

Serum Hyaluronic Acid (HA) and Soluble Intercellular Adhesion Molecule-1 (sICAM-1) as Non-Invasive Markers of Liver Fibrosis in Viral Hepatitis, Schistosomiasis *mansoni* and Co-infected Patients

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Abstract

Background: This study aimed to correlate serum Hyaluronic Acid (HA) and soluble intercellular adhesion molecule-1 (sICAM-1), with severity of liver fibrosis as clinically and histologically assessed in viral hepatitis, schistosomiasis *mansoni* and co-infected patients.

Methods: The study was performed on 4 groups: Group 1 (G1) 15 chronic hepatitis patients; Group 2 (G2) 15 chronic schistosomiasis *mansoni* co-infected with chronic hepatitis patients; Group 3 (G3) 15 chronic schistosomiasis *mansoni* without hepatitis patients; Group 4 (G4) 15 active schistosomiasis *mansoni* without hepatitis patients.

Results: The results showed a significant high level of HA and sICAM-1 in all groups compared to G4, while a significantly high level of HA in G2 compared to G3. There was a highly significant positive correlation between the level of HA and sICAM-1 and between both of them, and Child-Pugh clinical classification of patients with higher levels in Child-Pugh C. Also, serum level of both HA and sICAM-1 was positively correlated to the severity of liver fibrosis assessed by biopsy, with a highly significant higher level in advanced stages 4 and 5.

Conclusions: HA and sICAM-1 showed good diagnostic performance and could discriminate severe from mild liver fibrosis, enabling them to be used as valuable non-invasive markers to identify and follow up patients with liver fibrosis.

Keywords: Schistosomiasis *mansoni*; Hepatitis; Liver fibrosis; Non-invasive markers; Hyaluronic acid; Intercellular adhesion molecule-1

Introduction

Schistosomiasis *mansoni* is a chronic liver disease that is endemic in rural areas of Egypt. Some patients may acquire infection and develop minimal complications, while others may develop severe complications and progress to portal hypertension and cirrhosis, especially if co-infected with viral hepatitis [1,2]. Egypt has the highest worldwide prevalence for the concomitant infection of schistosomiasis and viral hepatitis, where the rates vary from 19.6%-64% for the hepatitis B (HBV), and 10.3%-67% for the hepatitis C virus (HCV) [3,4].

Liver fibrosis which results from chronic inflammation of hepatic parenchyma is a complex, dynamic process that includes an increase in extracellular matrix components, activation of cells producing matrix materials, cytokine release and tissue remodelling [5]. Liver biopsy is the gold-standard method for assessment of the liver fibrosis; however, it is an invasive procedure and is potentially dangerous [6].

Non-invasive markers have been developed to assess the severity of liver fibrosis. Among them, serum hyaluronic acid (HA) appears to be the most promising one [7]. HA is unbranched high-molecular-weight polysaccharide, and it represents a component of the extracellular matrix in virtually every tissue of the body. In the liver, HA is mostly synthesized by hepatic stellate cells and degraded by sinusoidal endothelial cells [8]. Some portion of tissue HA enters into the general circulation via lymphatics, and is mainly taken up by sinusoidal endothelial cells in the liver through hyaluronate receptors and degraded in lysosomes. Decreased function of sinusoidal endothelial cells in advanced liver diseases may raise serum levels of HA, through decreased number of hyaluronate receptors and reduced degradation of HA in the endothelial cells [9]. This marker is of interest in chronic liver diseases to avoid liver biopsy.

Granuloma formation is controlled and modulated by several cell

types and protein interactions, primarily CD4+ T-cells, Th1 cytokines and cell adhesion factors [10,11]. Intercellular adhesion molecule-1 (ICAM-1) is present on endothelial cells, antigen presenting cells and fibroblasts, and belongs to the immunoglobulin superfamily of proteins [10,12]. ICAM-1 mediates granulocyte extravasation, lymphocyte-mediated cytotoxicity and cell-cell interactions in immunologic responses [13]. Furthermore, the granulomatous response is mediated by eosinophils, the recruitment of which is critically dependent on ICAM-1 [14]. The soluble form of ICAM-1 (sICAM-1) may be shed from inflammatory cells, may be secreted by hepatocytes stimulated by inflammatory mediators, or may derive from passive liberation by necrotic hepatocytes [15]. The Th1 cytokines, IFN- γ and TNF- α , both trigger the release of sICAM-1 [16,17], and are known to play key roles in the modulation [18] and aggravation of hepatic fibrosis [19], respectively. The sICAM-1 is involved in lymphocyte and eosinophil recruitment and inflammatory, immune-mediated mechanisms [14,20]. Also, sICAM-1 plays a role in modulation of the schistosome granuloma [10]. Raised levels of sICAM-1 have been observed in the serum of patients with acute and chronic liver disorders [21]. Elevated expression has also been associated with intestinal schistosome infection and early granuloma formation in murine studies [22,23].

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Received October 08, 2012; **Accepted** October 22, 2012; **Published** October 25, 2012

Citation: Ismail KA, Ahmed SAEG, Elleboudy NAF (2012) Serum Hyaluronic Acid (HA) and Soluble Intercellular Adhesion Molecule-1 (sICAM-1) as Non-Invasive Markers of Liver Fibrosis in Viral Hepatitis, Schistosomiasis *mansoni* and Co-infected Patients. J Bacteriol Parasitol 3:155. doi:10.4172/2155-9597.1000155

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Human studies have also noted a significant increase in sICAM-1 levels in serum and plasma of hepatic schistosomiasis patients [24-26].

The aim of the study was to correlate serum levels of two non-invasive markers of fibrosis HA and sICAM-1, with the severity of liver damage as clinically and histologically assessed in viral hepatitis, schistosomiasis *mansoni* and co-infected patients, to determine the importance of those markers as a screening procedure to identify patients with liver fibrosis in an endemic area for schistosomiasis *mansoni* and viral hepatitis.

Subjects and Methods

Patient's selection

This study was carried out on 60 patients selected from Theodor Bilharz Research Institute in Giza, Egypt, during the period from January 2010 to January 2011. They were categorized (according to medical history and clinical evaluation) into 4 groups: Group 1 (G1) which included 15 chronic hepatitis patients (9 cases with HBV and 6 with HCV), as proved by positive hepatitis marker for at least 6 months, and negative serology for schistosomiasis by indirect haemagglutination test (IHAT); Group 2 (G2) included 15 chronic schistosomiasis *mansoni* co-infected with chronic hepatitis patients, as proved by past history of intestinal bilharziasis or anti-bilharzial treatment, positive IHAT for schistosomiasis and positive hepatitis marker (8 cases co-infected with HBV and 7 with HCV) for at least 6 months; Group 3 (G3) included 15 patients with chronic schistosomiasis *mansoni* without hepatitis, as proved by past history of intestinal bilharziasis or anti-bilharzial treatment, positive IHAT for schistosomiasis and negative hepatitis marker; Group 4 (G4) included 15 patients with active schistosomiasis *mansoni* as viable *Schistosoma mansoni* ova were detected in their stool by direct, and or Kato-Katz technique [27] with positive IHAT for schistosomiasis and negative hepatitis marker. Patients with the following conditions were excluded from the study: other causes of liver disease, liver transplantation, prior interferon therapy or immunosuppressive therapy, insufficient liver tissue samples for staging of fibrosis and alcohol consumption (>30 g/day).

Patients of the studied groups were subjected to:

Serum processing: Blood samples (4-5 ml) were obtained from the all studied subjects. Sera were separated within 12 h of collection, using standard procedures and stored at -70°C until assay. Many laboratory parameters as: HBsAg (Axiom, Germany), anti-HCV (Murex, South Africa), Bilirubin, Albumin (Beckman Coulter chemistry systems), Prothrombin time (Siemens healthcare) and IHAT (Bilharziose Fumouze Diagnostics/SERFIB, France) were measured, according to the manufacturer's instructions.

Determination of serum sICAM-1 and HA: The serum fibrosis markers HA and sICAM-1 were measured using two different commercially available enzyme linked immunosorbent assay kits, specific for each one, in accordance with the manufacturer's recommendations (HA-ELISA® and sICAM-1 ELISA®, R&D System, Inc, USA). Sample dilutions were 1/20, 1/10 for sICAM-1 and HA, respectively. Optical density (OD) was read at 450 nm. The serum levels were determined in one analytical batch in one working day.

Assessment of liver fibrosis severity: Liver fibrosis severity was assessed according to modified Child-Pugh classification, and liver biopsies results according to Ishak scoring system.

Modified Child-Pugh classification is based on the degree of ascites,

the plasma concentrations of bilirubin and albumin, the prothrombin time, and the grade of encephalopathy giving each point a score. A total score of 5-6 is considered grade A (well-compensated disease); 7-9 is grade B (significant functional compromise); and 10-15 is grade C (decompensated disease) [28].

Liver biopsy was carried out on 15 chronic hepatitis patients and 15 chronic schistosomiasis *mansoni* co-infected with chronic hepatitis patients (who were potential candidates for interferon), by the ultrasound-guided standard technique. Histological assessment of liver fibrosis was based on the Ishak scoring system [29], which is one of the commonly used scoring systems. The scoring is from 0 to 6, describing the architectural changes associated with different degrees of scar formation in the liver, which was done by a pathologist who was blinded to the results of serum indices in the study subjects.

Statistical analysis

Data management and analysis were made using SPSS version 15.0 for windows. Serum levels of HA and sICAM-1 are expressed in Mean \pm SD and range. One-way ANOVA test, t-test and Pearson correlation coefficient test were used to analyse the results. The diagnostic performance of HA and sICAM-1 to discriminate patients with severe (stage 3-5) from mild (stage 1-2) liver fibrosis were evaluated, using receiver operating characteristics (ROC) curve with calculation of the area under the curves (AUC), best cut-off point, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). AUC of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value. The diagnostic accuracy was calculated by sensitivity, specificity, positive and negative predictive values, considering fibrosis as the disease. The nearer a curve shifted to the top left-hand corner of the graph, the more useful marker was it for the diagnosis. We determined the turning point of the curve to the best cut-off value for the diagnosis, and it was a maximal value at the sum of the sensitivity and specificity. P values <0.05 were considered statistically significant, and P values <0.001 were considered as statistically highly significant.

Ethical consideration

An informed consent was taken from all patients before taking the samples. The study was approved by Research Ethics Committee, Faculty of Medicine, Ain Shams University.

Results

Analysis of the results showed that the percent of Child-Pugh classification B was higher (40%) in the hepatitis group (G1), while that of classification C was higher (20%) in chronic schistosomiasis co-infected with hepatitis group (G2), as compared to the other groups. But, Child-Pugh classification A was higher in the active schistosomiasis group (G4) in comparison to other groups. Meanwhile, analysis of liver biopsy stages showed that the percent of advanced stages 2, 3, 4 and 5 were increased in the chronic schistosomiasis co-infected with hepatitis group, as compared to the hepatitis group (Table 1). Regarding the serum level of HA and sICAM-1, there was significant difference between the studied groups ($p < 0.05$) by One-way ANOVA test, with significant higher level of sICAM-1 in the hepatitis, chronic schistosomiasis co-infected with hepatitis and chronic schistosomiasis without hepatitis groups, as compared to active schistosomiasis group. While, there was significantly higher level of HA in chronic schistosomiasis co-infected with hepatitis group, in comparison to chronic schistosomiasis without hepatitis group (Table 2). Furthermore, there was a highly significant ($p < 0.001$) positive correlation between the level of HA and sICAM-1

	Child-Pugh classifications			Stages of liver fibrosis				
	A	B	C	1	2	3	4	5
Hepatitis group (G1) n=15	7 (46.7%)	6 (40%)	2 (13.3%)	6 (40%)	3 (20%)	4 (26.7%)	0 0%	2 (13.3%)
Chronic schistosomiasis co-infected with hepatitis group (G2) n=15	7 (46.7%)	5 (33.3%)	3 (20%)	0 0%	4 (26.7%)	6 (40%)	1 (6.67%)	4 (26.7%)
Chronic schistosomiasis without hepatitis group (G3) n=15	10 (66.7%)	5 (33.3%)	0 0%	Not done				
Active schistosomiasis group(G4) n=15	15 (100%)	0 0%	0 0%	Not done				

Table 1: Child-Pugh classifications and liver fibrosis stages by biopsy in the studied groups.

	n	HA (ng/ml) ^a		sICAM-1 (ng/ml) ^a	
		Mean ± SD	Range	Mean ± SD	Range
Hepatitis group (G1)	15	1006.7 ± 1584.7 ^b	30-4900	156 ± 113.1 ^b	15.8-365
Chronic schistosomiasis co-infected with hepatitis group (G2)	15	880.7 ± 1070.7 ^{c,d}	60-3300	238.1 ± 197.5 ^d	17.8-605
Chronic schistosomiasis without hepatitis group (G3)	15	286 ± 321.6 ^e	40-1100	165.3 ± 82.7 ^e	17.8-290
Active schistosomiasis group(G4)	15	43.3 ± 32.4	10-100	80.8 ± 56.5	15.6-165

a=Significant difference between groups as regard level of HA and sICAM-1 (by Oneway of ANOVA test, p<0.05)

b=Significant difference G1 as compared to G4

c=Significant difference G2 as compared to G3

d=Significant difference G2 as compared to G4

e=Significant difference G3 as compared to G4

b,c,d,e as compared by student-t test

Table 2: Serum level of HA and sICAM-1 in the studied groups.

in the studied groups (Figure 1), while there was a non-significant negative correlation between both levels of HA, sICAM-1 and egg count by kato-katz (mean egg count 190.7 and range 40-600 egg/gm stool) in the active schistosomiasis group.

The results showed highly significant difference between Child-Pugh classification groups as regarding the levels of HA and sICAM-1, with higher levels in C classification followed by B (ANOVA test, p<0.001) (Table 3). As regard of the liver biopsy fibrosis stages groups, there was a highly significant difference between them in the levels of HA, with high level in stage 5 followed by 4, and in the level of sICAM-1 with high level in stage 4 followed by 5 (ANOVA test, p<0.001) (Table 4). Also, a highly significant positive correlation between both levels of HA and sICAM-1 and Child-Pugh classification of patients was found (Figure 2 and 3), and a highly significant positive correlation between the level of HA and the stage of fibrosis, while a significant positive correlation between the level of sICAM-1 and the stage of fibrosis (Figure 4 and 5).

The AUC (CI 95%, 0.641-0.938) for HA was 0.824, with p<0.001, giving a good diagnostic performance, discriminating severe from mild liver fibrosis with a best cut off value of >350 ng/ml, sensitivity of 82.35, specificity of 84.6, PPV of 87.5 and NPV of 78.6. While for sICAM-1, AUC (CI 95%, 0.590-0.909) was 0.778, with p<0.05, giving a good diagnostic performance discriminating severe from mild liver with a best cut off value of >54 ng/ml, sensitivity of 88.24, specificity of 53.85, PPV of 71.4 and NPV of 77.8. Pairwise comparison of ROCs of both markers (HA and sICAM-1) showed a non-significant difference (Figure 6).

Discussion

Fibrosis is the hallmark of chronic liver diseases, and it is one of the major causes of mortality and morbidity, related to both

schistosomiasis and hepatitis [30]. The assessment of presence and severity of liver fibrosis is essential in determining treatment strategies, response to treatment, prognosis and potential risk for complications in patients with chronic liver disease [31].

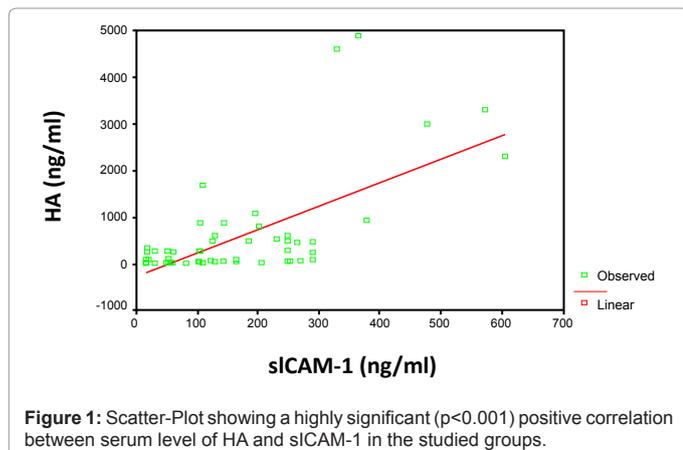
Liver biopsy, the gold-standard method for diagnosing the severity of fibrosis, is an invasive tool and it is associated with rare but serious complications, such as bleeding, pneumothorax and perforation of the colon and gallbladder [32]. Therefore, because of these risks, cost and inconvenience, liver biopsy is certainly not the ideal procedure for repeated assessment of disease progression, especially during chronic hepatitis [33,34].

Use of non-invasive markers to predict fibrosis severity is important for monitoring transition to the severe forms of the disease, prognosis of these patients, and evaluation of fibrosis regression after treatment, especially in the areas where access to health care services is limited [35].

Many parameters for non invasive diagnosis of liver fibrosis were studied extensively in the past [36-38]. These parameters include routine laboratory tests, serum markers of fibrosis and inflammation used, either individually or in combination, ultrasonography and radiological imaging studies [33,34,39].

Ideally, a marker of hepatic fibrosis should be liver specific. It should also be able to measure the activity of the matrix deposition, reflect the underlying fibrosis, irrespective of the cause, should be easy to perform and yet sensitive enough to distinguish between the different stages of fibrosis [40].

HA has been described as a component of several fibrosis indexes, or as a single parameter for the non invasive assessment of fibrosis in hepatitis and schistosomiasis [41]. Combining HA level with other serum markers for assessing liver fibrosis has been considered in some



Child-pugh classifications	n	HA (ng/ml) ^a		sICAM-1 (ng/ml) ^a	
		Mean ± SD	Range	Mean ± SD	Range
A	39	123.8 ± 137	10-600	101 ± 79.5	15.6-290
B	16	645 ± 403.3	50-1700	207.4 ± 81.6	102.4-378
C	5	3620 ± 1098.6	2300-4900	470 ± 121.8	330-605

a=Highly significant difference between Child-Pugh classifications as regard level of HA and sICAM-1 with higher level in C classification followed by B (ANOVA test, $p<0.001$).

Table 3: Relation between each one of the Child-Pugh classifications and level of HA and sICAM-1 in the studied groups.

Liver fibrosis stages	n	HA (ng/ml) ^a		sICAM-1 (ng/ml) ^a	
		Mean ± SD	Range	Mean ± SD	Range
1	6	131.7 ± 111.8	30-350	92.2 ± 103.4	15.8-270
2	7	328.6 ± 188.1	50-600	125.7 ± 103.3	17.8-290
3	10	569 ± 492.7	60-1700	146.1 ± 79	19.6-265
4	1	3300	3300	572	572
5	6	2705 ± 1827.1	480-4900	407.7 ± 115.2	290-605

a=Highly significant difference between liver fibrosis stages as regard level of HA with higher level in stage 5 followed by 4 and as regard level of sICAM-1 with higher level in stage 4 followed by 5 (ANOVA test, $p<0.001$).

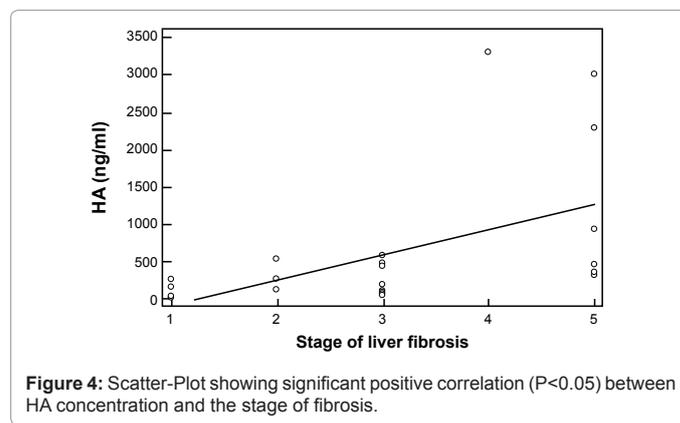
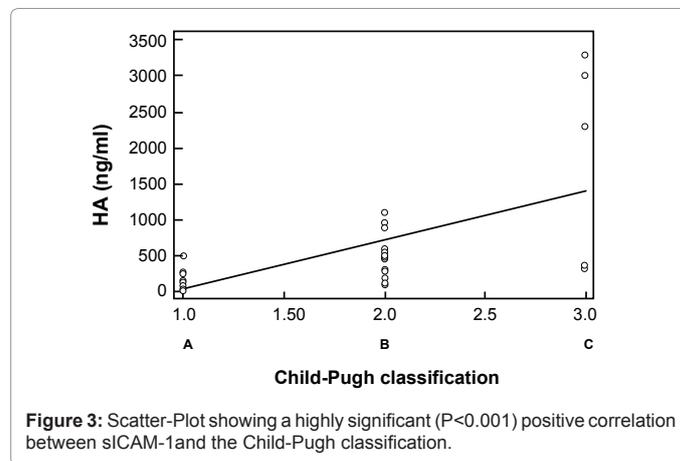
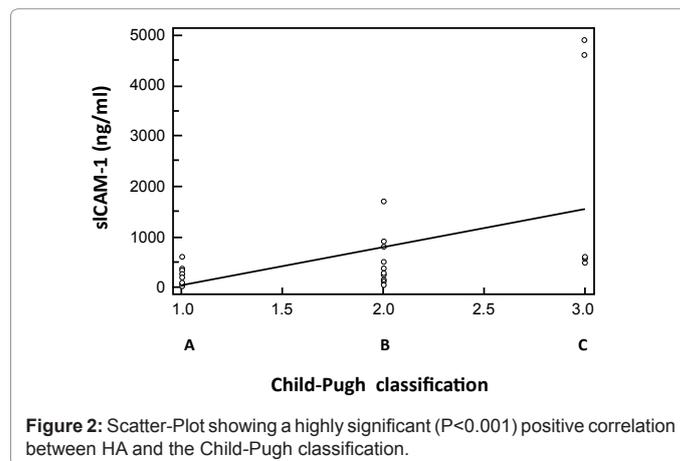
Table 4: Relation between each one of liver fibrosis stages and level of HA and sICAM-1 in the studied groups.

other studies [42,43]. Most of the studies regarding serum markers of fibrosis have focused on hepatitis or schistosomiasis, as an isolated disease. The main objective of this study was to correlate levels of serum HA and sICAM-1, with the prediction of significant liver fibrosis as clinically and histologically assessed in hepatitis and schistosomiasis, not only as isolated diseases, but also as co-infections.

The results showed that there was significant difference between the studied groups regarding the serum level of HA and sICAM-1, with significant higher level of both markers in all groups compared to active schistosomiasis group with no intergroup discrimination between the etiological causes of fibrosis studied, except for the significantly higher level of HA in chronic schistosomiasis co-infected with hepatitis in comparison to chronic schistosomiasis without hepatitis group, and this observation is acceptable as patients of schistosomiasis co-infections with hepatitis exhibit higher necroinflammatory and hepatic fibrosis, with a significant hepatic fibrosis progression rate compared with hepatitis infection alone [31,44,45].

Both markers were positively correlated to the severity of liver fibrosis, and both succeeded in the discrimination between the liver

fibrosis stages in the studied groups, as there was highly significant difference between the liver fibrosis stages regarding the levels of both markers. Patients with advanced stages of liver fibrosis had higher serum HA level, going in accordance with Sanvisens et al. [46], Saitou et al. [47] and Valva et al. [48]. This suggests that when liver damage develops, the liver more poorly metabolizes the HA, capillarization of the hepatic sinusoidal wall and the loss of the HA receptor of the sinusoidal endothelial cell occur, which is the main site of uptake and degradation of serum HA [49]. Also, endothelial function declines due to vascular obstruction by schistosoma eggs and chronic inflammation [50]. Patients in early stage of liver fibrosis possibly better metabolize HA in the liver than those with advanced liver fibrosis stages [51].



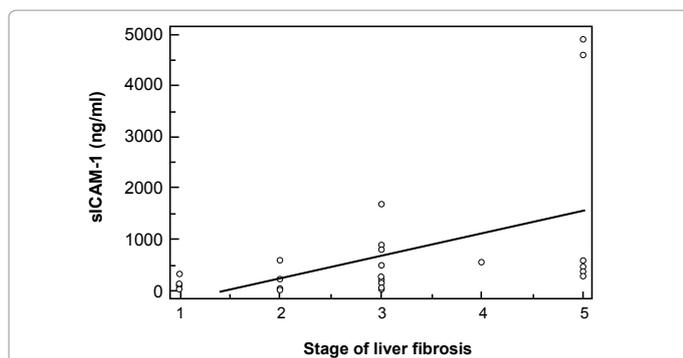


Figure 5: Scatter-Plot showing significant positive correlation ($P < 0.05$) between sICAM-1 and the stage of fibrosis.

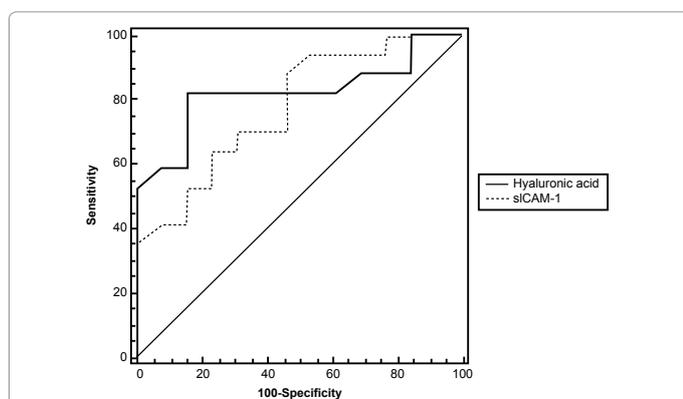


Figure 6: Receiver operating characteristics curve (ROC) of HA and sICAM-1. For HA, AUC= 0.824; at best cut off point of >350 ng/ml could differentiate between severe (stage 3-5) from mild (stage 1-2) liver fibrosis, while for sICAM-1, AUC= 0.778; at best cut off point of >50 ng/ml could differentiate between severe (stage 3-5) from mild (stage 1-2) liver fibrosis. Pairwise comparison of ROC curves of both markers shows non-significant difference.

We also observed the same pattern for sICAM-1 in the patient groups agreeing with Esterre et al. [25]. Most likely, elevated sICAM-1 levels are a result of the inflammatory responses leading to granuloma formation as inflammation and cytolysis lead to an up-regulation in the expression of tissue ICAM-1 derived from hepatocytes and vascular endothelium [52], with higher sICAM-1 levels in severe disease forms reflecting the more intense inflammation which may be occurring in these patients [24], and it could reflect the activation of undergoing immunological mechanisms [53]. This suggests that sICAM-1 may participate in the pathology associated with schistosomiasis infection and it could be employed as a potential morbidity marker in schistosomiasis *mansoni* infection.

Also, there was a highly significant positive correlation between the levels of HA and sICAM-1 in the studied groups. This relationship between HA and sICAM-1 levels was not unexpected, since both parameters were independently correlated with the disease severity [25].

Furthermore, both markers were highly significant and positively correlated to Child-Pugh classification of patients, and both succeeded in discrimination between Child-Pugh classification groups with higher levels in C classification followed by B. These results are supported by Thomson et al. [54], who found that sICAM-1 levels were positively correlated to summary assessment of primary biliary cirrhosis severity

by Child-Pugh classification. These suggest that both markers can be accurate indicators to assess the patient's clinical condition and prognosis. Körner et al. [55] concluded that a modification of the Child-Pugh classification of liver cirrhosis by inclusion of HA as a new marker, had significantly improved the predictive power of the classification. Our results suggest that the inclusion of both markers together can reinforce this predictive power.

When diagnostic accuracy of HA and sICAM-1 in discriminating severe from mild liver fibrosis in this study were assessed by ROC curve, AUC (CI 95%) for HA was 0.824, while for sICAM-1 was 0.778, with a non significant pairwise comparison between ROC curves of both markers, and these indicate a good diagnostic performance and introduce the availability of combining both markers. These observations are in agreement with Resino et al. [41] and Valva et al. [48]. Many fibrosis experts would consider non-invasive tests for fibrosis with an AUC of 0.85-0.90, to be as good as liver biopsies for staging fibrosis [36]. Some authors have argued that some non-invasive markers of fibrosis might be even more accurate than biopsies, and that most of the significantly discordant results between biopsies and non-invasive tests may be due to the method of obtaining biopsies that does not demonstrate the actual liver fibrosis state (sampling error when performing the biopsies) [56].

Furthermore, the results showed a non-significant negative correlation between both levels of HA, sICAM-1 and egg count by kato-katz in the active schistosomiasis group, and this is supported by Mwatha et al. [26] as their levels increase with fibrosis progression [57]. It is not surprising that no correlation between these morbidity markers and egg counting could be found, as egg counting represents a poor measure of parasite-associated morbidity [58].

It was concluded that measurement of serum HA and sICAM-1 levels can discriminate between patients with different degrees of liver fibrosis, as assessed by both histological and clinical diagnoses. Therefore, the results suggest the inclusion of both HA and sICAM-1 serum levels determination in clinical practice, prognosis and management of hepatitis, schistosomiasis and co-infected patients, and this may be helpful for the early diagnosis of fibrosis degree when liver biopsy is contraindicated, or when access to health care services is limited.

Acknowledgement

The authors are grateful to all the staff members at Tropical medicine and Pathology departments, Theodor Bilharz Research Institute in Giza for their kind help in clinical evaluation of the patients, collection of samples and histological assessment of liver fibrosis by biopsy.

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