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Differential Recruitment of Monocytes Subsets in Chronic Hepatitis C Patients

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Abstract
Background: Collagen producing myofibroblast activation of is critical for pathogenesis of liver fibrosis.
Aim of the work: To study the expansion of peripheral monocyte subsets in HCV patients.
Subjects and Methods: Sixty HCV patients were classified according to METAVIR score into 4 stages of liver fibrosis, 15 age and sex-matched controls were include. Flowcytometric analysis of peripheral blood monocytes subsets and CCR2^{+ve} cells was carried out using monoclonal anti-CD45, anti-CD14, anti-CD16, anti-collagen type I and anti-CCR2antibodies. MCP-1 and SAP levels were assessed using ELISA.
Results and Conclusions: A down regulation (p< 0.01) in the classical monocytes subset and an up regulation (p< 0.01) in both the non-classical monocytes and monocytes producing collagen subsets were notice in HCV patients compared to controls. A marked increase (p< 0.01) in the levels of MCP-1 and monocytes expressing CCR2 with a significant decrease (p< 0.01) in SAP levels, which paralleled the progression of liver fibrosis, were also noticed. MCP-1 and CCR2 may trigger monocytes recruitment to the injured liver promoting the development of collagen type I producing monocytes. The shift of classical monocyte subset towards the non-classical and collagen producing subsets may be present a predictive non-invasive biomarkers for progress of liver fibrosis.

Key Words: HCV, liver fibrosis, monocytes, CCR2, MCP-1, SAP.

INTRODUCTION

Hepatitis C virus (HCV) infection, a major health problem, is one of the main causes of liver diseases worldwide. The progress of the diseases is quite different, in the spectrum from acute hepatitis, through liver cirrhosis to hepatocellular carcinoma (1). A variety of phagocytes and cells with the typical characteristics of endothelial cells and fibroblasts were differentiated from committed precursors circulating monocytes (2-3). The extent of cell surface expression of CD14 and CD16 commonly defined and discriminated these cells, their associated differences in function and phenotype depend on the intensity of expression of these markers (4). Monocyte subsets show fundamentally different patterns in their migratory behaviour according to a distinct pattern of chemokine receptors expressed on their surface, (5). Monocyte emigration from the bone marrow and monocyte homing critically depend on C-C chemokine receptor (CCR2) (6, 7). Circulating peripheral blood monocytes comprise two major subsets, a classical monocytes ($CD14^{++}CD16^{-}$) and a non-classical monocytes ($CD14^{+}CD16^{++}$) subsets (8). A population of cells described as collagen-producing

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monocytes $CD45^{+}CD14^{+}$ $ColI^{+}$ subset is observed in the peripheral blood of fibrotic diseases patients (9, 10).
We aimed to assess the proportion of circulating monocyte subsets with hematopoietic progenitor origin in peripheral blood of patients with HCV induced chronic liver disease.

PATIENTS AND METHODS

Patients admitted to the Gastroenterology and Hepatology Departement, Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, were included in the present study. Seventy-five individuals were included, 60 patients with HCV induced chronic liver disease and 15 healthy age and sex-matched individuals served as normal controls. METAVIR score for hepatic activity index was used for scoring of necroinflammatory activity in patients with chronic hepatitis. Accordingly, studied cases were classified into 4 stages of fibrosis, 15 cases each; patients with stages F1, F2, F3 and F4.

The medical procedures used were approved by the Local Ethics Committee of Theodor Bilharz Research Institute (TBRI), Imbaba, Giza. A written informed consent was obtained from each participant prior to the medical examination. Patients were subjected to thorough clinical examination, laboratory investigations including urine and stool analysis, liver function tests, CBC, and serologic diagnosis of schistosomiasis and hepatitis markers and abdominal ultrasonography.

Liver function tests were carried out using commercially available kits. Hepatitis surface antigen and anti-HBs antibodies, total and IgM class antibodies against hepatitis B core antigen, hepatitis Be antigen, and anti-HBe antibodies, were tested using commercially available enzyme immunoassay kits (Abbott Laboratories; North Chicago, Illinois). Circulating anti-HCV antibodies were detected using Murex enzyme immunoassay kit (Murex anti-HCV, Version V; Murex Diagnostics; Dartford, England). The Amplicor test by real-time PCR was used to detect HCV-RNA in patient's sera (Roche Diagnostic Systems; Meylan, France). Controls had liver function tests values within the normal range and were serologically free from hepatitis B andr C viruses. Exclusion criteria include any parasitological, serological, histopathological, or ultrasonographic findings indicative of chronic viral diseases other than HCV, nonalcoholic steatohepatitis, autoimmune hepatitis, biliary disorders, malignancies, bacterial infection, *Schistosoma* infection.

Flow cytomertic analysis

Assessment of percentage of circulating peripheral blood monocyte subsets of hematopoietic originin different groups studied was carried out by immunophenotype characterization flow cytometric (EPICS® XL-MCL, Brea, CA, USA) analysis using fluorochrome labeled mouse anti-human monoclonal antibodies CD45 conjugated with ECD, CD14 conjugated with FITC, CD16 conjugated with PE (Beckman Coulter, Marseille, France) and purified type I collagen (1ry antibody) (Bio-Rad, Germany), and goat anti-mouse monoclonal antibody conjugated with PerCP (2ry antibody) (JacksonimmunoResearch, Baltimore, USA) as follow: for each case 2 test tubes with EDTA were prepared and 100 µl of fresh blood were dispensed. The 1st tube was set as the control for the procedure with no monoclonal antibody (mAb) to adjust the auto fluorescence region. Ten µl of the appropriate mAb (ECD-labeled anti CD45, FITC-labeled anti CD14 and PE- labeled anti CD16) were added to a 2nd tube, mixed gently and incubated in the dark, at room temperature, for 20 minutes. To each test tubes 25µl of PerFix-nc Buffer 1 (Fixative Reagent) (Beckman Coulter, Marseille, France) was added and immediately mixed gently and similarly incubated for 30 minutes, then 300µl of PerFix-nc Buffer 2 (Permeabilization Reagent) (Beckman Coulter, Marseille, France) was

added and immediately mixed well. In the 2nd test tube, 10 µl of primary mAb for collagen type I was added, mixed gently and similarly incubated for 30 minutes, then 10 µl of goat anti-mouse MAb (2ry antibody) for collagen type I 1ry antibody labeled with PerCP was added and mixed gently and similarly incubated for 30 minutes. Finally, 3 ml of pre-diluted PerFix-nc Buffer 3 (Final 10x Reagent) (Beckman Coulter, Marseille, France) was added and immediately mixed gently. The surface expression of CD14 & CD16 and detection of intracellular protein collagen type I of peripheral blood monocytes population were detected on gated CD45+ cells by flow cytometric analysis of peripheral blood monocytes in different groups of patients with chronic HCV related liver fibrosis. A multicolor analysis of immunofluorescence samples was done using a prism. A cell is either positive or negative for each of 2, 3 or 4 cell surface markers and each phenotype reflect a particular combination The Prism parameter permits displaying the percentages of all the phenotypic populations in a single histogram. Identification of monocytes activation in different groups studied was performed by assessment of the expression of CCR2 receptor by flowcytometric analysis using fluorochrome labeled rabbit anti-human polyclonal antibodies CCR2 conjugated with FITC (Novusbio LLC, Colorado, USA) as follow: In each of 2 test tubes prepared with EDTA, 50 µl of fresh blood were dispensed, the 1st tube is the control for the procedure with no MAb in order to adjust the auto fluorescence region. Ten µl of the appropriate polyclonal antibody (FITC-labeled anti-CCR2) were added to a 2nd tube, mixed gently and incubated in the dark, at room temperature, for 20 minutes. Five hundreds µl of RBCs lysis buffer (eBioscience, San Diego, CA, USA) were added to each tube for lysis of RBCs and were mixed gently then incubated at room temperature, for 10 minutes. The sample tubes (the 2nd tube) were then introduced and processed, the surface expression of CCR2+ve cells was detected on gated monocytes by flow cytometric analysis of peripheral blood monocytes in different groups of patients with chronic hepatitis C related liver fibrosis.

Enzyme linked immunosorbent assay (ELISA)

Quantitative determination of serum levels of monocytes chemoattractant protein-1 (MCP-1) (eBioscience, North America) and serum amyloid protein (SAP) (Assaypro LLC, Missouri, USA) was conducted using ELISA. The procedures were performed according to manufacturer's instructions. To distinguishing between false positive and negative results, the optimal cutoff point, a point at which the sum of the sensitivity and specificity values was highest, was determined using a receiver operating characteristic (ROC) curve analysis on ELISA results. The area under the ROC curve (AUC), a measure of the test accuracy, was calculated.

Statistical analysis

Data analyses 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.

RESULTS

Clinicolaboratory data (Table 1) in different groups studied are shown. The surface expression of CD14 & CD16 and detection of intracellular protein collagen type I of peripheral blood monocytes population were detected on gated CD45+ cells, by flow cytometric analysis of peripheral blood monocytes in different groups of patients with chronic hepatitis C related liver fibrosis. By using flow cytometry prism which reveals the percentage of different peripheral blood monocytes subsets that express $CD45^+$ $CD14^+$ $CD16^+$ and $COLI^+$ (Fig. 1), different combinations of peripheral blood monocytes surface markers and intracellular protein were noticed denoting the presence of different monocyte

subsets in the studied groups. Three monocyte subsets (Table 2) were detected in healthy subjects and patients with chronic hepatitis C.

Table 1: The clinicolaboratory data of healthy subjects and patients with HCV related chronic liver disease with different stages of fibrosis.

Groups			Controls (n=15)	Liver Fibrosis			
				F1 (n=15)	F2 (n=15)	F3 (n=15)	F4 (n=15)
Age			25-50	30-50	33-62	30-70	36-62
Sex	***Male***		6	8	9	11	11
	Female		9	7	6	4	4
Abdominal Ultrasonography	***Liver***	**Normal**	ND	15	12	12	10
		+		0	3	3	5
	Spleen	**Normal**	ND	15	12	12	10
		+		0	0	3	2
		++		0	0	0	2
	Ascites			0	0	0	1
Echogenic Pattern of the Liver	***Bright***		***ND***	15	13	11	10
	Diffuse		***ND***	0	2	4	3
	Coarse		***ND***	0	0	0	2
WBCs ($x10^9$/l)			6.72±1.70	4.65±2.34	8.19±1.77	8.06±2.57	5.49±2.03
***Mono* (%)**			5.33±1.59	6.33±1.88	5.33±1.71	5.47±2.07	6.14±2.214
Platelets ($x10^9$/l)			264.40±58.86	160.13±38.36	222.33±65.01	130.00±54.84	139.79±82.84
Total Protiens (g/dl)			7.20±0.40	7.00±0.45	7.10±0.51	7.21±0.1	7.33±0.87
Albumin (g/dl)			3.21±0.17	3.27±0.42	3.56±70.45	3.28±0.56	3.29±0.77
Total Billirubin (mg/dl)			0.50±0.20	1.00±0.40	0.99±0.46	2.90±1.70	5.10±2.11
AST(IU/l)			14.2±04.71	37.47±15.53	33.67±13.10	45.47±9.73	21.36±6.56
ALT(IU/l)			15.0±05.00	44.80±15.03	39.73±15.75	54.80±14.51	32.86±9.95
PT(sec)			11.12±0.16	11.38±0.36	12.53±1.02	13.56±1.71	15.74±3.77
INR			1.01±0.01	1.04±0.04	1.14±0.10	1.24±0.16	1.44±0.35

Flowcytometric analysis showed that the percentage of classical monocytes ($CD45^+$ $CD14^+$ $CD16^-ColI^-$) subset comprise about 80-90% of total peripheral blood monocytes in healthy subjects. This percentage, found to be down regulated ($p< 0.01$) in patients groups compared to healthy subjects, was mostly noticed in stage F4 fibrosis. Simultaneously, a marked progressive up-regulation ($p< 0.01$) in the percentage of non-classical monocytes ($CD45^+$ $CD14^+$ $CD16^+ColI^-$) subset was noticed in patients with different stages of liver fibrosis compared to healthy subjects. Concomitantly, a third subset of peripheral blood monocytes named monocyte producing collagen ($CD45^+$ $CD14^+$ $CD16^+ColI^+$) subset was revealed and found to be also up-regulated ($p< 0.01$) in all studied groups of HCV related chronic liver disease patients compared to healthy subjects.The marked ($p<0.01$) down regulation of circulating classical subset and up-regulation of circulating non-classical and collagen producing monocyte subsets in the HCV related chronic liver disease patients paralleled the severity of the disease and the stage of liver fibrosis.

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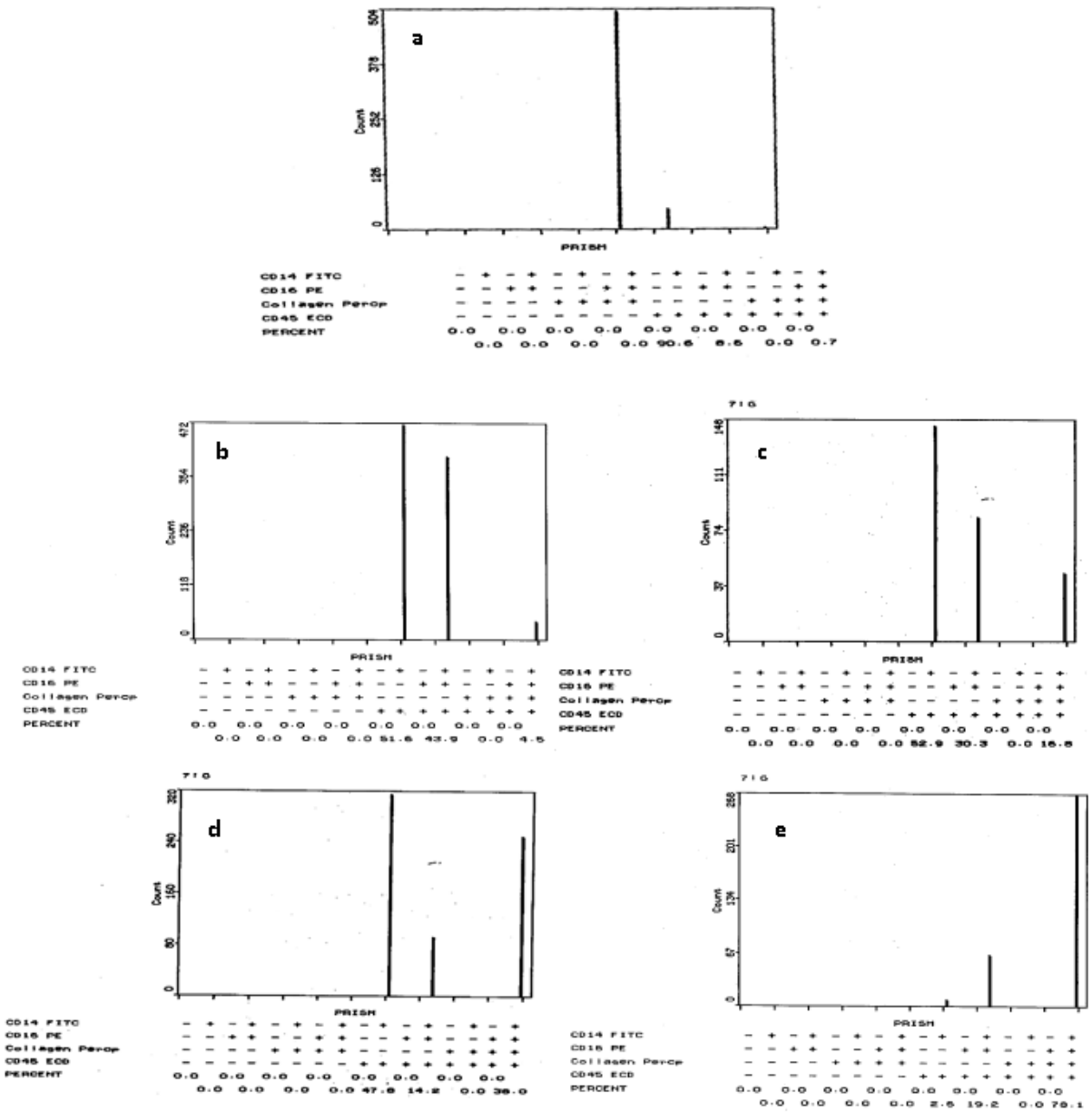

Figure 1: Flow cytometry prism shows the percentage of different monocytes subsets in a healthy subject (A) a patient with HCV-related stage F1 fibrosis (B) a patient with HCV-related stage F2 fibrosis (C) a patient with HCV-related stage F3 fibrosis (D) a patient with HCV-related stage F4 fibrosis (E).

Moreover, a significant up-regulation ($p< 0.01$) of the percentage of the monocytes population that positively expresses chemokine receptor CCR2 was detected in patients with HCV with different stages of fibrosis compared to healthy subjects and was found to match the progression of the disease. A marked increase ($p< 0.01$) in MCP-1 levels was detected in different groups of patients compared to controls and was also found to match the severity of the disease and the grade of liver fibrosis. In contrast, we found a marked progressive decrease ($p< 0.01$) in SAP circulating levels in four groups of patients compared to controls, and was mostly noticed advanced stage F4 fibrosis.

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Table 2: Different Monocyte subsets in healthy subjects and patients with HCV related chronic liver disease with different stages of fibrosis.

Groups	Control (n=15)	Liver Fibrosis			
		F1 (n=15)	F2 (n=15)	F3 (n=15)	F4 (n=15)
Classical monocytes	87.953±4.05	79.34 ± 4.401[a]	61.17±6.68[ab]	50.87±2.55[abc]	4.88±3.3 [abcd]
Non-Classical monocytes	11.62±3.96	13.5±4.78	20.96±10.29[ab]	16.95±2.71[abc]	30.03±5.68[abcd]
Monocytes producing collagen	0.4±0.189	7.15±4.05[a]	16.27±2.43[ab]	32.35±3.83[abc]	65.09±5.28[abcd]
CCR2	2.43±0.60	6.82±1.61[a]	12.37±1.97[ab]	21.47±1.77[abc]	42.26±4.09[abcd]
MCP-1	128.3±15.0	189.9±14.13[a]	257.1±22.2[ab]	329.4± 8.9[abc]	407.5±22.9[abcd]
SAP	38.88±1.42	30.49±1.148[a]	20.46±1.53[ab]	10.45±1.69[abc]	6.5±1.46[abcd]

Data are represented as mean ± SD, [a]p<0.01: Controls vs other groups, [b]p<0.01: Stage F1 vs other groups
[c]p<0.01: Stage F2 vs other groups, [d]p<0.01: Stage F3 vs other groups

By means of the ROC curves, the accuracy of MCP-1 and SAP ELISA test was evaluated and we found that, the specificity, sensitivity and AUC of the MCP-1 ELISA test was (100%, 100% and 1), respectively, for all stages of the liver fibrosis (Fig. 2), as well as the specificity, sensitivity and AUC of the SAP ELISA test was (100%, 100% and 1) respectively, in the liver fibrosis stages (F1, F2 and F3) (Fig. 3), while the specificity and sensitivity were decreased to 93% for the detection of stage F4 of liver fibrosis (Fig. 4).

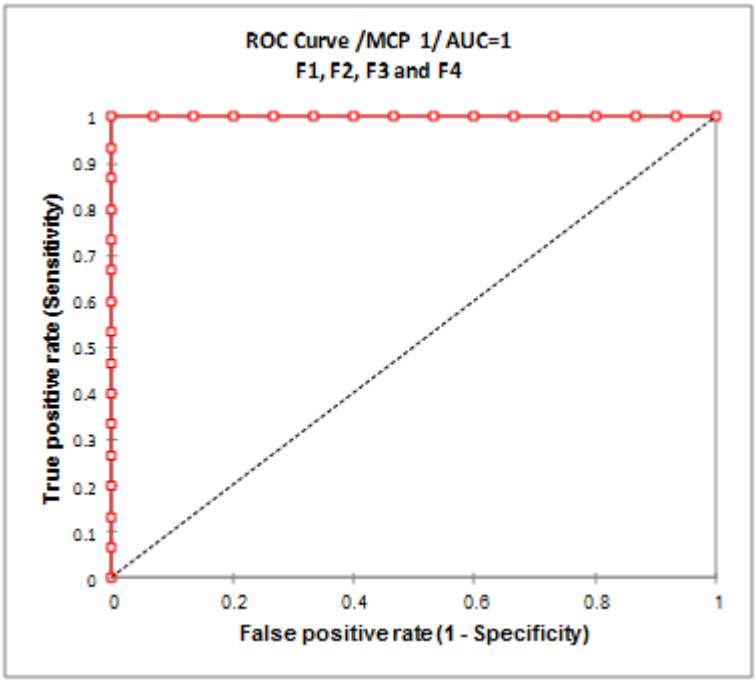

Figure 2: Represent the specificity, sensitivity and AUC of the MCP-1 ELISA test was (100%, 100% and 1), respectively, for all stages of the liver fibrosis

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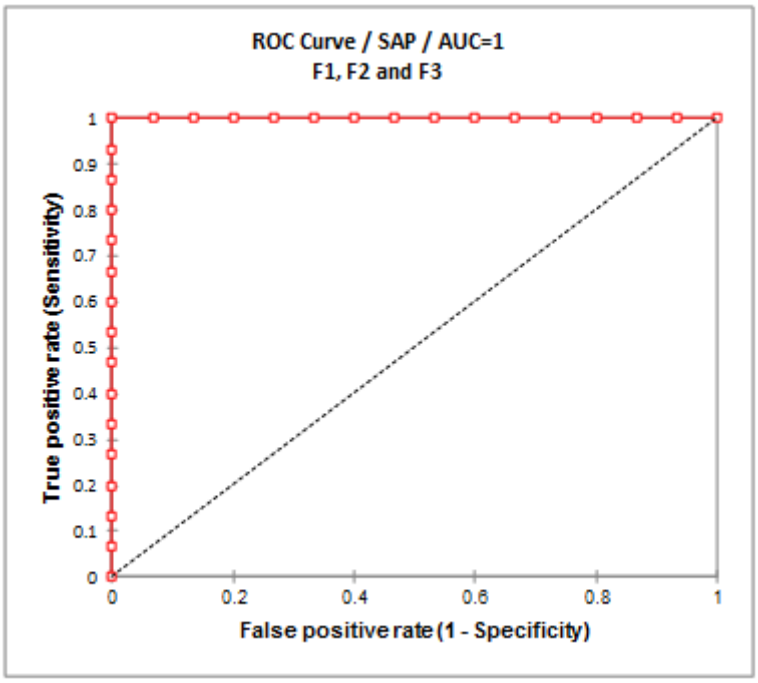

Figure 3: Represent the specificity, sensitivity and AUC of the SAP ELISA test in the liver fibrosis stages (F1, F2 and F3).

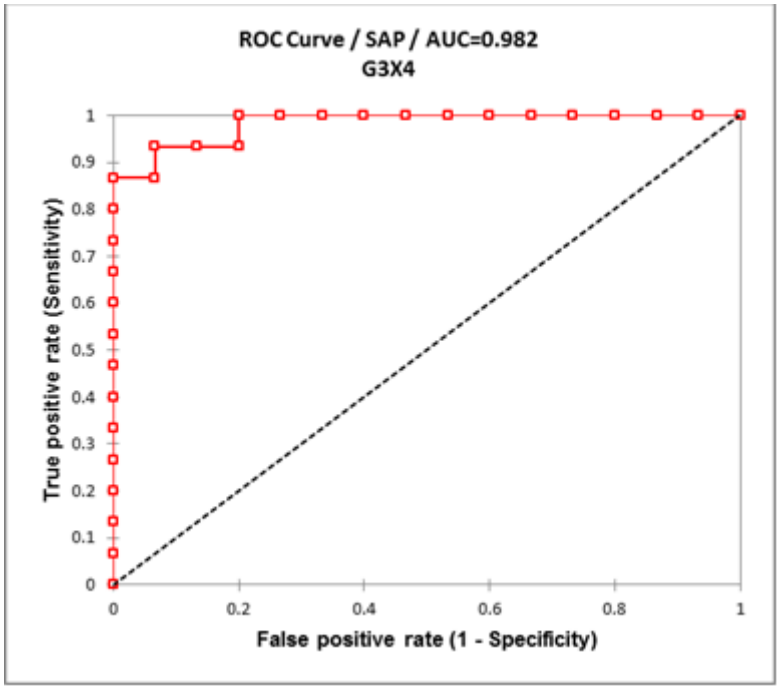

Figure 3: Represent the specificity and s sensitivity and AUC of the SAP ELISA test in stage F4 of liver fibrosis.

DISCUSSION

Ecessive production of extracellular matrix, mostly collagen type I, and scar formation result from liver fibrosis caused by desregulation of physiological wound healing. Collagen producing myofibroblasts activation is critical for the pathogenesis of liver fibrosis (11, 12).

The proportion of normal pattern of circulating peripheral blood monocytes that co-express $CD45^{+}$ $CD14^{+}$ $CD16^{-}ColI^{-}$, the classical monocytes subset,was found to be decreased in patients with HCV related chronic liver disease compared to healthy subjects,mostly among

patients with stage F4 fibrosis. These findings coincide with those of others who demonstrated a strong shift towards the non-classical monocytes $CD14^{+}CD16^{+}$ *subset* in patients with chronic liver disease, especially cirrhotic patients. Down regulation of classical monocytes may be due to the recruitment of classical monocytes into injured liver in response to chemokine MCP-1/CCR2 pathway (13) or to the differentiation of some classical monocytes into non-classical monocytes (7, 14).

Data also revealed that the proportions of circulating peripheral blood monocytes that co-express $CD45^{+}$ $CD14^{+}$ $CD16^{+}ColI^{-}$ increased in different groups of patients compared to healthy subjects.These findings confirm those of Liaskou *et al.* (15), who found that the frequency of non-classical monocytes were increased in liver tissue in patients with chronic inflammatory and fibrotic liver disease compared to controls. The percentage of non-classical monocyte subset was found to be increased during inflammatory diseases (16). The non-classical monocytes subset, which is able to activate primary human HSCs that in turn can secrete multiple chemokines for monocyte recruitment and trans-differentiate into myofibroblast that represents the central event in hepatic fibrosis (17, 18), is suggested to be crucial regulator in the pathogenesis of chronic liver disease induced liver fibrosis (13, 15, 19-22).

The proportions of circulating peripheral blood monocytes that co-express$CD45^{+}$ $CD14^{+}$ $CD16^{+}ColI^{+}$ was also found to be up-regulated in all patients groups compared to healthy subjects. Our findings agree with those of others who reported that $CD45^{+}$Pro-Col-$Ia1^{+}CD14^{+}CD34^{-}$ cells were increased by threefold to fourfold in the idiopathic pulmonary fibrosis and connective tissue disease-interstitial lung disease samples compared to non-fibrotic control (23). $CD14^{+}$ monocytes isolated from patients with systemic sclerosis were found to express higher levels of type-1 collagen than those isolated from healthy controls. Myofibroblasts were postulated to be chemokine-attracted extravasated cells potentially originating from the large pool of circulating peripheral blood monocytes and were often encountered perivascularly in systemic sclerosis tissue (24).

Fibroblasts, peripheral blood monocytes, macrophages, epithelial & endothelial smooth muscle, dendritic cells and microglial cells secrete MCP-1 (25). A marked increase in circulating level of MCP-1 was noticed in patients with HCV induced chronic liver disease compared to healthy subjects. Our findings are in accordance with those of others (26, 27) who found that MCP-1 levels were markedly elevated in culture supernatants from systemic sclerosis monocytes than in those from healthy control monocytes. However, a decrease in the serum level of MCP-1 was reported in patients with liver cirrhosis compared to healthy subjects, implicating that MCP-1 level did not reflect inflammatory activity in liver cirrhosis (28). In contrast, a marked rise in the serum level of MCP-1 was found in hepatitis C patients early after infection and sustained elevation of this fibrogenic mediator was detected in these patients 6 months after infection (29). It was, therefore, suggested that MCP-1 is likely to play an important role in the fibrogenic process initiated by inflammation. The increase of this biomarker matched the severity of the disease and the stage of liver fibrosis, which may, thus, implicate a role for MCP-1 in the mobilization of monocytes from the bone marrow and their homing in the liver during fibrogenesis. We may also suggest that the progress of liver fibrosis parallel the elevation in levels of MCP-1 in relevance to the fibrotic stage of liver disease. Sustained levels of CCL2 have been considered relevant in initiating liver injury and subsequent advancementt of fibrosis and may, therefore, be a good predictor of progression towards cirrhosis (30).

An increase in the percentage of circulating peripheral blood monocytes that express CCR2 was detected in patients with HCV related chronic liver disease compared to controls. Our results are in agreement with those of others (30; 22; 13). Data also revealed a significant up-regulation in the percentage of peripheral blood monocytes that express chemokine receptor CCR2 with the disease progression which corroborate the findings of Asselah *et al.*,(31) and Zhdanov *et al.*,(32) who suggested that high CCL2 and its receptor CCR2 mRNA transcripts in HCV infected liver tissues matched the disease severity. Chemokine receptor CCR2 is crucial for peripheral blood monocytes infiltration of injured liver implicating its regulation of monocyte entry into the inflamed tissue by promoting the egress of immature monocytes from the bone marrow into the circulation. The deficiency of CCR2 was also suggested to attenuate liver inflammation and fibrosis (33; 30, 22, 15). The percentage of circulating peripheral blood monocyte that express CCR2 was found to be strongly correlated with that of monocytes producing collagen subset in studied patients suggesting that these receptors may modulate monocytes expansion in the bone marrow, egress from the bone marrow, and travelling from the blood stream into the fibrotic liver. MCP-1/CCR2 play a critical role in fibrogenesis and trigger monocytes trafficking to the injured liver initiating their differentiation into collagen type I producing monocytes, supporting that monocytes may become a novel target for anti-fibrotic therapy.

SAP is a serum protein which inhibits inflammation and fibrosis. Its recombinant form is tested as an anti-fibrotic agent (34). A severe decrease in SAP levels was found in studied groups compared to healthy subjects. The decrease of this biomarker is inversely proportional with the severity of the disease and the progression of liver fibrosis. These findings confirm the results of Verna *et al.* (34) who showed that the serum levels of SAP were significantly lower in patients with non-alcoholic fatty liver disease and any stage of fibrosis compared to normal subjects; similar findings were found in patients with advanced fibrosis compared to patients with mild-moderate fibrosis. The authors have, therefore, suggested that the down-regulation of SAP may be due to its recruitment to the site of injury to bind to monocytes preventing their differentiation to myo-fibroblast like cells. The recruitment of SAP to the site of injury may further explain the decline of SAP plasma concentrations in patients with inflammatory diseases. The decrease may more likely be due to its consumption (35). It was also noticed that the SAP plays an important role in HSCs activation inhibition in addition to fibrogenesis and wound healing regulation (36, 37), therefore, repletion with recombinant protein is a promising therapeutic target. It was also found that SAP initiated the release of the macrophages anti-inflammatory IL-10 which inhibits the production of pro-fibrotic collagen by myofibroblasts (37). The replacement of SAP may be considered as a promising new anti-fibrotic strategy for hepatic fibrosis management and may, thus, play a dual role as a biomarker of profibrotic response to tissue injury, and a therapeutic target for fibrotic disease management.

In conclusion, our findings revealed a down regulation of the classical monocytes subset which was simultaneously conjugated with an up regulation of both the non-classical monocytes and monocytes producing collagen subsets in HCV related chronic liver disease patients, and that the differential circulating peripheral blood monocytes recruitment in these patients matched the advancement of the disease and the progression of liver fibrosis. The present study has revealed different combinations of peripheral blood monocytes that express monocyte surface markers and intracellular protein, denoting the occurrence of differential recruitment of circulating peripheral blood monocyte subsets in HCV related chronic liver disease patients with compared to healthy subjects. The pathogenesis of HCV-induced hepatic injury may be attributed to cytopathic damage by HCV and/or immune-mediated

hepatic injury, especially *via* cellular immunity. Hepatic fibrogenesis represents a wound-healing response of liver to a chronic liver injury. Different monocytes subsets expansion to the circulation of chronic hepatitis C related liver disease patients upon disease progression implicate their participation in the intra-hepatic inflammation and pro-fibrogenic HSCs activation in liver cirrhosis through their recruitment into the liver via chemokines/chemokine receptors.

The shift of classical monocyte subset towards the non-classical and collagen producing subsets may present a predictive non-invasive biomarker for progress of liver fibrosis. MCP-1 and CCR2 may trigger monocytes recruitment to injured liver promoting their development into collagen type I producing monocytes. Monocytes may be also implicated s a potential target for anti-fibrotic therapy. Replacement of SAP may also be further studied as a novel anti-fibrotic strategy.

Ethical Standards:
The guidelines of TBRI Institute's Human Research Ethics Committee were followed; all subjects have given their informed consents before inclusion in the study.

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Conflict of interest:
None of the authors had conflict of interests.

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