Diagnostic Values of Some Non- Invasive Biomarkers in Patients with Different Stages of Chronic Hepatitis C

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Abstract: Liver biopsy is still a disturbing method subjected to spectator inconsistency for staging hepatic fibrosis. The study was conducted to evaluate procollagen III peptide, hyaluronic acid, and fibronectin as diagnostic biomarkers in hepatic fibrosis among HCV patients. Serum and/or plasma were collected from 38 patients with different grades of hepatic fibrosis, portal, portolobular fibrosis and cirrhosis were investigated. All patients with fibrotic liver had significantly higher levels of both procollagen III peptide and hyaluronic acid than healthy controls (n=16). Theses markers were higher in patients with hepatic fibrosis (n=22) than the remaining non fibrotic cases. Regarding fibronectin plasma levels, in significant difference was found between normal subjects and patients with grade 0, 1 or 2 fibrosis. Higher positive and negative predictive values for diagnosis of fibrosis and cirrhosis were observed with hyaluronic acid (83% and 82%) respectively, while lower predictive values were observed with fibronectin (60% and 63%) respectively. The diagnostic value of serum hyaluronic acid is greater than both serum Procollagen III peptide and plasma fibronectin for different grades of lever fibrosis. Therefore, procollagen III peptides should be preferred as a non-invasive as a biomarker test for monitoring the early fibrotic process in HCV patients where fibronectin was detected only in the advanced cirrhostic cases.
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1. Introduction

Chronic hepatitis C (CHC) is a slow, systematic inflammatory liver disease causing both acute and chronic hepatitis infections (Zampino et al., 2013). Up to date, liver biopsy is the golden standard technique for grading and assessing liver diseases impairments for histological examination and assessing liver lesions. They became an invasive method subjected to sampling errors, intra and/or inter observer variability and potentially associated with many complications. Furthermore, ethically they cannot be repeated to monitor liver status (Mendes et al., 2018). Still, patients can have no clinical signs of cirrhosis and fibrosis accompanied by no evidence of the disease from non-invasive tests such as liver function test, platelet counts, serum albumin levels, hepatoplasmin test and ultrasonography (Holmberg et., 2013).

Hepatic fibrosis (HF) stimulates expression and accumulation of extracellular matrix proteins via breakdown of the matrix proteins by matrix metalloproteinases (Fontana et al., 2010). These matrix proteins can be divided into collagens (types I and III being predominant in the liver, with lesser amounts of types IV, V, and VI), non-collagenous glycoproteins (fibronectin, laminin, entactin, osteonectin, and elastin), proteoglycans (heparan, dermatan, and chondroitin sulfates), and polysaccharides (hyaluronan and hyaluronic acid (Mouw and Weaver 2011). Mainly, fractions of the newly synthesized biomatrix proteins escape into the systemic circulation (El Guesiry et al., 2015). Therefore, it is necessary to find a sensitive and specific direct diagnostic test as an indicator for HF in HCV patients (Liakina et al., 2015).

Different separate research studies accepted particular procollagen III peptide (PIIIP), fibronectin (FN) and hyaluronic acid (HA) biomarkers as indices of the extent of HF in chronic liver diseases. They became measurable in elevated levels in patients sera (Nassef et al., 2013, Abdel-Latif, Elesawy et al., 2014 & Deng et al., 2014). Serum biomarkers levels have been proposed as useful parameters for studying the fibrotic process in chronic liver diseases. In other words, they may be useful as non-invasive approaches to distinguish cirrhotic from non-cirrhotic patients (Attallah et al., 2015).

Therefore, the current study was performed to characterize the diagnostic application of serum PIIIP, HA and plasma FN biomarkers as non-invasive tools for diagnosis of HF, and to investigate their relations to morphological features of liver disease in a group of patients suffering from CHC. It is claimed that the present study is the first of its kind that combines these as biomarkers in one study.

2. Material and Methods

A-Subjects and Sampling

Whole venous blood samples were collected from 38 patients ranging in age between 25 and 45 (33±7.6 years), recruited from outpatient clinics of Suez Canal University hospital. They were presented to the center for viral hepatitis C management and follow up. All the selected cases had persistently high serum ALT (more than twice the upper limit of normal values) for at least 6 months and three determinations. All had anti-hepatitis C virus antibodies (positive by 2nd-generation ELISA). Average viral load was 4×10^5 HCV RNA (IU/ml). All had elevated hepatic enzymes for more than 6 months. None had been previously treated for hepatitis C. Patients with evidence of hepatitis B virus infection (detectable serum HBV surface antigen) or bilharziasis were excluded from the study. Sixteen subjects with normal liver function tests, had no history suggestive of liver disease, bilharziasis, gallbladder stones, negative serological test for B and C viruses, and matched for age and sex, were considered as a control group. None of the patients and health subjects had clinical, biochemical, or histological evidence of chronic renal disease, pulmonary fibrosis or arthropathy. Serum and plasma samples (using EDTA as an anticoagulant) were stored at –20^{o}C till biochemical measurements.

Ethical consideration: Informed consent was obtained from all patients and volunteers. The aim and the value of the current work were explained in a simplified manner for them. The methodology and ethical aspects were approved by the research committee.

B- Liver function tests

All participants underwent a baseline medical history, clinical examination and biochemical study. Initial screening with laboratory tests were commonly performed. AST, ALT, albumin (ALB), bilirubin (BIL), alkaline phosphatase (ALP) and international normalized prothrombin (INR) were measured by means of the Automated Analyzer Hitachi 704 and employing their own specific reagents furnished by Boehringer Mannheim GmbH, Gemany (Lentjes et al., 1987).

Serum ALB (gm %) was determined by colorimetric method with bromocresol green at 340 nm (Doumas et al., 1971). BIL (U/L) was measured using the colorimetric method, in which BIL reacts with the diazotised sulphanilic acid in the presence of caffeine, resulting an azobilirubin pigment and which was measured at 546 nm (Doumas et al., 1973). Serum ALT and AST levels (IU/L) were measured by the enzymatic optimized standard method, in which transferring of an amino group from ALT or AST forms pyruvate or oxaloacetate, respectively. The developed color was measured at 520 nm (16. Gella et al., 1985). Finally an enzymatic method was used for the determination of ALP based detection of yellow color for p-nitro-phenylphosphate (Ishak 2000).

C- Investigated fibrosis biomarkers

Biomarkers of liver fibrosis include PIIIP, HA and FN for detecting HF. Serum PIIINP was measured according to Galambos et al. 1985 by commercially available radioimmunoassay kit (Procollagen III Peptide RIA, ALPCO, Catalog Number #72-OCFK07-PIIIP, (RIA-gnost® PIIIP, Behringwerke AG, Marburg, Germany).

Plasma FN levels were assessed using commercially available ELISA purchased from (Bender Medsystem GmbH, Code No. BMS2028, Vienna, Austria, Europe). The ELISA assay was processed according to the manufacturer's protocol. FN was captured from plasma with first polyclonal antibody coated polyvinyl solid surface, then bounded to a biotin-conjugated polyclonal anti-fibronectin antibody, then captured by streptavidin- horseradish peroxidase to bind conjugated anti-fibronectin, then detected with a chromogenic substrate (3,3',5,5'-Tetramethylbenzidine/H_2O_2). The developed color was measured at 450 nm (Fouad et al., 2013).

Serum HA levels were detected with ELISA kit, purchased from (Corgenix, Broomfield, CO, USA). The ELISA assay was processed according to the manufacturer's protocol. The HA test based an enzyme-linked binding protein assay that based capturing of HA-binding protein (HABP), then conjugated with horseradish peroxidase to be detected with a chromogenic substrate (3,3',5,5'-Tetramethylbenzidine/ H_2O_2) The developed color was measured at 450 nm (Theise 2007).

D-Histopathological examination

The liver histopathological changes were assessed and graded by independent pathologist experts using formalin-fixed, paraffin-embedded liver biopsies. Fibrosis staging was semi-quantitatively assessed according to the METAVIR system (Brunt 2000 and Theise 2007). Fibrosis were classified into grade 0 for no fibrosis, grade 1 for fibrous within and around the portal tracts (mild fibrosis), grade 2 for bridging fibrosis, thick fibrosis in the portal tracts with bridging between portal tracts and central veins (severe fibrosis) and grade 3 for nodular regeneration (cirrhosis).

E-Statistical analysis

Data were presented in terms of mean and standard deviation (SD) of the mean, and percentages. Statistical analysis was carried out by (SPSS Ver. 20). Student-t test, Chi-square, and correlation tests were used to evaluate the results. *P* value was set at <0.05 for statistically significant results and <0.001 for highly significant results. Comparison among subgroups was performed by analysis of variance (ANOVA). The correlation among the various

parameters were assayed by linear regression analysis (for continuous variables) and by means of Kendall Rank correlation test. Receiver operating characteristic curves were performed to obtain the cut-off values. Diagnostic sensitivity, specificity and predictive values were defined as well (Replogle et al., 2009).

3. Results

The patients and the control subjects were well matched regarding age, sex, and body mass index (BMI) as shown in table (1). There were no significant differences between both groups regarding to age and BMI. Biomarkers of liver function tests, serum ALP, ALT, and AST levels were significantly higher in the patient group than for the control group as shown in table (2). No significant differences observed between both groups regarding BIL, and ALB levels. Table (3) shows that, out of the 38 patients with CHC, 16 patients had no fibrosis (grade 0, 42.2%). Twenty two patients had a fibrotic liver (57.8%), 50% of them had grade 1, 27% had grade 2, and 23% had grade 3. Serum PIIIP concentration in the patient group ranged from 15.2-98.4 ng /ml (39.5±12.2), significantly higher than that in the healthy subjects (14.8±5.2). Stratification of patients into subgroups (CHC with and without histological features of HF), only the groups with grade 2 (severe fibrosis), and grade 3 (cirrhosis) had a higher PIIIP than controls. The CHC patients without fibrosis had a lower PIIIP than those with fibrosis (24.6±4.8 vs 52.2±15.7), which did not significantly differ from the control group. On the other hand, plasma FN was not significantly different from the control subjects (26.2±6.3) and in CHC with or without fibrosis (47.9±9.9 vs 36.1±8.2). A significant difference was observed only between the control group and those CHC patients with histologically features of cirrhosis (26.2±6.3 vs 65.2±13.2).

Serum HA concentration in the patient group ranged from 22.6–107.5 ng/ml (48.4+26.9) being significantly higher than that in the healthy subjects (18.2+7.2). All patients with various grades of fibrosis (grade 1, 2, 3) had a higher serum HA than either controls or patients without fibrosis (grade 0). The CHC patients without fibrosis had a higher level of HA, but did not significantly differ from the control subjects (27.3±9.4 vs 18.2±7.2).

Table (4) represents the correlation between the investigated biochemical markers (serum PIIIP, serum HA and plasma FN) and the biochemical liver function tests. It appears that PIIIP and HA correlate significantly only with ALT, AST (P<0.01) and ALP (P<0.05). No significant inter-correlations were observed between PIIIP, FN and HA.

The predictive values, sensitivity and specificity of each marker is shown in tables 5 and 6 for diagnosis of both HF and cirrhosis. The selected cut-off values for diagnosing HF were 55.2, 42.7, and 76.2 ng/ml for FN, PIIIP, and HA, respectively. The selected cut-off values for diagnosing cirrhosis were 63.2, 51.3, and 84.3 ng/ml for FN, PIIIP, and HA, respectively. Higher predictive values for fibrosis and cirrhosis diagnosis were observed for HA (83% and 82%), while the lowest predictive values were observed for FN (60% and 63%).

Table (1): Age, sex and BMI in both control and patient groups.

		Age (years)	Sex (male %)	BMI %
Control (N=16)	Mean ±SD	34.4 ±5.2	87.5	23.1 ±1.1
Patients (N=38)	Mean ±SD	35.2 ±7.2	89.5	23.5 ±0.8
P value		n.s	n.s	n.s

SD: standard deviation, BMI: body mass index, n.s: non significant, N: number of cases

Table (2): Liver function tests (ALB, BIL, ALP, ALT, and AST) in both control and patient groups.

		ALB (gm%)	BIL (U/L)	ALP (U/L)	ALT (U/L)	AST (U/L)
Control (N=16)	Mean ±SD	4.7 ±0.7	3.2 ±0.2	54.3 ±12.5	22.3 ±6.4	25.3 ±7.2
Patients (N=38)	Mean ±SD	3.9 ±0.6	5.3 ±2.1	87.2 ±22.9	109.3 ±32.4	105.4 ±43.7
P value		n.s	n.s	<0.05	<0.01	<0.01

ALB: albumin, BIL: bilirubin, ALP: alkaline phosphatase, ALT: alanine transaminase AST: aspartate transaminase, SD: standard deviation, n.s: non significant

Table (3): Mean values of the biochemical markers in CHC patient group (with different grades of hepatic fibrosis) and the control subject.

		Control (N =16)	CHC without fibrosis (N=16)	CHC with hepatic fibrosis (n=22)			
				Grade1 (N=11)	Grade2 (N=6)	Grade3 (N=5)	Total (N=22)
PIIIP (ng/ml)	Mean	14.8	24.6	39.5	70.2	84.3	52.2
	SD	±5.2	±4.8	±11.2	±17.2	±21.2	±15.7
	P	--	n.s	n.s	<0.01	<0.01	<0.05
FN (ng/ml)	Mean	26.2	36.1	42.1	46.2	65.2	47.9
	SD	±6.3	±8.2	±11.1	±14.2	±13.2	± 9.9
	P	--	n.s	n.s	n.s	<0.05	n.s
HA (ng/ml)	Mean	18.2	27.3	61.1	78.3	89.2	77.3
	SD	±7.2	±9.4	±14.2	±16.3	±21.2	±22.3
	P	--	n.s	<0.01	<0.01	<0.01	<0.01

PIIIP: procollagen III peptide, FN:fibronectin, HA: hyaluronic acid, SD: standard deviation, N: number of cases, n.s: non-significant, *P*: *P* value

Table (4): Correlation between the investigated biochemical markers (PIIIP, FN, and HA) and the liver function tests (ALB, BIL, ALP, ALT, and AST) in CHC patient group

		ALB	BIL	ALP	ALT	AST	PIIP	FN	HA
FN	r	0.32	0.44	0.52	0.61	0.63	0.31	--	0.41
	P	n.s	n.s	n.s	n.s	n.s	n.s	--	n.s
PIIIP	r	0.42	0.55	0.77	0.85	0.81	-- --	0.31	0.38
	P	n.s	n.s	<0.05	<0.01	<0.01		n.s	n.s
HA	r	0.61	0.42	0.46	0.76	0.75	0.38	0.41	--
	P	n.s	n.s	<0.05	<0.01	<0.01	n.s	n.s	--

ALB: albumin, BIL: bilirubin, ALP: alkaline phosphatase, ALT: alanine transaminase AST: aspartate transaminase, PIIIP: procollagen III peptide, FN: fibronectin, HA: hyaluronic acid, n.s: non significant

Table (5): Sensitivity, specificity, and predictive values of the biochemical markers (PIIIP, FN, HA) for the diagnosis of fibrosis in CHC patient group.

	Cut-off value	Sensitivity%	Specificity%	PPV (%)	NPV (%)
FN (ng/ml)	55.2	61	58	60	59
PIIIP (ng/ml)	42.7	79	76	77	76
HA (ng/ml)	76.2	86	80	83	79
FN+PIIIP		84	79	83	81
FN+HA		89	84	87	82
PIIIP+HA		91	85	88	87
FN+PIIIP+HA		93	87	91	89

PIIIP: procollagen III peptide, FN: fibronectin, HA: hyaluronic acid, PPV: positive, predictive value, NPV: negative predictive value

Table (6): Sensitivity, specificity, and predictive values of the biochemical markers (PIIIP, FN, HA) for the diagnosis of cirrhosis in CHC patient group.

	Cut-off value	Sensitivity %	Specificity %	PPV (%)	NPV (%)
FN (ng/ml)	63.2	62	64	63	62
PIIIP (ng/ml)	51.3	77	74	75	76
HA (ng/ml)	84.3	81	82	82	81
FN+PIIIP		79	77	79	79
FN+HA		84	87	87	85
PIIIP+HA		88	89	89	87
FN+PIIIP+HA		92	91	93	88

PIIIP: procollagen III peptide, FN: fibronectin, HA: hyaluronic acid, PPV: positive predictive value, NPV: negative predictive value

4-Discussion

CHC prognosis is closely related to the development of HF. As the disease progresses, the liver parenchyma is replaced at first by connective tissue, then becomes fibrotic, and finally cirrhotic (Fernandes et al., 2015 and Zhu et al., 2015). Current assessments of HF necessitate histo-pathological examinations of percutaneous biopsy specimens. However, they are invasive and questionable because of the heterogeneous distribution of pathological changes in the liver (Lurie et al., 2015). On the contrary, the assessment of biochemical markers in blood is rapid, non-invasive and inexpensive. Recent studies explained a high predictive identification of the progression of the fibrotic process and hence its correct treatment. As a result, non-invasive biochemical markers for assessing HF in chronic hepatitis are being actively sought to help the evaluation of histological damage and to monitor fibrosis progression (Gökcan et al., 2016 and Veidal et al., 2010).

In the current study, the diagnostic application of serum PIIIP, HA and plasma FN measurement in CHC was evaluated. Different grades of HF were investigated, portal, portolobular fibrosis and cirrhosis. Data analysis had revealed that, the serum of the patients with fibrotic liver had significantly higher levels of both PIIIP and HA than control subjects. Also, patients with hepatic fibrosis had higher serum levels than the remaining non fibrotic ones. Different studies have found PIIIP to be associated with inflammation and necrosis, and histological activity (Giannini et al., 2001). The current study investigated a statistical significant correlation with ALT, AST, and ALP values that suggest that cytolysis and steatosis may be involved in the increase of PIIIP in the patients as a whole (Fontana et al., 2014, Abdel-Latif et al., 2014 and Attallah et al., 2013).

No significant correlations between FN and ALT, AST, ALP were present. Also, the current study data demonstrated that, FN does not distinguish between normal subjects and patients with grade 0, 1 or 2 fibrosis, confirming that FN was diagnostic only in advanced cases (cirrhosis). Therefore, FN measurement may be a useful test for monitoring the development of early grades of HF in CHC patients. On the other hand, a research study reported that, FN can be a useful marker for showing hepatic inflammation, fibrosis in cases of CHC, and evaluation of the response to the treatment plans. [30]

Differently from FN, serum PIIIP was significantly higher in CHC patients with fibrosis than in control subjects. Patients with minor fibrosis had a normal PIIIP values, excluding that the virus may affect the level of serum PIIIP. A similar note was already suggested by (Maxwell and Flisiak 2005). In contrast, an earlier study reported that there is no correlation between PIIIP and liver histology. Such conflict results may reflect differences in the selection of patients, also some unavoidable subjects in quantifying the histological features by means of numerical score (Colombo et al., 1985).

Being one of the glycosaminoglycans polysaccharide, HA close correlation to different grades of fibrosis in this study is an observation that was recorded in other studies (Guechot et al., 1996, Iushchuk 2005 and El-Kamary et al., 2015). Raising serum HA levels have been shown in different liver diseases. This is attributed to impairment of their clearance from the circulation by damaged liver endothelial cells (Valva et al., 2016).

Cut-off values of detected PIIIP, HA and FN concentrations for diagnosis of HF and/or liver cirrhosis were selected as the values that maximized the sum of sensitivity and specificity in a clinical situation where false-negative classifications could be considered as harmful as false-positive classifications (Griner et al., 1981 and Hawass 1997). At cut-off values of 42.7 for serum PIIIP, 76.2 for serum HA, and 55.2 ng/ml for plasma FN, sensitivities were 79%, 86%, 61%, specificities were 76%, 80%, 58 and predictive values were 76.5%, 81%, 59.5%, respectively, for discriminating patients with HF from those without fibrosis. However, at the cut-off values of 51.3 for serum PIIIP, 84.3 for serum HA, and 63.2 ng/ml for plasma FN, sensitivities were 77%, 81%, 62%, specificities were 74%, 82%, 64% and predictive values were 75.5%, 81.5%, 62.5%, respectively for discriminating patients with and without cirrhosis. These data revealed that serum HA had the greatest diagnostic performance, both for discriminating patients with HF from those with no fibrosis or for discriminating patients with cirrhosis from those without cirrhosis, where FN had the lowest one. However, other authors found a positive correlation between PIIIP and histological HF in patients with various types of chronic liver diseases arising from different etiology might further suggest that serum PIIIP is also a dependable marker of hepatic fibrogenesis (Attallah 2015 and Nassef et al., 2013).

Finally, other studies suggested that serum biomarkers are useful non-invasive methods for diagnosing severe fibrosis and cirrhosis and for excluding significant fibrosis in HCV patients thus reducing the need for liver biopsy. In addition, they are marked by safety, cost-effectiveness, and widespread accessibility (Crisan et al., 2012 & Stasi and Milani 2016). Further comparative research

studies are recommended to assess fibromarkers in relation to fibroscan.

Conclusions

The present evaluation study settled that (i) the diagnostic value of serum HA is greater than that of both serum PIIIP and plasma FN as a marker of HF. Therefore, serum HA should be preferred as a non-invasive biochemical test for monitoring early fibrotic processes. (ii) HA is more strongly correlated with histological grades of HF than serum PIIIP, this suggests that serum HA may be preferable for discriminating patients with cirrhosis from those without cirrhosis. (iii) Measurements of both HA and PIIIP together may be more useful in evaluating the hepatic fibrosis in CHC. In addition, measurement of both in sera is relatively easy to be performed. Overall, using combined biomarkers will be more diagnostic utilities, but the matter possibly will be of a further extra-cost. There are other extracellular matrix components and a combination of several serum biomarkers could increase their diagnostic values, therefore further studies are needed to confirm that. The current study was not designed to evaluate the possible role of PIIIP, HA, and FN in the follow up of the patients. Further studies are necessary in order to elucidate whether these biochemical markers have any value in this aspect or not. The main limitation of the present study is the sample size. A wide study using larger sample size is recommended.

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