Diagnostic and Prognostic Value of Direct and Indirect Non-Invasive Fibrosis Bio-Markers versus Liver Biopsy to Stage- Hepatic Fibrosis in Patients with Chronic HCV Infection with and without Schistosomiasis

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Back ground: HCV and Schistosomiasis are the most serious health burden in Egyptian community. **Aim:** we aimed to compare the value of direct (serum Hyaluronic acid (HA)) and indirect fibrosis markers versus liver biopsy to stage liver fibrosis and activity in patients with chronic HCV infection either co-infected with bilharziasis or not, and to study the effect of the current standard of care therapy on the serum level of these biomarkers. **Subjects and methods: 100** patients with chronic HCV (50 patients were co-infected with Bilharziasis (they were positive for anti biharzial antibodies), the other 50 patients were negative for anti-bilharzial antibodies), all were eligible for anti viral therapy according to the National Egyptian Program for treatment of chronic hepatitis C, all patients were investigated for serum alanine transaminase (ALT), aspartate transaminase (AST), albumin, total bilirubin, prothrombin time and concentration, complete blood count, hepatitis B surface antigen (HBs Ag), HCV Abs, HCV-RNA by quantitative polymerase chain reaction, abdominal ultrasound and ultrasonic-guided liver biopsy. The following ratios, scores and indices were calculated and compared with the results of liver biopsy, AST/ALT ratio (AAR), age platelet index (API), AST to platelet ratio index (APRI**)** and serum Hyaluronic acid (HA) before and after end of therapy. **Results:** HA is a good sensitive, bad specific to diagnose fibrosis and cirrhosis (73.9%, 66.7%). AST is a good sensitive, bad specific to diagnose fibrosis and cirrhosis (73.9, 49.1). Hyaluronic acid /platelets ratio is a good sensitive, bad specific to diagnose fibrosis and cirrhosis (73.9%, 59.3%). There is no statistical significant difference between both HCV group with bilharsiasis and HCV without bilharziasis regarding HA (*p*- value > 0.05). There is highly statistical significant difference between basal and follow up of serum HA (*p*- value < 0.01). There is no statistical significant association between HA and activity grade in both groups (*p*- value > 0.05). There is statistical significant association between HA and sustained viological response to interferon treatment in both groups (*p*- value < 0.05).
Conclusions: The use of indirect and direct (HA) biomarkers may reduce the need for liver biopsy, HA could also predict the sustained virological response to interferon in the studied patients with chronic HCV with and without bilharziasis

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Key words: Hyaluronic acid-Liver fibrosis

Introduction:

Ten years ago, it was stated that Hepatitis C virus (HCV) infection is a worldwide disease that affects more than 170 million people, now the accurate number of infected people is not exactly known but it is expected to be rising. Most of patients (80-90) % of them develop chronic hepatitis which may progress to cirrhosis and hepatocelluar carcinoma [1].

Assessment of the severity of liver injury is mandatory for lot of decisions: first we need to categorize the patients according to the severity of liver injury, second we are in need to plan for treatment by current anti viral therapy, third we have to expect the possible HCV induced cirrhosis and its complication that might be fatal for patients as HCC and haematesis. Follow up of those patients depends also on the degree of severity of liver injury.

Therefore, panels of investigations have been tried ranged from simple non-invasive imaging and laboratory investigation to liver biopsy [2].

However a lot of direct and indirect biomarkers were studied to replace or help the liver biopsy to give the accurate diagnostic stage of liver fibrosis in different causes of liver injuries.

Till now liver biopsy is the gold standard diagnostic tool to give the accurate diagnosis of liver fibrosis, it has many advantages over any other diagnostic modality. It gives the stage of fibrosis, activity of liver injury and it can give an idea about the possible etiological factors or associated pathologies as auto-immune hepatitis or steato-hepatitis [3].

However many complications or controversies were reported with liver biopsy; it is an invasive procedure with many reported complication that

ranged from minor bleeding or pain to reported cases of mortalities, not applicable for all individuals as in presence of coagulation defects, not real time procedure (gives an idea only at the time of examination), variation in reading between different investigators, cannot be easily repeated, costly and requires hospitalization for at least 4-6 h in our center and sampling errors [3.4]. This initiated the development or innovation of many new non invasive diagnostic tools.

Egypt has the highest prevalence of HCV worldwide (15%) and the highest prevalence of HCV genotype 4, which is responsible for almost (90%) of HCV infections [6].

Aim of the study:

We aimed in the present study to compare the direct (serum HA) and indirect fibrosis markers versus liver biopsy to stage liver fibrosis and activity and to study the effect of the current standard of care therapy on the serum level of these biomarkers. We compared these data between patients with chronic HCV infection with and without bilharziasis.

Subjects and methods:

From May 2010 to January 2012, 100 patients suffering from chronic HCV were evaluated in the Interferon outpatient clinics-Hepatology department-National Liver Institute- Menoufiya University (a tertiary referral center for liver disease), patients were prospectively evaluated for eligibility for Interferon therapy. One handed patients were selected within the criteria of Egyptian Ministry of Health (the National Treatment Program for HCV), they were 65 male patients and 35 females (age range; 21-60 years). The patients were prospectively followed before, during, after the end of course of therapy and 6 months later. Patients were divided into 2 groups according to their Anti-Bilharzial sero-status each group included 50 patients.

Exclusion criteria:

We excluded patients with positive HCV anti bodies and negative HCV PCR, we also excluded patients with cirrhosis, combined HCV and hepatitis B virus and patients with an associated diseases (as autoimmune hepatitis and HCV).

Specimen Collection:

Under complete aseptic technique, 10 ml of blood was taken from all subjects. Blood was allowed to clot naturally in a test tube after taking 2 ml for complete blood count. Serum was separated, divided into small aliquots and stored at – 60 °C till being tested.

Investigations:

According to the National Egyptian Program for the treatment of chronic HCV infection: we reviewed all studied subjects with history taking and we examined them for clinical features of liver disease. Liver function tests: HCV anti bodies using 4th generation ELIZA. Total and direct bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total proteins and serum albumin, Prothrombin time and INR, HBV sAg, Anti Nuclear Antibodies (ANA), Alpha feto-protein (AFP), anti bilharzial anti body, TSH, Fasting blood sugar, quantitative HCV PCR, pregnancy test in married females in child bearing period and liver biopsy. Serum HA was measured in all patients.

Statistical Methods

Data was statistically analyzed using SPSS (statistical package for social science) program version 20 for windows and for all the analysis a p value < 0.05 was considered statistically significant:

- Data are shown as mean, range or value and 95% confidence interval (95% CI) and frequency and percent.

Student t- test was done for normally distributed quantitative variables to measure mean and standard deviation and p-value < 0.05 was considered significant.

Mann-Whitney test was done for quantitative variables which are not normally distributed and p-value < 0.05 was considered significant.

Roc curve (Receiver operating characteristic curve) : was done to detect cut level of any tested variable where at this level there is a the best sensitivity and specificity cut off values of the variables for the presence of the disease moreover, they were used to identify the cut off the prevalence adjusted negative and positive values for the presence of the disease . The validity of the model was measured by means of the concordance © statistic (equivalent to the area under the Roc curve). A model with a c value above 0.7 is considered useful while a c value between 0.8 and 0.9 indicated excellent diagnostic accuracy.

Paired t test was done to detect mean and standard deviation of normally distributed pre and post values of the same variable of the same group of patients and p-value < 0.05 was considered significant.

All data are tested with kolmogorov-Smirnov Z test and most of them were found normally distributed and so presented with mean ± SD and using parametric testes on doing association.

Results:

Table 1: Descriptive personal and laboratory data of both groups:

Variable	**HCV group (n= 50)**	**HCV + Bilharzial co-infection (n= 50)**	***p*- value**
Age (yrs)	43.73 ± 8.47	36.29 ± 9.61	< 0.05*
Gender (M/F)	M35: F15	M44 :F6	
Weight(kg)	79.12 ± 10.26	74.63 ± 7.72	0.715 NS
Height(Cm)	171.62 ± 21.87	172.99 ±14.70	0.289 NS
BMI	28.85 ± 13.33	25.47 ± 5.78	0.104 NS
Fibrosis F1/F2/F3/F4/F5/F6	14/17/4/10/3/2	20/13/6/7/3/0	0.479 NS
Activity of fibrosis A1/A2	1/49 (2%/98%)	2/47 (4%/94%)	0.503 NS
Glucose	101.96 ± 28.39	97.94 ± 25.20	0.456 NS
Creatinine	0.80 ± 0.09	0.78 ± 0.13	0.328 NS
AST (U/L)	44.29 ± 19.43	45.10 ± 20.12	0.838 NS
ALT (U/L)	44.20 ± 19.14	44.67 ± 18.76	0.901 NS
T. bilirubin (TlBL)	0.88 ± 0.12	0.89 ± 0.20	0.873 NS
Albumin (g/dl)	4.32 ± 0.33	4.42 ± 0.38	0.174 NS
WBCs (10^3/mm^3)	4.30 ± 0.89	4.55 ± 1.09	0.219 NS
Hb (g/dl)	11.96 ± 2.27	11.88 ± 1.49	0.848 NS
Platelets (10^3/mm^3)	176.37 ± 55.06	171.70 ± 51.58	0.663 NS
HAB	40.96 ± 19.18	36.65 ± 17.89	0.256 NS
HAA	32.63 ± 12.23	31.92 ± 13.57	0.788 NS
A.F.PT	8.95 ± 14.28	6.95 ± 8.00	0.390 NS
TSH	1.11 ± 0.37	1.27 ± 0.72	0.167 NS
ALP (U/L)	114.88 ± 33.75	144.53 ± 39.17	< 0.05*
PT	13.32 ± 0.56	13.35 ± 0.42	0.732 NS

Data are expressed as mean ± SD or number (%).
M= male; F= female. NS=*p*> 0.05= not significant.
**p*< 0.05= significant.
Alkaline phosphatase was significantly high in patients with bilharziasis.

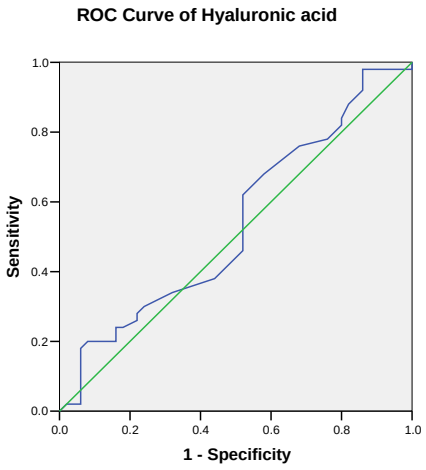

Figure (1): ROC of sensitivity and specificity of HA in different studied groups

Table 2: AUC, cut-off values, sensitivity and specificity of HA marker for HCV versus HCV and Bilharzial co-infection

	AUC	Cut-off	Sensitivity (%)	Specificity (%)
HA	0.54	28.5	76.0	32.0

AUC- area under the curve

Hyaluronic acid is a good sensitive, bad specific to diagnose Fibrosis HCV with bilharziasis.

Table 3: Sensitivity, specificity and positive (PPV) and negative (NPV) predictive values of different biomarkers to diagnose severe fibrosis or cirrhosis (stages 4–6) based on its observed prevalence of 23%

Cut-off value for biomarkers	Sensitivity	Specificity	PPV	NPV
Hyaluronic acid (HA)				
32.5	73.9	66.7	33.3	26.1
AST index/platelet count ratio				
250.2	73.9	49.1	50.9	26.1
HA/platelet count ratio				
169.69	73.9	59.3	40.7	26.1

Hyaluronic acid is a good sensitive, bad specific to diagnose severe fibrosis or cirrhosis.

AST is a good sensitive, bad specific to diagnose severe fibrosis or cirrhosis.

HA/platelets ratio is a good sensitive, bad specific to diagnose severe fibrosis or cirrhosis.

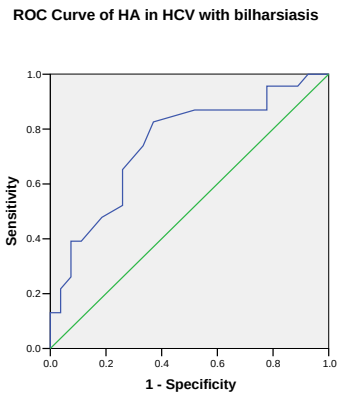

Figure 2: ROC of HA in HCV with bilharziasis:

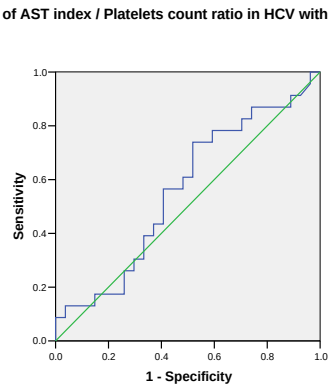

Figure 3: ROC of AST/platelet count between studied groups:

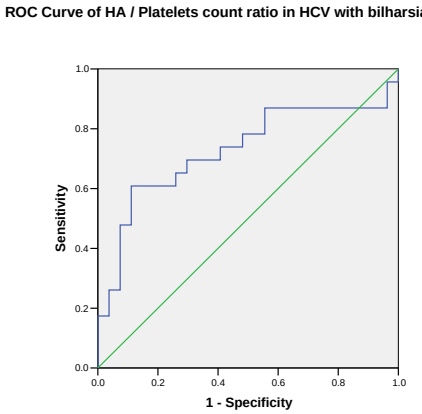

Figure 4: ROC of HA /platelet ration between studied group

Table (4): The base line serum HA and after end of treatment.

	Mean ± SD	Paied t- test	*P*- value
Hyaloronic acid basal Hyaloronic acid follow up	40.24±19.16 32.36± 12.18	5.36	< 0.01

There is highly statistical significant difference between basal and follow up of HA (*p*- value < 0.01).

Table (5): Differences between HCV with no bilharsiasis and HCV with bilharsiasis regarding HA.

	Group2	Mean ± SD	Mann Whitney test	*p*- value
Hyaloronic acid	HCV with no bilharsiasis HCV with bilharsiasis	40.24±19.16 37.00±17.87	0.67	> 0.05

There is no statistical significant difference between both HCV group with bilharsiasis and HCV with no bilharziasis regarding HA (*p*- value > 0.05).

Table (6): Association between HA and early virological response to interferon in both group

Hyaluronic acid	Groups	Mean ± SD	Mann Whitney test	*p*- value
HCV group without bilharziasis	Responders Non responders	35.83±19.96 44.31±17.81	2.64	< 0.01
HCV group with Bilharziasis	Responders Non responders	37.63±22.44 36.61±14.81	0.81	> 0.05

There is statistical significant association between HA regarding early virological response in patients without bilharziasis (*p*- value > 0.05) while there was no significant association in patients with bilharziasis (*p*- value > 0.05).

Table (7): Association between HA and fibrosis grade in each HCV group with bilharziasis and HCV group with no bilharziasis.

Group	Fibrosis grade	Mean ± SD	Mann Whitney test	*p*- value
HCV without bilharziasis	F1, F2, F3 F4, F5	29.15±18.83 40.79±19.74	2.02	< 0.05
HCV with bilharziasis	F1, F2, F3 F4, F5	30.37±9.34 44.78±22.16	2.97	< 0.01

There is statistical significant association between HA and fibrosis grade in HCV group with and without bilharziasis (*p*- value < 0.05 and 0.01).

Table (8): Association between HA and activity grade in each HCV group with bilharsiasis and HCV group with no bilharziasis.

Group	Activity grade	Mean ± SD	Mann Whitney test	*p*- value
HCV with no bilharsiasis	A1 A2	55.00±0.0 39.94±19.24	1.08	> 0.05
HCV with bilharsiasis	A1 A2	34.00±5.57 37.19±18.39	0.08	> 0.05

There is no statistical significant association between HA and activity grade in each group of HCV (*p*- value > 0.05)..

Table (9): Association between SVR and HA in each group with bilharziasis and HCV group with no bilharziasis.

Group	Sustained virological response	Mean ± SD	Mann Whitney test	*p*- value
HCV without bilharziasis	Responder Non responder	27.94±5.39 51.63±28.63	2.09	< 0.05
HCV with bilharziasis	Responder Non responder	33.00±18.23 62.33±30.83	2.36	< 0.05

There is statistical significant association between HA and response to interferon treatment in each group (*p*- value < 0.05).

Discussion:

Tarter emetic was used since 1921 as anti-bilharzial therapy. From the 1950s until the 1980s, the Egyptian Ministry of Health initiated the mass treatment program for bilharziasis control using intravenous tartar emetic (10- to12-dose course), This was accused by many investigator as a major source to infect a lot people at this time with HCV and possibly with other blood born viruses. The newly infected people represent the reservoir for newly developed cases for decades later [8].

A second link between HCV and Bilharziasis is that the Bilharzisis can cause imbalance in HCV-specific T-cell responses leading to increased viral load, higher rates of HCV chronicity, more liver injury and rapid progression to cirrhosis in co-infected persons [9].

In Egypt, parenteral anti bilharzial therapy was not the only risk factor to get chronic HCV infection and other blood porn viruses, high prevalence rate of HCV infection was seen in older age, male sex, blood transfusion – before nineties-, contact with hospital or clinics with invasive medical procedures, injections, circumcision of boys by "informal" health care providers [9 and 10].

Direct biomarkers are defined simply as serum components with direct relation to the mechanism of fibro-genesis, either as a matrix-related components of activated hepatic stellate cells and fibroblasts or as mediators of Extra-Cellular Matrix synthesis or turnover (e.g. hyaluronan, laminin, type IV collagen, matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-1, and amino-terminal peptide of pro-collagen III) [10].

Liver is proved to be one of the organs which contain the highest hyaluronidase activity, so liver damage by different etiologies including HCV and Bilharziasis might decrease the degradation of HA and hence increases its serum level [11]. It is found that the serum HA level was related to the degree of liver fibrosis, it is more accurate than serum amino-terminal pro-peptide of type III pro-collagen to assess the degree of fibrosis and can be used as a marker for studying the liver fibrosis, It can be used to monitor the response to anti viral therapy.

Our study was a prospective controlled study; we evaluated all patients attending the interferon clinics for evaluation for eligibility of the current standard of care therapy in Egypt (Interferon based therapy). We selected 100 patients who were eligible for therapy according to the National Egyptian Program for treatment of HCV, 50 patients were co-infected with HCV and Bilharziasis (with positive anti Bilharzial antibodies) and the rest were mono-infected with HCV (negative for anti-Bilharzial antibodies).

In our series we followed the patients prior, during, after the end of treatment by IFN and 6 months later by routine tests plus the serum level of HA, we found that the HA was a good sensitive marker for diagnosis of liver fibrosis. However no significant difference was noted between both groups. This might reflect that the serum HA level is correlated to the degree of liver fibrosis regardless the etiological agents.

However the use of HA in assessment of activity of liver injury was not significantly good, so we could consider the HA is a reflection of degree of liver fibrosis but not the activity.

In addition we studied the effect of antiviral therapy on the serum level HA prior and after end of treatment, a significant difference was noted, this might argued to the positive impact of antiviral therapy leading to decrease liver injury and increase the liver capacity for degradation of the HA.

In our subjects a significant drop of the serum HA in the responder group than non responders, this might explained by that in the responder side either mono or co-infected with Bilharziasis, the clearance of the virus has better impact on the liver histology and function leading to increase production of enzymes responsible HA degradation.

Similarly in 1995 Guechot, et al. evaluated the potential value of serum HA and liver fibrosis, inflammation, and necrosis, before and after IFN-a therapy, they found a significant correlation between serum HA levels and liver histology using Knodell scores, serum HA levels were significantly lower after treatment only in non relapsers [12].

Liver injury leads to hepatic stellate cell activation and transformation to active myofibroblastic phenotype, and secrete a large amount of collagen with inhibition of collagenase activity [13]. Hence, activated stellate cells are the predominant source of the fibrillar ECM proteins characterizing hepatic fibrosis.

In 2004 the effect of HA was studied on hepatic stellate cells which were isolated from male Sprague–Dawley rats, the authors found that HA inhibits adhesion of quiescent or activated HSCs in spite of its stimulation of DNA synthesis, this is indicative that HA might has a role in the process of liver fibrosis [14].

More recently, Attallah et al. studied HA and its degradation products in sera of 153 patients, they concluded that HA can differentiate between chronic HCV with and without liver cirrhosis with accuracy up to 96% of the cirrhotic patients at a cut-off score=2.5 (i.e. less than 2.5 greater than 2.5 indicated liver cirrhosis). The positive predictive and negative predictive values were also high (95% and 97%, respectively) [15].

Lazarova et al. studied the serum HA alone or included in Hepascore showed a good ability to detect all stages of fibrosis in chronic liver disease. The correlation between HA. With these preliminary data, it appears that HA could exclude cirrhosis from advanced fibrosis. Therefore, the developed HA assay can be applied in clinical practice to evaluate liver fibrosis, but they did not differentiate between responders and non responders or the follow up after the end of treatment [16].

In 2005, Calès et al. constructed a series of tests called FibroMeters, they are direct, non-invasive quantitative tests to measure liver fibrosis [17].

Veillon et al. assayed the serum HA in patients with liver fibrosis and its impact on adding it to fibro Meter, it was very good. However with little impact on adding it to the Fibro Meter [18].

Serum HA was not used only in adult for evaluation of liver fibrosis but it was utilized in children with non alcoholic steato-hepatitis, the authors concluded that HA was useful predictor in staging hepatic fibrosis: values of HA≥1200ng/ mL made the absence of fibrosis (F0) is unlikely whereas values of HA≥2100 ng/mL made F2, F3, or F4 fibrosis likely, however further work is needed to support these data [19].

Many authors revised HA in combination with other suggested serum fibrosis markers to improve its diagnostic accuracy; Valva et al. applied serum TGF-ß1, tissue inhibitor of matrix metalloproteinase inhibitor-HA and amino-terminal peptide of pro-collagen type III and compared them to liver biopsy in 22 pediatric and 22 adult HCV patients, the authors concluded that HA and the other used fibrosis parameters can predict fibrosis and the liver biopsy could be avoided, the authors suggested an important notice that; serum fibrosis markers can be applied as diagnostic mirror to follow liver fibrosis (20). However further work is needed in long term study and in addition we should compare between responders and non-responders as in non-responders the progression of liver fibrosis is strongly expected

Arain et al. evaluated another group of suggested variables including: the patient age and serum fibrosis markers (alanine transaminase, gamma-glutamyltranspeptidase, apolipoprotein A-1, alpha-2 macroglobulin and hyaluronic acid). and collectively named them Liver-score. The authors applied these investigations to patients with chronic HCV in total of 98 patients, the area under the curve was found to be 0.813. On a 0-1 scale, negative predictive value at a cutoff level of ≤ 0.40 was 83%, while positive predictive value at ≥ 0.80 remained 89%. Altogether, 61% of the patients had these discriminative scores. These data can be applied in the clinical practice to avoid liver biopsy (21)

Lia et al. added the serum laminin to HA in assessment in patients with chronic HBV infection as serum markers for predicting significant fibrosis in 87 patients and 19 blood donors versus liver biopsy. The diagnostic performances of all indices were evaluated by the receive operating characteristic (ROC) curves. Serum HA and LN concentrations increased significantly with the stage of hepatic fibrosis, which showed positive correlation with the stages of liver fibrosis. Serum HA and Laminin showed positive correlation with the stages of liver fibrosis (22).

Conclusion and recommendation:

We concluded from our study that the HA assessment could be useful in evaluation of liver fibrosis as a direct biomarkers and could be useful as a predictor of response to current standard of care therapy, it can be also applied in the situations when liver biopsy is contraindicated, a very good role could be suggested for HA as a long term quantification of liver fibrosis, however further work is needed to evaluate its role. The presence or absence of Bilharziasis has no impact on the assessment of HA as HA might reflect the liver fibrosis stage, this suggestive that HA reflect the degree of liver fibrosis regardless the etiology of liver disease.

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