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Phonotyping Transition of Infiltrating Hepatic Monocytes to Fibroblasts In CHC Patient's Induced Liver Cirrhosis

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Abstract

Monocytes differentiate into other cell types, including fibroblasts which infiltrate the damaged tissue and participate in the fibrotic processes. We aim to assess the possible contribution of circulating monocytes and infiltrating hepatic monocytes in hepatitis C virus (HCV)-induced expansion of the myofibroblastic pool resulting in liver fibrosis. 105 subjects with HCV-induced chronic liver disease were grouped into three groups according to METAVIR score. And assessed by Lab investigations, abdominal ultrasonography, CT and Ultrasound-guided liver biopsy for histopathological and immunohistochemical studies. Flow cytometric and immunohistochemical analysis of (circulating and tissue) monocyte markers using anti- (CD45, CD14, and CD16 and α-SMA). Determination of serum levels of α-SMA and TGF-β was also conducted using ELISA. CD14+, CD16+ and α-SMA+ Cells were upregulated with the increase in hepatitis and fibrosis grade activity ($p< 0.01$). CD45+cells showed higher expression in grades II&III fibrosis compared to grade I. however,CD45+ cells was not correlated with the stage of hepatic fibrosis. CD14+, CD16+ and α-SMA+ cells were significantly elevated in cirrhotic tissues compared to non-cirrhotic. A significant up regulation of CD14+ CD16+ monocytes observed in circulation with increased serum levels of α-SMA and TGF-β along with the down regulation of CD14+ monocytes which paralleled the severity of liver disease and the progression of liver fibrosis. Infiltrating hepatic monocytes may have undergone a phenotypical transition to fibroblasts. CD14, CD16, and α-SMA can serve as predictive biomarkers, and further measurement of their soluble forms can be used as a non-invasive tool for the diagnosis of progression of liver fibrosis.

Keywords: Liver cirrhosis; HCC and (CD14, CD16, CD45, TGF-β, and α-SMA).

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1. Introduction

Hepatitis C virus infection is considered one of the common deriving factors of hepatic fibrosis, and cirrhosis. [1] Fibrosis is characterized by extensive extracellular matrix (ECM) deposition, mostly collagen I as a result of repeated epithelial injury, and so resulting in the collection and activation of mesenchymal cells like fibroblasts and myofibroblasts. Furthermore, it promotes the invasion of inflammatory cells, such as macrophages [2]. Monocytes circulate and localize into damaged tissues following liver injury, where they develop into diverse myeloid cell types capable of phagocytosis, antiviral immunity, antigen presentation, immune suppression, and tissue healing [3]. Hepatic macrophages arise from two distinct sources; peripheral blood monocytes, which are recruited to the liver in case of injury through chemokine signals, and Kupffer cells which are self-renewing embryo-derived local macrophages [4]. Accumulating studies in murine models have shown that infiltration of monocyte into the liver is a key component in the pathological mechanism of chronic hepatic inflammation and fibrosis [5-9]. Under normal circumstances, fibroblasts play a key role in mediating ECM turnover. Additionally, they are found in all body organs' interstitial spaces. They generate a variety of chemokines and cytokines in response to tissue damage, including transforming growth factor-beta1 (TGF-1), tumor necrosis factor-alpha (TNF-), interleukin-1 beta (IL-1), and transforming growth factor-beta1 (TGF-1).[10-12] These

elements attract macrophages to inflamed regions and stimulate the development of fibroblasts into myofibroblasts, which synthesize and secrete a large amount of ECM components[13-15]. Apart from fibroblasts and myofibroblasts, it is becoming clear that monocyte-derived cells, like monocytes, macrophages, as well as fibrocytes contribute to fibrosis pathogenesis by additional mechanisms that widen the pool of myofibroblasts in the fibrosing liver [9,16-19]. TGF-β is secreted by various cell types after acute and chronic hepatic damage; it is triggered by deposits in the ECM. TGF-β promotes the transition of hepatic stellate cells (HSCs) into myofibroblasts. Myofibroblasts exhibit stellate-like morphology. Also, they up-regulate the expression of non-muscle myosin, fibronectin, *and alpha-smooth* muscle actin (α-SMA)[20]. So, increased numbers of myofibroblasts are associated with the progression of the disease in subjects with liver fibrosis [21]. Consequently, myofibroblasts draw attention as a potential anti-fibrotic therapy, which aims to eliminate the driving factors of activated hepatic myofibroblasts [22,23].
This study evaluates the possible contributing role of the peripheral blood monocytes and infiltrating hepatic monocytes in HCV patients in the expansion of the myofibroblastic pool inducing liver fibrosis.

2. Subjects and Methods

2.1 Ethical approval

The study protocol and the suggested informed consent were approved by the Institutional Review Board (IRB) of Theodor Bilharz Research Institute. An informed consent was obtained in a written form from each participant in the study in accordance with the 1975 Declaration of Helsinki's standards.

2.2 Subjects

A cohort of 105 subjects with HCV-related different liver diseases was recruited from Theodor Bilharz Research Institute, Hepato-Gastroenterology Department, (TBRI), Giza, Egypt). Patients were classified into three groups according to METAVIR score for the staging of hepatic fibrosis and scoring of necro-inflammatory activity. [24]. CHC patients with stage 0 fibrosis (control group): It comprised 15 cases. CHC without cirrhosis (F1, F2, and F3): It comprised 66 cases. CHC with cirrhosis (F4): It comprised 24 cases. Exclusion criteria were etiologies for CLD other than HCV. Patients were subjected to the followings; clinical examination, laboratory investigations including urine analysis, serologic diagnosis of schistosomiasis, stool analysis, INR, complete liver function tests, hepatitis markers, complete blood count, abdominal ultrasound and CT as well as ultrasound-guided liver biopsy. Hepatitis B markers, comprising HBsAg, HB core Ab, HBe antibodies, and the hepatitis Be antigen were determined using commercially available enzyme immunoassay kits (Abbott Laboratories; North Chicago, Illinois). Anti-HCV Abs were identified using a Murex enzyme immunoassay kit (Murex anti-HCV, Version V; Murex Diagnostics; Dartford, England). Real-time PCR using the Amplicor test was used to identify the presence of HCV-RNA in patients' serum (Roche Diagnostic Systems; Meylan, France). Alpha-fetoprotein (AFP) levels in serum were determined using a Eurogenetics enzyme immunoassay kit (Eurogenetics, NV; Tessenderlo, Belgium).

2.3 Flow cytometric analysis

The proportions of various subsets of circulating monocytes in the several groups investigated were determined by immunophenotyping with flow cytometric analysis using mouse anti-human (CD45/ECD, CD14/FITC, and CD16/PE, Beckman Coulter, Marseille, France). Ten microliters of the suitable conjugate were added to 100LμL of peripheral blood from each sample and incubated in the dark for 15 minutes at room temperature. To remove erythrocytes, 500 μL of red blood cell lysing buffer (ebioscience, USA) was added for 10 minutes. Leukocytes were washed twice in phosphate buffer saline following incubation (PBS). The cells were then re-suspended in 300 μL PBS. Cells were investigated using a Beckman Coulter apparatus equipped for four-color flow cytometry (Coulter, Coultronic, Margency, France). The Kaluza analysis program was used to examine the data (Beckman Coulter Inc., USA).

2.4 Immunohistochemical (IHC) technique

De-paraffinization and rehydration of paraffin segments from hepatic lesions were done. To inhibit endogenous peroxidases, methanol with 3% hydrogen peroxide was employed. The sections were microwaved in citrate buffer (pH 6.0) to regain the antigens. They were then incubated overnight at 4oC in a humid chamber with the primary antibodies: Anti-CD14 (monoclonal antibody, Beckman Coulter, clone RMO52), Anti-human α-SMA (monoclonal mouse IgG, catalog number: MAB1420), Anti-human CD45 purified (eBioscience, catalog number: 14-9457), and Anti-CD16 (Novus Biologicals, NB100-64346) in an optimal dilution of 1:100-1:400, with an application of ultravision detection system. The addition of a 3,3'diaminobenzidine tetrahydrochloride (DAB) substrate chromogen solution resulted in antigen localization (Universal Detection Kit, Dako, Denmark). Finally, hematoxylin was used to counterstain the slides, which were then dehydrated in alcohol and mounted. Each setting had both positive and negative control slides. The negative control sequences contained the same processing of the hepatic tissue as the preceding sequences, but with the addition of non-immune immunoglobulin G antibodies in place of the primary antibodies (IgG; DAKO, Glostrup, Copenhagen, Denmark).

2.5 Interpretation of immunostaining and scoring analysis

Two pathologists independently measured immunohistochemistry analysis of liver tissue sections. A light microscope was used to examine the sections (Scope A1, Axio, Zeiss, Germany). A microscope camera was used to capture photomicrographs (AxioCam, MRc5, Zeiss, Germany).

2.6 Circulating Human α-Smooth Muscle Actin (α-SMA) and Transforming Growth Factor Beta (TGF- β)

Identification of myofibroblasts activation was performed by quantitative determination of circulating levels of TGF-β and α-SMA using Immunoassay ELISA kit (CUSABIO, BIOTECH Co., Ltd., PRC).

2.7 Statistical analysis

A data analysis was conducted using statistical SPSS version 18.0 for windows (SPSS Inc., Chicago, IL).

Diagnostic parameters of subjects were compared using the sample (t) test, a p-value less than 0.05 was considered statistically significant.

3. Results and discussion

The clinical and laboratory data for all studied cases were presented in (Table 1).

3.1 Flow cytometric assessment of CD14+ and CD16+monocytes

The surface expression of CD14 and CD16 was determined on gated CD45+ monocytes.

3.2 Flow cytometric assessment of CD14+classical monocytes

The analyzed data have shown a statistically significant decrease ($p< 0.01$) in the CD14+ classical monocytes in patients with CHC without cirrhosis and CHC with cirrhosis compared to the control group. Data also revealed a marked progressive decrease ($p<0.01$) in the percentage of CD14+ classical monocytes in patients with CHC without cirrhosis compared to CHC with cirrhosis, and the marked reduction ($p<0.01$) of this subset was mostly noticed among patients with CHC with cirrhosis (Fig.1, Table 2).

3.3 Flow cytometric assessment of CD14+CD16+non-classical monocytes

Data revealed a marked up-regulation of the non-classical monocytes in different groups of patients. A marked significant increase ($p< 0.01$) in the percentage of the CD14+CD16+ non-classical monocytes was noticed in patients with CHC without cirrhosis and CHC with cirrhosis compared to the control group. Patients with cirrhotic livers had the highest up-regulation ($p< 0.01$) of the non-classical monocytes (Fig.1, Table 2).

3.4 Immunohistochemical expression of CD14, CD16, CD45, and α-SMA in liver tissues

Our results showed a gradual increase in cells expressing α-SMA, CD14, and CD16 ($p< 0.01$) with increasing grade of hepatitis activity. CD45+ cells showed significantly higher expression ($p< 0.01$) in grades II & III hepatitis compared to grade I (Fig 2, Table 3). In this study, CD45 showed positive expression by many mononuclear cells. The percentage of the CD45+ hepatic mononuclear cells was found to be upregulated in CHC with cirrhosis compared to that with CHC without cirrhosis, but the results were comparable. The expression of CD45 showed a non-significant correlation with the stage of hepatic fibrosis (Fig 3, 5; Table 4). A positive expression of CD14 appears in few hepatic mononuclear cells and on the surface of adjacent hepatocytes, and this expression was significantly increased ($p< 0.01$) with the progression of liver fibrosis stages (Fig.3, 6). The percent of hepatic mononuclear cells that express CD16 (Fig. 7) and α-SMA (Fig. 8) was significantly increased ($p< 0.01$) in all groups compared to the control group, and this up-regulation was parallel to the severity of the disease.

3.5 Circulating levels of TGF- β

The results of patients with CHC without cirrhosis showed a marked increase ($p<0.01$) in the circulating levels of TGF- β compared to healthy subjects. Additionally, a progressive marked increase in the levels of TGF-β in patients with cirrhotic livers compared to patients with CHC without cirrhosis and controls (Fig. 4, Table 5).

3.6 Circulating levels of α-SMA

Our data also revealed a significant increase in circulating levels of α-SMA in patients with CHC without cirrhosis ($p<0.05$) and cirrhotic ($p<0.01$) patients compared to healthy subjects. The increase in circulating levels of α-SMA was mostly encountered among cirrhotic patients (Fig. 4, Table 5). A downregulation of the percentage of CD14+ classical monocytes in this study in all studied subjects groups compared to the controls was observed and this reduction was found to match the progression of the disease and the stages of liver fibrosis. These findings are in agreement with those of Liaskou *et al.* [27] and El Bassiouni *et al.* [9] who reported that the classical monocytes constitute approximately 80% of peripheral blood monocytes. Their percentage was significantly down-regulated in all stages of liver fibrosis compared to controls. Moreover, the marked decrease of their percentage was mostly noticed among cirrhotic patients. This finding agrees with those of others who demonstrated a strong shift towards the non-classical monocytes in patients with chronic liver disease, especially those with established cirrhosis. It suggested that this downregulation may be due to the differentiation of some classical monocytes into non-classical monocytes [9, 25, 28] or to the recruitment of classical monocytes into the injured liver in response to chemokine MCP-1/CCR2 pathway. [9, 29] Our immunohistochemical assessment of the CD14+ hepatic mononuclear cells confirmed these results as we found that the downregulation of peripheral blood classical monocytes (CD14+) was associated with up-regulation in the proportion of CD14+ hepatic mononuclear cells that parallel the severity of the disease. These results were in agreement with those of Liaskou *et al.* [27] and Mukherjee *et al.* [30] who showed that the percentage of CD14+ cells was increased in patients with chronic inflammatory and fibrotic liver disease, and this up-regulation may be due to their recruitment from the peripheral blood into the injured liver in response to chemokine MCP-1/CCR2 pathway. [9,29] Moreover, our results showing a progressive increase in cells expressing CD14 with an increasing grade of hepatitis activity. This was in accordance with those of Ogawa *et al.* [31] who showed that the serum sCD14 levels are strongly associated with the grade of liver inflammation and reflecting the important role of CD14+ hepatic mononuclear cells in inflammation. A marked progressive up-regulation in the percentage of CD14+CD16+non-classical monocytes and CD16+ hepatic mononuclear cells was noticed in all studied groups compared to the control group. This up-regulation was noticed mostly encountered among patients with cirrhotic livers. Our findings agree with those of Mukherjee *et al.* [30] who suggested that the percentage of non-classical monocyte subset increases during inflammatory diseases and also were consistent with those of Liaskou *et al.* [27] who found that the proportion of non-classical monocytes were increased in the liver tissue in patients with chronic inflammatory and fibrotic liver disease compared to controls.

Table 1: Clinical and laboratory Data of all studied cases

Parameters			Controls(n=15)	CHC without cirrhosis (n=66)	CHC with cirrhosis(n=24)
Age			30-50	33-62	36-70
Male/Female			6/9	39/27	19/5
Abdominal Ultrasonography	***Liver***	Normal	ND	57	16
		+		9	8
	Spleen	Normal	ND	61	18
		+		5	4
		++		0	2
	Ascites			0	2
Laboratory investigation	**AST(IU/l)**		17.5 ±4.3	38.88 ±12.5	24.36 ±4.2
	ALT(IU/l)		15.8 ±5	46.5 ±15.4	34.76±8.65
	Total Billirubin (mg/dl)		0.53±0.25	1.9 ±0.86	4.9±1.89
	Albumin (g/dl)		4.2 ±0.5	3.35 ±0.46	3.26 ±0.72
	PT(sec)		12.13±0.17	16.78±3.86	26.37 ±1.3
	INR		1.03±0.02	1.3±0.09	1.68±0.53
Hepatitis Markers	***HBs Ag***		-ve	-ve	-ve
	HCV Ag		-ve	+ve	+ve
	HCV Ab		-+ve	+ve	+ve

Data are represented as mean ± SD.

Ab, antibody; Ag, antigen; ALT, alanine aminotransferase; AST, aspartate amino transferase; dl, deciliter; g; gram; HBs, hepatitis B virus; HCV, hepatitis C virus; INR, international normalized ratio; IU/l, international unit/liter; mg, milligram; ND, not done; -ve , negative; positive, +ve. PT, prothrombin time.

Table 2: The percent of CD14+ and CD16 + CD45 + peripheral blood monocytes by flow cytometry in all studied group

Groups	Control (n=15)	CHC without cirrhosis (n=66)	CHC with cirrhosis (n=24)
Classical monocytes (CD14+)	88.1±0.27	63.78±0.2a	5.9±0.04ab
Non-Classical monocytes (CD14+ CD16+)	12.08±0.25	36.6±0.2a	90.1±0.07ab

Data are represented as mean ± SE.
ap<0.01: control vs other groups.
bp<0.01: CHC without cirrhosis vs CHC with cirrhosis

Table 3: CD14+ CD16 + CD45 + α-SMA +infiltrating monocytes in active hepatitis in all studied groups

Groups	Active Hepatitis	
	Low grade (GI, GII)	**High grade (GIII, GIV)**
CD14+	**29.1667±3.16**	**38.6538±2.4a**
CD16+	**1.3333±0.12**	**16.2692±2.6a**
CD45+	**2.3333±0.18**	**58.2692±2.2a**
ASMA+	**1.1667±0.17**	**13.6538±1.8a**

Data are represented as mean ± SE.
ap<0.01 high grade vs low grade

Table 4: CD14+ CD16 + CD45 + α-SMA+ infiltrating monocytes by IHC in all studied groups

Groups	Control (n=15)	CHC without cirrhosis (n=66)	CHC with cirrhosis (n=24)	
CD14+	**14.0±1.4**	**36.88±2.25a**	**49.17±.3.8ab**	
CD16+	**1.60±046**	**13.47±3.63a**	**20.50±3.19ab**	
CD45+	**63.40±6.07**	**47.78±3.34**	**52.50±4.9**	
ASMA+	**0.80±0.1**	**11.31±0.90a**	**30.83±5.21ab**	

Data are represented as mean ± SE.
ap<0.01: control vs other groups.
bp<0.01: CHC without cirrhosis vs CHC with cirrhosis.

Table 5: Circulating levels of TGF-βandα-SMA (ng/ml) by ELISA in all studied groups

Groups	Control (n=15)	CHC without cirrhosis (n=66)	CHC with cirrhosis (n=24)
α-SMA	2.44±0.02	22.03±0.3 a	61.8±0.27 ab
TGF-β	2.1±0.02	5.32±0.06 a	20.7±0.3 ab

Data are represented as mean ± SE.

ap<0.01: control vs other groups.
bp<0.01: CHC without cirrhosis vs CHC with cirrhosis

Our findings also confirm the results of Zimmermann *et al.* [29] and El Bassiouni *et al.*[9] who demonstrated the expansion of non-classical monocytes in the circulation and livers of patients with chronic liver disease-upon disease progression and suggested their functional contribution to the perpetuation of intrahepatic inflammation and profibrogenic HSC activation in liver cirrhosis. Up-regulation of circulating and liver tissue non-classical monocytes subset was observed in patients with advanced fibrosis implicating a pro-inflammatory and pro-fibrogenic role of non-classical monocytes in advanced fibrosis. [29] Non-classical monocytes play a critical role in hepatic inflammation due to their ability to phagocytose foreign materials, the present antigen to T cells, and generate various cytokines, including TNF-α, IL-1, and IL-6 [32,33] Additionally, non-classical monocytes stimulated primary human HSCs, which can produce various chemokines to attract monocytes and trans-differentiate into myofibroblasts, the main event in hepatic fibrosis [34,35] As a result, it has been hypothesized that non-classical monocytes are critical regulators in the development of chronic liver disease-induced liver fibrosis. [8,9,27,33,36-37].

CD45, commonly known as leukocyte antigen, is one of the highly expressed leukocyte cell surface glycoproteins. It is only expressed by hematopoietic cells. [38] Our results revealed that the CD45+ hepatic mononuclear cells showed significantly higher expression in grades II & III hepatitis than grade I, which may refer to the presence of one or more types of inflammations associated with the increased severity of the disease. In contrast, the percentage of the CD45+ hepatic mononuclear cells was found to be upregulated in CHC with cirrhosis compared to that with CHC without cirrhosis, but the results were comparable. The expression of CD45 showed a non-significant correlation with the stage of hepatic fibrosis. This result was in contrast with those of De Vito *et al.* [39] They discovered that the proportions of CD3, CD45, and CD163 liver-resident cells changed in individuals with more severe histological activity and were associated with the degree of chronic liver damage, as shown by the presence of fibrosis.
Profibrotic mediators like TGF-1 and platelet-derived growth factors are secreted by macrophages. They stimulate the proliferation of fibroblasts, which develop into myofibroblasts [40,41]. There was a marked increase in the circulating levels of TGF- β and α-SMA in different groups studied, namely patients with CHC without cirrhosis compared to healthy subjects with a marked progressive increase in their levels in patients with cirrhotic livers compared to patients with CHC without cirrhosis and controls. The increase in serum levels of α-SMA was in conjunction with up-regulation of the expression of α-SMA+ hepatic mononuclear cells that were found to be parallel with the progression of the disease. These findings are in harmony with other studies [41,42] who found that the percentage of α-SMA+ HSCs was significantly higher in HBV and HCV cirrhosis groups. They also demonstrated that HSCs activation was positively correlated to both the percentage of α-SMA+ HSCs and the extent of fibrosis which found to be in agreement with that of Dong *et al* [43] who said that the amount of α-SMA in biliary atresia was positively correlated with liver fibrosis scores. Upon chronic injury occurrence, mobilization of lymphocytes and monocytes takes place, thus supporting the persistence of an inflammatory response. Macrophages produce profibrotic mediators, among them, TGF-β, which is responsible for

activation, and trans-differentiation of HSCs to a myofibroblast phenotype; this may explain the marked upregulation of TGF-βlevels present in this study. They also trans-differentiate into myofibroblasts and smooth muscle-like cells during the repair process [44, 45]. Recent data suggests that many of TGF-'s pathological effects may be attributed to its capacity to mediate cell plasticity, which ultimately results in phenotypic changes in many liver cell populations. Cell plasticity is described as the inter-conversion between distinct stem cell populations, the dedifferentiation, the activation of facultative stem cells, and trans-differentiation, or phenotypic transition of differentiated cells within a tissue. [46] Additionally, inflammatory macrophages have been shown to upregulate fibroblast and myofibroblast markers such as Col I and α -SMA in damaged regions. [47] The presence of α-SMA+ hepatic mononuclear cells suggesting that the infiltrating hepatic monocytes may have undergone a phenotypical transition to fibroblasts that can lead to an increase of the myofibroblastic pool in liver fibrosis and clarified the important role of infiltrating of α-SMA+ cells in the initiation and progression of liver fibrosis, these findings were in accordance with Yang et al. [48] discovery that adiponectin promoted the production of α-SMA and extracellular matrix proteins in cultured bone marrow-derived monocytes in a time- and dose-dependent manner. The mechanical force generated by myofibroblasts, dependent on the neo expression of α-SMA in these cells' stress fibers, governs critical tissue remodeling processes like cytokine synthesis and ECM component manufacturing. [49] In chronic hepatitis C, oxidative stress resulting from hepatic and mitochondrial damage and enhanced lipid metabolism leads to the development of fibrosis via HSC activation and production of α-SMA. [50, 51] The increased serum α-SMA levels and α-SMA+ hepatic infiltrating monocytes suggest that hepatic infiltrating monocytes may be activated early in HCV-mediated injury, recruited to the site of injury via the chemokine MCP-1/CCR2 pathway in response to inflammation and lipid metabolism, and may transdifferentiate into myofibroblasts, the primary event in hepatic fibrosis. Additionally, elevated levels of α -SMA may be used as an early biomarker of hepatic fibrogenesis and as a measure of treatment effectiveness.

4. conclusion

Monocytes are active members in the pathogenesis of liver injury. They may potentially induce matrix production, which may initiate the inflammatory and immunological mechanisms in liver fibrosis. Additionally, in individuals with chronic hepatitis C virus, upregulation of monocyte tissue markers may play a crucial role in magnifying these inflammatory and immunological processes. In these individuals, gentle circulation in the liver results in prolonged exposure of monocytes to inflammatory mediators in the liver microcirculation, activating monocytes and increasing tissue factor expression, therefore enhancing hepatic fibrogenesis. Our findings showing monocytes undergo phenotypic conversion to fibroblasts and the increased positivity of both monocytic and fibroblastic markers in cirrhosis further establish the involvement of monocytes in the development of HCV-induced liver fibrosis. CD14, CD16, and α-SMA can serve as a predictive biomarker, and further measurement of their soluble form can be used as a non-invasive tool for the diagnosis of progression of liver disease and development of liver fibrosis. The preliminary clinical findings suggest that comprehending and interpreting mononuclear cell biology during liver fibrosis may result in the development of new anti-fibrotic treatments.

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