblast like cells are possible [17].

On one hand, TGF- $\beta$ 1 could activate HSCs and induce their transformation into MFs and modulate the activity of MMPs and TIMPs, in this way controlling the ECM homeostasis [18]. On the other hand, TGF- $\beta$ 1 is produced by HSCs and exerts an autocrine control in HSCs activation and their involvement in progression of fibrosis during the disease development [19].

In normal liver, TGF- $\beta$ 1 mRNA was reported to be expressed in Kupffer cells, no expression being found in endothelial cells and hepatocytes [20]. The hepatocytes of normal liver contain TGF- $\beta$  but they don't synthesize this protein [21] which is taken up and further released in the environment [22].

In fibrotic liver, TGF- $\beta$ 1 mRNA was strongly expressed in all the sinusoidal cells. When compared to the level of expression in normal stellate cells, the level of TGF- $\beta$ 1 increased

12-fold in stellate cells from fibrotic livers, and 6-fold in endothelial cells while in Kupffer cells, the level of expression remained unchanged [20].

In our studies, we observed TGF- $\beta$ 1-positive cells in the periportal and perisinusoidal areas, precisely in the same zones where fibrosis started, as well as in some cells from the portal spaces for the F2 and F3 specimens and in isolated cells from the biliary ducts in cirrhosis samples. TGF- $\beta$ 1 protein was inconstantly present in hepatocytes, a chess table aspect being noticed for the liver parenchyma [18]. Most of the hepatocytes from these specimens were damaged, possibly apoptotic or necrotic cells able to release mature TGF- $\beta$ 1 and to stimulate its signaling pathways.

In liver fibrogenesis, TGF- $\beta$ 1 is believed to be involved in the synthesis and deposition of collagenous and non-collagenous ECM proteins like fibronectin, type I, III, and IV collagens via an autocrine/paracrine signaling but the detailed mechanism still remains unclear [23].

Production of fibronectin and type I/III collagen are markers of epithelium undergoing EMT in addition to loss of E-cadherin and *de novo* expression of FSP-1, vimentin and  $\alpha$ -SMA [24].

FSP-1, also named S100A4, a small protein that belongs to the S100 superfamily of calcium-binding proteins was discovered by Strutz et al., in 1995 in their attempt to find a marker specific for MFs [25]. These proteins don't exhibit enzymatic activity and it is considered that they are involved in controlling various biological functions of other proteins [12]. FSP-1 positive fibroblasts represent a class of fibroblasts present in many tissues [14, 26, 27].

In normal liver sections, FSP-1 showed no expression associated with epithelial biliary duct cells or the surrounding hepatic parenchyma, except for occasional expression in immune cells (monocytes, macrophages, neutrophil granulocytes, Kupffer cells) [15, 28].

As we observed in our selected specimens, FSP-1 displayed a heterogeneous distribution among liver cells. Immune cells were constantly positive for FSP-1 regardless the fibrosis degree while in samples with advanced stages of fibrosis fibroblasts, biliary duct cells and endothelial cells positive for FSP-1 increased. Hepatocytes located nearby fibrotic septa were inconstantly FSP-1 positive. Our results are in accordance with several reported data of *in vivo* studies which indicated that hepatocytes or cholangiocytes thought to undergo EMT express

FSP-1 [14, 15, 29]. Since human normal cells express FSP-1 and recent emerging data indicate only a subpopulation of macrophages positive for FSP-1 in liver fibrosis [30], the outcomes should be interpreted cautiously due to its low specificity.

## Conclusions

Nowadays it is well known that liver fibrogenic cells are heterogeneous in their origin. Besides activated HSCs, other liver cells able to undergo EMT seem to be involved in initiation and progression of fibrosis in chronic HCV infection. Future studies are expected to uncover the precise origin of collagen producing cells within the liver in order to target EMT events for new therapeutic approaches.

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DANIELA ELISE TACHE and Ș.G. FIRU equally contributed in the research and drafting of this paper.

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*Corresponding Author: Ana Marina Andrei, University of Medicine and Pharmacy of Craiova, Petru Rares Street, no.2-4, Craiova, Romania; e-mail: marinafusaru@yahoo.com*