JMSCR Vol||04||Issue||12||Page 14773-14780||December 2016

www.jmscr.igmpublication.org
Impact Factor 5.244
Index Copernicus Value: 83.27
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossref DOI: https://dx.doi.org/10.18535/jmscr/v4i12.75

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Noninvasive Markers for the Assessment of Liver Fibrosis and Cirrhosis in Egyptian Patients with Chronic Hepatitis C and Schistosomiasis Coinfection

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ABSTRACT
To assess the role of noninvasive biomarkers in patients with chronic hepatitis C virus (HCV) and schistosomiasis coinfection and to correlate them with hepatic fibrosis and activity.
This study was carried out on one hundred HCV positive Egyptian patients with detectable serum HCV RNA. All patients were positive for anti-schistosomal antibodies prior to HCV treatment. Serum cryoglobulins, serum autoantibodies; ANCA, AMA, ASMA and expression of CD 25 on CD4+ T cells were measured. APRI score, Fibrotest and Actitest scoring systems using computed algorithm were done to assess the degree of liver fibrosis.
A high prevalence of cryoglobulins and autoantibodies was detected. There was a statistical difference between cryopositivity and the degree of liver fibrosis and cirrhosis. Autoantibody positive patients were found to have a significantly higher Fibrotest and Actitest score as well as APRI score than autoantibody negative patients. There was a significant reduction in CD4+ T cells % while CD4+25+ T cells % were significantly higher in HCV patients than controls.
Serum cryoglobulins and autoantibodies were related to the degree of liver fibrosis and cirrhosis based upon Fibrotest and Actitest and thus may serve as non invasive indices for assessment of hepatic fibrosis. There was a significant up-regulation in $CD4^+$ $CD25^+$ T cells but no correlation was found between APRI score and CD4+T cell % or CD4+25+T cell %. Thus CD25 expression can only be considered as a critical marker for evaluating the immune regulation in patients with coinfection of HCV and schistosomiasis but not as a marker to measure the degree of liver fibrosis and necro inflammatory activity in these diseases.
Keywords- *Noninvasive; markers; liver; fibrosis; HCV; schistosomiasis; coinfection.*

Introduction

Egypt has the largest epidemic of HCV in the world with an overall serum positive prevalence of 14.7% as reported by the Egyptian demographic health survey [1]. Schistosomiasis is also significantly endemic in Egypt [2]. In Egypt; patients with HCV are commonly found to be co-infected with schistosomiasis (bilharzias). Both conditions can

augment portal hypertension with its consequences which includes splenomegaly, hypersplenism with pancytopenia and portal varices with and without bleeding [3]. Patients with coinfections of both HCV and Schistosoma have been shown to have higher HCV RNA titers, increased histological activity, greater incidence of cirrhosis/hepatocellular carcinoma, and higher mortality rates than patients suffering from single infections. Liver biopsy remains to be the gold standard in staging the degree of liver fibrosis and can also guide about the treatment response [4]. Being invasive, relatively expensive with higher chances of technical errors during biopsy procedure have led to the search for other safer, non-invasive and cheaper modalities with comparable effectiveness [5]. One of the potential alternatives to liver biopsy is APRI score, however, in patients with schistosomiasis, the platelet count is inversely related to the degree of periportal fibrosis and spleen size which might alter the reliability of APRI score in patients with HCV infection who are also co-infected with schistosoma infection [6]. Among the non invasive alternatives, there are also two combinations of simple serum biochemical markers; Fibrotest; for the assessment of fibrosis and Actitest; for the assessment of necroinflammatory activity [7]. It has been shown that in patients with chronic HCV infection, HCV-associated cryoglobulins, are of clinical significance especially that the reactivities of IgG and IgM in cryoglobulins against specific HCV antigens has not been extensively studied [8]. The prevalence of non–organ-specific autoantibodies NOSAs in chronic HCV-infected patients varied from 25% to 66%. Different mechanisms have been implicated in the development of NOSAs during chronic hepatitis C with clear evidence of altered immune system homeostasis in chronically infected patients. Since dysfunction of CD4+ and CD8+ T cells is closely associated with HCV-specific immune escape and HCV persistence, therefore cytokine receptors as CD25, CD122 and CD132, required for activation, proliferation, and survival of T cells should be recognized as another indicator for estimating disease progression and severity [8].

The aim of the present study was to detect the prevalence of cryoglobulins, serum autoantibodies; ANCA, AMA, ASMA and **e**xpression of CD 25 on CD4+ T cells in patients with concomitant chronic HCV and schistosomiasis infection and to correlate them with the degree of hepatic fibrosis and activity.

Material and Methods

This study was carried out on one hundred HCV positive Egyptian patients with detectable serum HCV RNA, admitted to the hepatology and tropical medicine inpatient departments between January 2014 and December 2015. All patients were also positive for anti-schistosomal antibodies prior to HCV treatment. Patients were excluded from the study if they had any other concomitant chronic liver disease, including; chronic hepatitis B infection, human immunodeficiency virus co-infection, autoimmune hepatitis, decompensated liver disease, hepatocellular carcinoma, history of previous immunosuppressive, antiviral or interferon therapy. Patients were subdivided according to the degree of liver fibrosis into; group Ia; thirty five patients with no or minimal fibrosis and group Ib; sixty five patients with significant fibrosis. Those patients were scheduled to receive treatment for HCV infection. Fifty apparently healthy subjects were selected from the outpatient clinics as the control group II. All patients were subjected to full history taking, thorough clinical examination, and abdominal ultrasound; focusing on liver echopattern and splenomegaly. Serum anti HCV antibodies using the third-generation enzyme-linked immunosorbent assay ELISA and serum HCV RNA level were performed to all cases using quantitative PCR (Cobas Amplicor HCV monitor test, Roche molecular systems, Banchbug NJ, USA). Diagnosis of schistosomal co-infection was based on positive antischistosomal antibody titer equal to or more than 1:160 (Fumouze Diagnostics, Levallois-Perret, France). Complete blood picture, alanine aminotransferase ALT, gamma glutamyl transferase GGT, bilirubin and prothrombin time were done to assess liver functions. APRI score was calculated

according to the formula: [(AST of the sample/ reference AST) x 100] / platelets.The reference value for aspatate aminotransferase AST was considered to be 40 IU, which is the upper normal limit in our laboratory. Quantitation of the $CD4^+CD25^+$ markers in the peripheral blood lymphocytes using flow cytometry (Becton Dickinson, FACS caliber flow cytometer equipped with Cell Quest software USA). Using two flurochrome-conjugated antibodies, one is specific for identification & numeration of cell populations expressing the CD4 antigen (fluorescein isothiocyanate FITC) and the other is specific for identification and numeration of cell populations expressing the CD25 antigen (Phycoerythrin PE) that presented in the peripheral blood. Fibrotest and Actitest biochemical markers were performed to group I. The markers include; ά macroglobulin, apolipoprotein A1 and haptoglobin assays done by nephelometry (BN system, Dade Behring GmbH, Marbuing,USA) as well as total bilirubin, GGT and ALT assays done on Hitachi 912 analyzer (Boehringer Mannheim, Germany). Group I was then subdivided according to the degree of liver fibrosis into; group Ia (no or minimal fibrosis; F0-F1) and group Ib (significant fibrosis; >F1). Serum cryoglobulins were assessed by a semiquantitative method. The cryocrit level was estimated by measuring the height of the column of precipitated protein relative to the total height of the serum column after incubation at 4°C for 72 hours and was expressed as a percentage. Serum autoantibodies (antineutrophil cytoplasmic antibodies ANCA c, ANCA p, anti-smooth muscle antibodies ASMA, anti-mitochondrial antibodies AMA) were measured by indirect immunofluorescence technique (IMMCO Diagnostics, 14228 Buffalo, NY, USA). Patients' sera were incubated on optimized preparations of human neutrophils substrate slides for detection of ANCA (ImmuGlo IFA, IMMCO Diagnostics) and mouse kidney/stomach substrate slides for detection of ASMA and AMA (ImmuGlo IFA, IMMCO Diagnostics) to allow binding of antibodies to the substrate. Bound antibodies of the IgG class were detected by incubation of the substrate with fluorescein-labeled, antihuman IgG conjugate. Reactions were observed under fluorescence microscope equipped with appropriate filters. The presence of ANCA is demonstrated by an apple green fluorescence either with cytoplasmic staining (ANCA c) or perinuclear staining (ANCA p). The presence of AMA and ASMA is demonstrated by an apple green fluorescence of specific histologic structures in the tissue. The titre is then determined by testing serial dilutions of the patient serum.

The study was approved by the medical ethics committee and informed consents were obtained from all participants involved in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

Statistical Analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (SPSS Inc., Chicago, IL, USA). Comparisons between groups for categorical variables were assessed using Chi-square test (χ^2). Student t-test was used to compare two groups for normally distributed quantitative variables. Mann- Whitney test and Kruskal-Wallis test were used to compare two or more groups for abnormally distributed quantitative variables. Paired t-test and Wilcoxon signed ranks test were assessed for comparison between different periods. Spearman coefficient was used to correlate between quantitative variables. Significance of the obtained results was judged at the 5% level.

Results

Patients were subdivided according to the degree of liver fibrosis according to Metavir scoring system into; group Ia; thirty five patients (no or minimal fibrosis; F0-F1) and group Ib; sixty five patients (significant fibrosis; >F1) and fifty apparently healthy subjects as a control group II. Cryoglobulinemia was detected in forty five patients; group I (45%), while fifty five patients

were negative for cryoglobulins (55%), and no one showed cryopositivity in group II. Cryopositivity was significantly higher in group Ib (88.9%) than group Ia (11.1%), P<0.05. Serum ALT, GGT, total bilirubin and PT showed no statistically significant difference between cryopositive patients (mean 102±67.533 U/L. 192.778±201.691 U/L, 1.203±0.648 mg/dl and 14.022±10351 s respectively) and cryonegative patients (mean 88.64±97.33 U/L, 100.273±103.329 U/L, 0.762±0.561 mg/dl and 1.245±0.23 s respectively). Meanwhile, there was a statistically significant difference between group Ia, Ib and II as regards the degree of liver fibrosis, P<0.05.

Cryopositivity was observed in five patients with F0-F1 (11.1%), in five patients with F2-F3 (11.1%) and in thirty five patients with F4 (77.8%). The increase in cryoglobulins frequency was statistically significant when patients with F1-F2 and F2-F3 were compared to F4, P< 0.05 as shown in table 1. As regards the relation of the cryocrit level and the stage of liver fibrosis (Fibrotest score); a low cryocrit level prevailed in patients with F0-F2 and F3-F4 (5% and 40% respectively). Increased fibrosis (F3-F4) is correlated with a higher prevalence of high cryocrit level. As regards the different cryocrit levels and the necroinflammatory activity (Actitest), it was found that among cryopositive patients; five patients were A0 (5%), five patients were A1 (5%), five patients were A2 (5%) and thirty patients were A3 (30%). Meanwhile, cryonegative patients; twenty were A0 (20%), fifteen were A1 (15%), no one was A2 (0%) and twenty were A3 (20%). A high cryocrit was more frequent in patients with a higher Actitest score. A statistically significant difference can be detected on comparing patients with A0 and patients with A2-A3, P<0.05 as shown in table 2.

Patients who had at least one non organ specific autoantibody were 85 at a titer higher than 1:40. ASMA and ANCAc were the most frequent autoantibodies (65%), followed by AMA (35%). ANCAp was only detected in 10% of the patients. Serum ALT and total bilirubin in those patients (mean 85.53±59.58 U/L and 0.98±0.64 mg/dl respectively) showed no statistically significant difference with patients who were negative for all the studied autoantibodies (mean 146±181.51 U/L and 0.84±0.69 mg/dl respectively. However, a statistically significant difference was found between both groups as regards; serum 154.82±167.82 U/L versus 68.67±49.94 U/L respectively), P<0.05. Autoantibody positive patients were found to have a significantly higher Fibrotest score than autoantibody negative patients (mean 0.75±0.21 and 0.73±0.32) versus (mean 0.42±0.28 and 0.40±0.22) respectively, P< 0.05 as shown in table 3.

There was a significant reduction in CD4+ T cells % in group I than group II, p = 0.022. CD4+25+ T cells % was significantly higher in patients than controls p <0.05 as shown in figure 1. APRI score was significantly higher in group I than group II, p <0.001 as shown in figure 2. No correlation was found between APRI score and CD4+ T cells or CD4+25+ T cells %, p = 0.451 and 0.855 respectively as shown in table 4.

Table 1: Prevalence of cryoglobulinemia and stage of fibrosis

Stage of fibrosis	Cryopositive patients	Cryonegative patients
F0-F1	63.6%	11.1%
F2-F3	27.3%	11.1%
F4	9.1%	77.8%
X^2	9.9	
P	0.007*	

Table 2: Cryocrit level and necroinflammatory activity of the liver

Cryocrit level (%)	A0 (No activity) and A1 (Minimal Activity) (n=45)		A2 and A3 (Moderate and severe activity) (n=55)		Total (n=100)	
	n	%	n	%	n	%
2-5	10	10	5	5	15	15
5-10	0	0	15	15	15	15
>10	0	0	15	15	15	15
Total cryopositive patients	10	10	35	35	45	45
Total cryonegative patients	35	35	20	20	55	55
P	0.0126*					

Qualitative data were described using number and percent and was compared using Chi square test.*: Statistically significant at $p \leq 0.05$

Table 3: Patients with and without serum autoantibodies (ASMA, AMA, ANCA (p), ANCA(c) comparison of biochemical parameters.

	Autoantibody positive patients	Autoantibody negative patients	P
ALT (U/L) Range Mean±S.D.	 14 – 217 85.53±59.58	 25 – 355 146.33±181.51	0.31
GGT (U/L) Range Mean±S.D.	 12 – 668 154.82±167.82	 23 – 122 68.67±49.94	0.05*
Total bilirubin (mg/dl) Range Mean±S.D.	 0.26 – 2.55 0.98±0.64	 0.39 – 1.63 0.84±0.69	0.31
Fibrotest score Range Mean±S.D.	 0.04 – 0.98 0.75±0.21	 0.11 – 0.97 0.42±0.28	0.012*
Actitest score Range Mean±S.D.	 0.05 – 0.99 0.73±0.32	 0.11 – 0.92 0.40±0.22	0.048*

Normally quantitative data was expressed in mean ± SD and was compared using student t-test.*: Statistically significant at $p \leq 0.05$

Table 4: Correlation between APRI score with CD4+ T cell %, and CD4+ 25+ T cell %

	APRI score	
	r_s	P
CD4+ T cells %	-0.143	0.451
CD4+ 25+ t cells %	-0.035	0.855

r_s: Spearman coefficient *: Statistically significant at $p \leq 0.05$

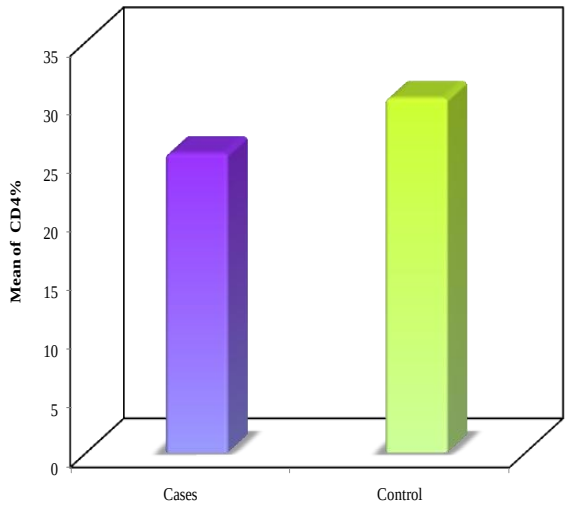
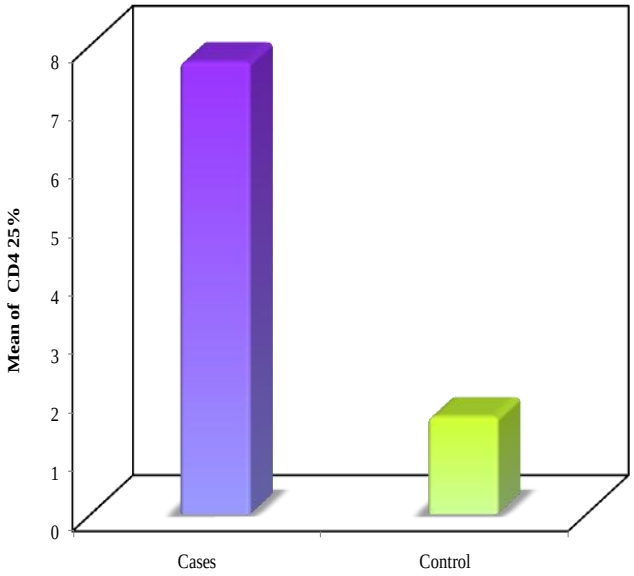


Figure (1): Comparison between the two studied groups as regards CD4+ cells and CD4+ 25+ T cells %



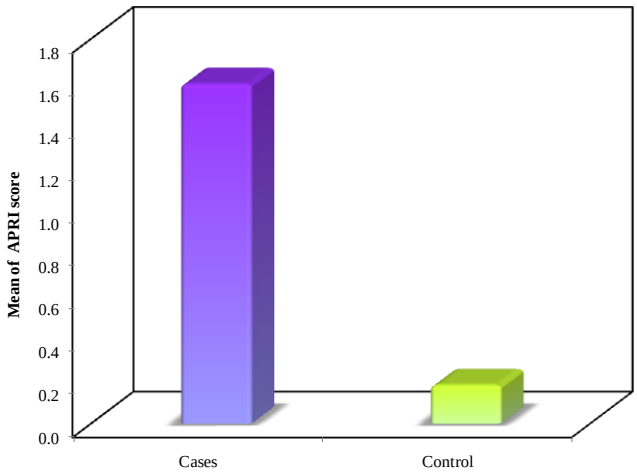


Figure (2): Comparison between the two studied groups as regards APRI score.

Discussion

Egypt has the highest and devastating prevalence of HCV in the world, amounting to 14–20%. In Egypt, the major route of exposure is considered to be due to receiving medical injections and inefficient infection control practices. Historically, Schistosomiasis used to be a common endemic disease in Egypt and during the 90s, only glass syringes were available for treatment of schistosomiasis. Therefore, despite improvement in schistosomiasis-related morbidity during 90s, such treatment campaigns have primarily triggered the current large hepatitis disease burden in Egypt [9]. The assessment of the presence and severity of liver fibrosis is essential in determining treatment strategies,

response to treatment, prognosis and the potential risk for complications in patients with chronic liver disease. Owing to its risks, cost and inconvenience, liver biopsy is not the ideal procedure for repeated assessment of disease progression, especially during chronic liver disease [10]. Developing non-invasive tests that can accurately predict fibrosis stage and progression over time is a high priority and a growing medical necessity to undertake the current study.

Mixed cryoglobulinemia MC is a systemic vasculitis that is considered the most common extrahepatic manifestation of HCV infection [11]. The data of the present study demonstrated a high prevalence of cryoglobulins (45%) which is in agreement with the results reported by Ramos-Casals et al (50%) [12]. On the other hand, Abbas et al detected cryoglobulins in only (15.1%) of their patients [13]. This discrepancy may be caused by strong regional differences and the reduction in the incidence of HCV-related MC associated with higher incidence of *S. mansoni* coinfection was explained by an apparent protective role of *S. mansoni* infection against the development of immune-mediated diseases such as MC in chronic HCV-infected patients [13]. The present study revealed that high levels of cryoglobulins were associated with high Actitest score. It is controversial whether MC is an independent risk factor for the development of cirrhosis. It was reported that MC was a risk factor for cirrhosis and a meta-analysis of studies predominantly carried out in areas where > 40% of HCV^+ individuals have MC has shown MC to confer an OR 4.87 for cirrhosis, independent of age, gender, and estimated duration of HCV infection [14]. The high cryoglobulins found in the studied patients can be explained by the fact that liver cirrhosis per se can induce cryoglobulins. Moreover, the reduction in hepatic perfusion and the alterations of kupffer cells present in liver cirrhosis may delay the clearance of circulating immune complexes [15]. The current study revealed that cryoglobulins were significantly higher in patients with F4 than in those with F0, F1, F2 and F3. (40%, 0%, 0%, 5%, 0% respectively).

The present study showed a total of (85%) of the studied patients had at least one NOSA, with ANCAc and ASMA showing the highest prevalence rates (70% and 65% respectively), while AMA and ANCAp have been rarely found, which agrees with Acay et al [16]. Most of the autoantibody positive patients in the current study have exhibited a higher biochemical and histological activity and it was found that they exhibit a high Fibrotest and Actitest score ($P<0.05$). Therefore, Fibrotest and Actitest were found to be an alternative to liver biopsy which is consistent with Yakoob et al who reported that Fibotest and Actitest appear to be a good non invasive marker for assessment of liver fibrosis and inflammatory activity [17]. In addition, it was demonstrated that Fibrotest had a better 5-year prognostic value than liver biopsy, Child-Pugh or other scores for predicting complications and death related to chronic hepatitis C [18]. The present study revealed that APRI score was significantly higher in patients than controls, $p < 0.05$. This is concordant with Derbala et al who concluded that APRI score is sensitive and specific in diagnosing the degree of fibrosis and cirrhosis in patients with coinfection of HCV and schistosomiasis as compared to biopsy [19]. The present study also revealed a significant reduction in CD4+ T cells % in group I than group II, $p = 0.022$. Furthermore, a significant up-regulation in the relative proportion of $CD4^+$ $CD25^+$ T cells% in the studied patients was observed in comparison to the uninfected controls so that group I was significantly higher than group II as regards CD4+25+ T cells %, $p <0.05$. These results are in agreement with Shen et al who examined the phenotypic characteristics of circulating $CD4^+$ T cells and the level of CD25 expression in $CD4^+$ T cells in chronic HCV-infected patients. Surface CD25 expression of the described CD4+T cell subsets was investigated following chronic HCV infection [20]. However, no correlation was found between APRI score and CD4+T cells % or CD4+25+T cells %, $p= 0.451$ and 0.855 respectively. Therefore, CD25 expression can only be considered for evaluating the immune regulation in patients with coinfection of HCV and

schistosomiasis via maintenance and development of T cell homeostasis.

Conclusions

Serum cryoglobulins and autoantibodies were related to the degree of liver fibrosis and cirrhosis based upon Fibrotest and Actitest and thus may serve as non invasive indices for assessment of hepatic fibrosis. There was a significant up-regulation in $CD4^+$ $CD25^+$ T cells but no correlation was found between APRI score and CD4+T cell % or CD4+25+T cell %. Thus CD25 expression can only be considered as a critical marker for evaluating the immune regulation in patients with coinfection of HCV and schistosomiasis but not as a marker to measure the degree of liver fibrosis and necroinflammatory activity in these diseases.

Conflict of Interest

Authors have no conflict of interest.

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