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EVALUATION OF KALLISTATIN AS A BIOMARKER IN CHRONIC HEPATITIS C PATIENTS

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ABSTRACT

Early outcome prediction after hepatitis C viral infections has a great interest. This study aims to evaluate kallistatin in the prediction of liver fibrosis in chronic hepatitis C Egyptian patients. This study included 62 patients with chronic HCV infection (30 patients suffering from early liver fibrosis, 16 with advanced liver fibrosis, 16 with HCC) and 14 healthy subjects. Serum liver function tests were determined by colorimetric methods, HBsAg, HCVAb, AFP, CRP and kallistatin were investigated by ELISA, HCV-RNA and the expression of SERPINA4 gene were determined by qRT-PCR assay, abdominal ultrasound and ultrasonic-guided liver biopsy were done to determine the stage of fibrosis. Serum kallistatin level was significantly lower in patients with chronic liver disease (CLD) than healthy subjects at the gene expression and protein levels (P=0.003 & 0.001, respectively). Also, there was a significant difference in kallistatin concentration in early and advanced fibrosis, (p=0.044). Serum kallistatin had greater sensitivity and NPV values than did AST/ALT ratio (AAR) and AST/platelet ratio index (APRI) in patients with CLD compared with healthy subjects with sensitivity 95.1%, specificity 50%, PPV 89.2%, and NPV 70%. Moreover, Kallistatin could significantly distinguish patients at early stage of liver fibrosis from healthy subjects with sensitivity 96.7% and specificity 50%. Compared with single detection, combined measurement of the AAR, APRI and kallistatin markers showed 90% sensitivity and 78.6% specificity, 90% PPV and 78.6% NPV. These data support that kallistatin may be an efficient

biomarker in early detection of fibrosis. Also, it suggested that combination of kallistatin with AAR and APRI could improve the sensitivity and specificity for the diagnosis of CLD and this can be used as a practical method for clinical diagnosis for the early stage of liver fibrosis.

KEYWORDS: Chronic liver disease, Kallistatin, SERPINA4, qRT-PCR, ELISA.

INTRODUCTION

HCV is one of several factors inducing liver fibrosis and its consequences as liver cirrhosis (LC) and hepato-cellular carcinoma (HCC) over several decades.[1] Recent evidence indicates that around 71 million people are infected with HCV globally.[2] In Egypt, hepatitis C viral infection is endemic with the prevalence rate of 14.7% and this rate is considered the highest prevalence rate around the world.[3]

Hepatic fibrosis occurs due to the wound-healing response of the liver to repeated injuries, which contribute to the occurrence of chronic damage accompanied by activation of the innate immune system and progressive accumulation of extracellular matrix (ECM) proteins, including collagen type I and III.[4,5] As liver fibrosis advances, the bands of collagen, bridging fibrosis and frank cirrhosis forms and this is an important risk factor for portal hypertension, liver failure and development of primary liver cancer.[5]

Early detection of liver fibrosis is a vital need for successful management and prevention of the health problems associated with advanced liver cirrhosis and HCC, evaluating the therapeutic indications and improving the treatment regimens for better health community.

Liver biopsy (LB) is the gold standard for appraising hepatic fibrosis. However, this procedure has several drawbacks including being invasive test with the risk of complications, high cost, high rate of refusal by patients and sampling errors, which led to approximately 10–30% false negative result in cirrhotic patients.[6]

Nowadays, use of non-invasive approaches could be recommended as a screening tool, which allows physicians to determine patients who need further assessment of fibrosis by liver biopsy.[7]

The complexity of liver cirrhosis and the influence of a variety of genetic and environmental factors on the progression of disease and response to therapy are the major obstacles for

discovering a specific marker for non-invasive staging and early detection of fibrosis. Routine assessment of liver enzymes (ALT and AST) can increase the sensitivity and specificity of currently diagnostic and prognostic markers.

C-reactive protein (CRP) is a non-specific acute-reactant secreted by the liver in response to infections.[8] CRP levels are elevated during viral and bacterial infections, non-infectious inflammatory diseases and malignancies.[9] Alpha-fetoprotein (AFP) is a 72 kD glycoprotein produced normally by the liver and the fetal yolk sac. AFP levels are elevated in HCC, other neoplastic and non-neoplastic conditions.[10]

Kallistatin (Kal) is a 58-60 kD inhibitory protein of the serine proteinase inhibitors (SERPIN) family. In human, it is encoded by the SERPINA4 gene.[11] Kallistatin is widely expressed in organs such as the kidney, eye, liver, heart, body fluids, blood cells and blood vessel but liver is the main primary source of its production and secretion. Therefore, it was believed that the levels of kallistatin in plasma reflect its production in the liver.[12-14] Kallistatin has valuable effects as anti-inflammatory, antioxidant, anti-fibrotic and anti-tumor growth protein by its heparin-binding domain.[15] It is also a negative acute phase protein, whose expression in the liver is rapidly decreased after lipopolysaccharide-induced inflammation.[16]

More recent evidence[17] highlights that kallistatin levels could be considered as a potential biomarker for liver cirrhosis in a Chinese population. However, there is no study indicated its diagnostic role in the Egyptian population. Thus, this work aims to identify and appraise the diagnostic value of kallistatin to predict liver fibrosis in chronic hepatitis C (CHC) Egyptian patients. Also, to investigate whether combination between kallistatin and other routine liver function tests will improve the sensitivity and specificity of its diagnostic value.

SUBJECTS AND METHODS

Subjects

This study included (76) subjects divided into (62) clinically and laboratory confirmed HCV-Induced chronic liver disease patients (CLD) and 14 matched healthy controls. CLD patients were selected from the outpatient clinic of hepatology department of Kasr El Aini Hospital, Cairo, Egypt, from October 2015 to December 2016. The study was approved by the Ethics Committee of National Research Centre, Cairo, Egypt (Code No. 15208). An informed consent was taken from each individual participated in the present study and all were fully informed concerning the nature of the disease and the diagnostic procedures.

All patients and controls included in the present study were subjected to full medical history and clinical examination. The HCV infection was diagnosed based on serologic detection of hepatitis C antibodies with positive serum HCV-RNA by polymerase chain reaction and they were negative for hepatitis B virus testing. None of the patients had a history of habitual alcohol consumption. These chronic liver disease patients were divided into patients with early liver fibrosis (F0-F2) (n=30) and patients with advanced liver fibrosis (F3-F4) (n=16) and HCC (n=16). Liver fibrosis was diagnosis by using liver biopsy. Also, spiral CT-scan of the abdomen (triphasic study) was performed to confirm diagnosis of HCC.

Liver histology and quantification of liver fibrosis

For patients who had liver biopsy, liver biopsy was fixed in formalin and paraffin embedded. All biopsy specimens were analyzed independently by two experienced pathologists, who were blinded to the patients' clinicopathological data. Liver biopsies that contained less than 10 portal tracts (except for cirrhosis) were excluded from the histological analysis. Fibrosis was staged according to the METAVIR scoring system as follows: no or mild fibrosis (no fibrosis or portal fibrosis without septa, F0-F1), moderate fibrosis (portal fibrosis and few septa, F2), severe fibrosis (numerous septa without cirrhosis, F3) and cirrhosis F4.

Exclusion criteria

1) Patients with age <18.

2) Patients with chronic liver disease due to causes other than HCV.

3) Patients suffering from renal or cardiac disease, hypertension, diabetes, community acquired pneumonia, any other malignant disease except HCC, or patients with current infection that might affect the Kallistatin level.

Methods

Samples collection

Eight ml of blood were collected from each individual (patient and control) by vein-puncture then divided into three portions; 2 ml of the blood was treated immediately with EDTA-K_2 for routine blood pictures (CBC) by Sysmex the automated hematology analyzer SF-300, which produced by Sysmex Corporation, Japan, 2 ml of the blood was treated immediately with EDTA-K_2 for RNA extraction. Sera were separated from the rest of blood samples and biochemical analysis including aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, serum albumin, creatinine and random blood sugar were done according to

the manufacturer's instructions. The reagents were purchased from Spectrum Company, Cairo, Egypt.

The AST-ALT ratio was calculated as [AST/ ALT]. The AST/Platelet ratio index (APRI) was calculated as [AST/ (upper limits of normal) / platelets count 10^9/L] × 100.

Quantitation of CRP antigen by (ELISA)

Serum CRP levels were measured using an ELISA kit purchased from (IMMUNOSPEC CORPORATION, 14155 Farmington Rd. D, Livonia, MI 48154 USA), according to the manufacturer's instructions.

Quantitation of AFP by (ELISA)

Serum AFP levels were measured using an ELISA kit purchased from (IMMUNOSPEC CORPORATION, 7018 Owensmouth Ave. Suite 103 Canoga Park, CA 91303, USA), according to the manufacturer's instructions and values were reported as ng/ml.

Quantitation of kallistatin by (ELISA)

Serum kallistatin was measured in all enrolled subjects using ELISA kit supplied by (NOVA, No. 18, Keyuan Road, DaXing Industry Zone, Beijing, China). The assay is based on a double-antibody sandwich ELISA technique for the quantitative assay of human kallistatin in samples. The assay was performed according to the manufacturer's instructions and values were reported as pg/ml.

Gene expression of SERPINA4 by quantitative real time–PCR (qRT-PCR)

To confirm the obtained results of protein levels of kallistatin with its expression status, the expression profile of its gene (SERPINA4) was analyzed at messenger RNA (mRNA) in peripheral blood samples of forty one patients and correlation between protein and gene expression levels was detected.

Samples were taken from fourteen healthy controls and eight patients with early fibrosis, ten patients with advanced fibrosis and nine patients with HCC (five with AFP concentrations of 20 ng/mL or less and four with concentrations higher than 20 ng/mL).

Total RNA was extracted from peripheral blood samples using RNA extraction kit according to the manufacturer's instructions of the kit (Direct-zol™ RNA MiniPrep, ZYMO RESEARCH CORP.,) then the concentration for extracted RNA was detected using Nano-

drop2000 spectrophotometer (Thermo Fisher Scientific, USA). Eluted RNA was stored in -80°C till further processing. Secondly, PCR quantitation experiments were performed by using SensiFAST SYBR Green PCR Master Mix Kit, (BIOLINE GmbH, Germany) on the Rotor-Gene Q instrument (Qiagen, USA), to determine the expression of SERPINA4 and housekeeping gene Glyceraldehyde–3–phosphate dehydrogenase (GAPDH) (used as an endogenous control) for normalization. The working master mix was prepared according to manufacturer's protocol. Fluorescence measurements were made in every cycle and the thermal profile used as follows: Reverse transcription 1 cycle 45°C for 10 min; polymerase activation 1 cycle of 95°C for 2 min; cycling 45 cycles of 95°C for 5 sec and 60°C for 20 sec. The expression levels of SERPINA4 in tested samples were expressed in the form of ΔΔCT (cycle threshold) value which calculated on the basis of threshold cycle (Ct) values, corrected by Glyceraldehyde–3–phosphate dehydrogenase expression, with the following equation: the relative amount of SERPINA4 = $2^{-\Delta\Delta Ct}$; ΔΔCt = [ΔCt of cases - ΔCt of control]; [ΔCt=Ct (SERPINA4) – Ct (GAPDH)]. The following primers were used in the quantitative real-time PCR analyses: SERPINA4 forward primer: 5′- GTGGGCACAATCCAGCTTAT-3′, reverse primer: 5′- ACCCGGACTGTTGTGTTCTC -3′; GAPDH forward primer: 5′- GTCCACTGGCGTCTTCACCACC -3′, reverse primer: 5′- AGGCATTGCTGATGATCTTGAGGC -3′.

Statistical Analysis

In the present study, statistical analyses of data were carried out using SPSS version 23. Shapiro –Wilks test was used to test normal distribution of variables. Quantitative variables were described in the form of mean ± SD or median and range, while qualitative variables were described as number and percent. The significance of the difference between groups for quantitative variables was determined by the student's t-test and Mann-Whitney (U-test) as appropriate. Categorical variables were compared using the chi-squared ($\chi 2$) test. Correlations between different parameters were done using spearman's correlation coefficient. The Receiver Operating Characteristic (ROC) was plotted to assess the diagnostic power of kallistatin in different CLD patients and controls. Also, it was constructed to obtain the most sensitive and specific cutoff value for serum kallistatin in diagnosing liver fibrosis and cirrhosis, and the area under the curve (AUC) greater than 0.5 was considered to be statistically significant. The probability (P) values of ≤0.05 were considered statistically significant indicated *, while P> 0.05 was considered statistically not significant.

RESULTS

Overall, 76 participants were recruited in this study (40 male and 36 female); Sixty two (62) patients were divided into (48.4%) with early liver fibrosis (F0-F2) (n=30), (25.8%) with advanced liver fibrosis (F3-F4) (n=16) and HCC was present in 16 (25.8%) of Patients; with mean age 47.85 ± 12.13, 60.6 ± 8.48 and 57.62 ± 11.22 years (Y) respectively; and 14 matched healthy controls with mean age 41.93 ± 15.69 years. Studied patients displayed a significant trend of elder age with the progression of liver disease from early fibrosis to advanced fibrosis and HCC.

By considering the hematological characteristics of the studied groups; there were significant differences among certain hematological parameters including red blood cells count (RBCs), hemoglobin and platelets count in different CLD patients when compared to controls. Patients with advanced liver fibrosis and HCC had significantly lower mean value of hemoglobin and platelet count compared to both control subjects and early fibrosis patients ($P \leq 0.001$). However, there was no significant difference in the mean value of WBCs between all groups ($P > 0.05$) (**Table.1**). The variation in routine clinical investigations of liver function among different groups was shown in **Table. 1**. Patients with CLD had significantly higher CRP levels than those without ($P \leq 0.05$) (**Table 1 & Figure1A**). Although the median concentration of serum AFP was increased for HCC patients than its level in healthy controls, as expected ($P<0.0001$), significant increases were also detected in patients with early and advanced liver fibrosis than healthy participants ($P = 0.014$ and $P<0.0001$ respectively, **Table 1 & Figure 1B**).

It is interesting to note that serum Kallistatin was significantly lower in cases with chronic liver disease than those without ($P=0.001$), suggesting a potential link between serum Kallistatin levels and CLD (**Table 1&Figure1C**). This results confirmed by qRT-PCR, as it was detected that the expression levels of SERPINA4 were frequently lower in patient suffering from CLD than healthy controls ($P=0.003$) **(Figure 2)**. Furthermore, when early liver fibrosis patients compared with advanced fibrosis in eighteen cases, there was no significant difference at the gene expression or protein levels ($P>0.05$). Moreover, a moderate positive correlation was found between serum protein level of kallistatin and its gene expression($r = 0.328$, $P = 0.036$, **Figure 2C**). A noticeable finding is that when serum Kallistatin concentration was measured in a large number of patients (forty six) by ELISA, the median level of kallistatin was 32.69% lower in patients with early liver fibrosis

[734.4(154.7 – 1322.96) (pg/ml)], 62.3% lower in patients with advanced liver fibrosis [413.17(110.1 – 1230.3)] and 44% lower in HCC patients [610.15(165.7 – 1506.8] than the healthy subjects. [1091.1(298.4 – 10859.3) (pg/ml)]p=0.047, p=0.005 and P=0.042; respectively). A significant decrease 43.74% in serum kallistatin levels was observed when patients with advanced liver fibrosis compared to patients with early stage of liver fibrosis (p=0.044). While there was no significant difference in the median levels of kallistatin in HCC patients when compared to both early and late stages of liver fibrosis (P >0.05) (**Table 1 & Figure 1 C**).

Receiver operating characteristic curves for predicting early fibrosis, advanced fibrosis, and HCC

To detect whether serum Kallistatin and its combination with APRI and AAR could be used as a diagnostic biomarker for chronic liver disease, non-parametric receiver operating characteristic (ROC) curve analysis was performed. ROC curve showed the optimum cutoff for Kallistatin was 1253.5 (pg/ml) for distinguishing patients with chronic liver disease from healthy subjects with sensitivity 95.1% and specificity 50%; an area under the ROC curve (AUROC) 0.725(95% CI: 0.561-0.890) (**Figure 3A, Table2**). In the assessment of differential diagnostic accuracy, serum kallistatin had greater sensitivity and NPV values than did AAR and APRI in patients with chronic liver diseases compared with healthy subjects (**Table2**). Values of specificity, PPV and NPV elevated when the three tests were combined. Furthermore, ROC curve also showed that at a cutoff value of 1224.89 (pg/ml), Kallistatin could significantly distinguish patients at early stage of liver fibrosis from healthy subjects with sensitivity 96.7% and specificity 50%; an area under the ROC curve (AUROC) 0.688(95% CI: 0.496-0.880) (**Figure 3B, Table2**). These data supported that kallistatin may be an efficient biomarker in early detection of fibrosis. According to data mentioned in table 2, the sensitivity and NPV for kallistatin were also better than those for different studied parameters. However, values of specificity and PPV improved when 3 different parameters combined as showed in (**Table2**). On the other hand, there was no statistically significant difference in the AAR between the ‘healthy subject’ group and the ‘early liver fibrosis’ group (AUC 0.598, P =0.302).

Moreover, the area under the receiver operating characteristic (ROC) curve for distinguishing between early and advanced stage of liver fibrosis using serum kallistatin level was 0.686 (**Fig. 3C**) and using a cutoff level of 586.49 (pg/ml) for serum kallistatin level yielded

sensitivity and specificity values of 73.3% and 70%, respectively. Whereas, APRI showed high sensitivity (92.3%) for differentiating between different stages of liver fibrosis but combination between all studied parametres increased specificity and PPV to 96.7% and 91.7% respectively.

In patients with HCC, the AUC for kallistatin was 0.719 (95% CI: 0.534–0.904) with sensitivity of 87.5% and specificity of 50%, compared with controls (**Table 2**). ROC analysis showed that testing of kallistatin, AAR and APRI increased the diagnostic accuracy for HCC compared with either test alone (AUC 0.945, 95% CI: 0.839–1, sensitivity 92.3% and specificity 100%) and this is very similar to the sensitivity and specificity of AFP (93.8% & 91.7% respectively).

Correlation between serum kallistatin, CRP, AFP and other parameters including hematological, hepatic functional capacity and liver damage parameters

The correlation between kallistatin, CRP, AFP and biochemical liver function tests is mentioned in (**Table 3&Figure 4).**

Results of laboratory tests indicated a weak inverse correlation between serum kallistatin and CRP (r = -0.244, P = 0.037, Fig. 4A), AFP (r = -0.232, P = 0.05, Fig. 4B), AST (r = -0.285, P = 0.016, Fig. 4C), APRI (r = -0. 293, P = 0.014 Fig. 4D) or prothrombin time (r = -0.42, P = 0.002, Fig. 4E). Also, Serum kallistatin decreased in parallel with hemoglobin level (r =0.374, P = 0.001, Fig. 4F), platelet count (r = 0.232, P = 0.046, Fig. 4G) and serum albumin, (r = 0.305, P = 0.018, Fig. 4H).

However, serum kallistatin was not significantly correlated with Age (r = -0.122, P = 0.313), WBCs (r = -0.212, P = 0.069), RBCs (r = 0.179, P = 0.125) total bilirubin (r = -0.212, P = 0.096), ALT (r = -0.106, P = 0.378) or AST/ALT ratio (r = -0.144, P = 0.232).

Also, CRP and AFP correlated with all studied parameters except WBCS and ALT for CRP WBCS only for AFP as mentioned in **table 3.**

Table 1: Demographic data and biochemical parameters of the patients and controls.

Variable / Groups	Healthy Controls(n=14)	Early fibrosis patients Group(n=30)	Advanced fibrosis patients Group(n=16)	HCC patients Group(n=16)
Age(Yrs.)	41.93 ± 15.69	47.85 ± 12.13	60.6 ± 8.48$^{ab:***}$	57.62 ± 11.22^{a**,b*}
Gender Male/Female Percentage of Male	2/12 (14.3%)	14/16 (46.7%)	11/5^{a**} (68.8%)	13/3^{a***} (81.2%)
RBCs (10^6/µL)	4.89 ± 0.63	4.72 ± 0.59	3.57 ± 0.68$^{ab:***}$	3.49 ± 0.76$^{ab:***}$
Hemoglobin(g/dl)	13.92± 1.08	12.75 ± 2.07	10.44 ± 1.86$^{ab:***}$	9.89 ± 2$^{ab:***}$
Platelets count (10^3/µL)	283 ± 28.93	208.7 ± 90.64^{a***}	93.87 ± 41.54$^{ab;***}$	187.57 ± 96.49$^{ac:***}$
WBCs (10^3/µL)	6.66 ± 1.65	7.04 ± 2.37	8.29 ± 5.29	8.53 ± 4.18
ALT (U/L)	30 (20 – 39)	44.5(12 – 222)^{a**}	29 (7 – 53)b*	56 (26 – 152)^{a***,c**}
AST(U/L)	28(16 – 39)	42(18 – 136)^{a**}	59.5 (27 – 255)^{a***}	100 (26 – 386)^{a***,b**}
AST/ALT ratio (AAR)	0.904(0.552 – 1.56)	1.05(0.388 – 1.643)	1.85(1.032 – 36.43)$^{ab:***}$	1.67(0.68 – 6.031)$^{ab:**}$
APRI	0.224(0.157-0.417)	0.574(0.099-2.252)^{a***}	1.126(0.5-6.73)$^{ab:***}$	1.123(0.139-9.95)$^{ab:***}$
Total bilirubin (mg/dl)	0.45(0.05 – 0.7)	0.7(0.2 – 1.3)^{a**}	1.6(0.3 – 15.9)$^{ab;***}$	2.15(0.3 – 25.4)^{a***,b**}
Prothrombin time (sec.)	12.5(11 – 14.6)	13.4(12.2 – 18.4)^{a*}	21.1(15.2 – 31.3)$^{ab:***}$	17.1(14.8 – 34)$^{ab:***}$
Serum albumin (g/dl)	4.05(3.7 – 4.5)	4(2.7 – 4.7)	2.2(1.7 – 3.4)$^{ab;***}$	2.6(1.7 – 3.4)$^{ab:***}$
AFP (ng/ml)	1.49(0.79 – 2.2)	1.99(0.5 – 95.63)^{a*}	2.5(1.33 – 198.04)^{a***}	14.58(1.58 – 426.56)$^{ab:***,c**}$
CRP (mg/l)	6.55(2.38 – 9.96)	30.46(2.17 – 99.63)^{a**}	54.71(7.1 – 125.1)^{a***,b**}	88.33(32.5 – 99.63)$^{ab:***}$
Kallistatin (pg/ml)	1091.1(298.4 – 10859.3)	734.4(154.7 – 1322.9)^{a*}	413.17(110.1 – 1230.3) ^{a**,b*}	610.15(165.7 – 1506.8)^{a*}

Parameters are presented as means ± SD for normally distributed and medians (range) for non-normally distributed variables, and as total number (%) for categorical variables. Units are in parentheses.

- a: significant difference from Healthy Controls, b: significant difference from Early fibrosis patients, c: significant difference from Late fibrosis patients.

- *: P ≤0.05, **: P ≤0.01, ***: P ≤ 0.001.

Abbreviations: RBCs, red blood cells; WBCs, white blood cells; AST, aspartate aminotransferase; ALT, Alanine aminotransferase; PT, prothrombin time; APRI: [(AST/ 40) / platelets count 109/L]×100; CRP, C reactive protein; AFP, alpha fetoprotein.

Table 2: ROC analysis of kallistatin, AAR and APRI in different studied groups.

Test	Best cut off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC (95%CI)
Healthy control vs chronic liver disease patients						
Kallistatin	1253.5	95.1	50	89.2	70	0.725 (0.561-0.890)
APRI	0.422	82.1	100	100	58.3	0.923(0.86-0.987)
AAR	0.977	75.4	78.6	93.5	44	0.737(0.61-0.865)
Kallistatin+ APRI		92.9	57.1	89.7	66.7	0.945(0.894-0.997)
Kallistatin+ APRI+AAR		94.6	78.6	94.6	78.6	0.955(0.905-1)
Healthy Control vs Early Stage of Fibrosis						
Kallistatin	1224.89	96.7%	50%	80.6	87.5	0.688 (0.496-0.880)
APRI	0.324	83.3	92.9	96.2	72.2	0.89 (0.792-0.988)
AAR	0.977	60	78.6	85.7	47.8	0.598(0.417-0.779)
Kallistatin +APRI		86.7	78.6	89.7	73.3	0.917(0.836-0.997)
Kallistatin +APRI+ AAR		90	78.6	90	78.6	0.931(0.851-1)
Early Stage vs Advanced Stage of Fibrosis						
Kallistatin	586.49	73.3	70	55	84	0.686(0.513-0.858)
APRI	0.685	92.3	66.7	54.5	95.2	0.833(0.709-0.957)
AAR	1.41	85.7	90	80	93	0.929(0.842-1)
Kallistatin +APRI		53.8	93.3	77.8	82.4	0.856(0.743-0.969)
Kallistatin +APRI+ AAR		84.6	96.7	91.7	93.5	0.969(0.924-1)
Advanced Fibrosis Patients vs Healthy Control Subjects						
Kallistatin	577.88	73.3	78.6	78.6	73.3	0.807 (0.647-0.967)
APRI	0.458	100	100	100	100	1
AAR	0.993	100	78.6	82.4	100	0.964(0.906-1)
Kallistatin +APRI		100	100	100	100	1
Kallistatin +APRI+ AAR		100	100	100	100	1
HCC Patients vs HC						
Kallistatin	1246.96	87.5	50	66.7	77.8	0.719 (0.534-0.904)
APRI	0.468	92.3	100	100	93.3	0.923(0.778-1)
AAR	0.977	84.6	78.6	78.6	84.6	0.816(0.636-0.996)
AFP	1.836	93.8	91.7	93.8	91.7	0.977(0.932-1)
Kallistatin +APRI		92.3	100	100	93.3	0.945(0.839-1)
Kallistatin +APRI+ AAR		92.3	100	100	93.3	0.945(0.839-1)

Table 3: Correlation between serum kallistatin, CRP, AFP and other parameters including hematological, hepatic functional capacity and liver damage parameters.

Parameters	Concentration of kallistatin (pg/ml)		Concentration of CRP (mg/l)		concentration of AFP (ng/ml)	
	r	P-value	r	P-value	R	P-value
kallistatin (pg/ml)	1.000	-	-0.244*	0.037	-0.232*	0.05
CRP (mg/l)	-0.244*	0.037	1.000	-	0.322**	0.006
AFP (ng/ml)	-0.232*	0.05	0.322**	0.006	1.000	-
Age(Yrs.)	-0.122	0.313	0.298*	0.012	0.489***	0.000
WBCs(10^3/µL)	-0.212	0.069	0.127	0.285	0.098	0.417
RBCs(10^6/µL)	0.179	0.125	-0.412***	0.000	-0.307**	0.009
Hemoglobin(g/dl)	0.374***	0.001	-0.552***	0.000	-0.332**	0.005
platelet count(10^3/µL)	0.232*	0.046	-0.336**	0.004	-0.290*	0.014
total bilirubin (mg/dl)	-0.212	0.096	0.422***	0.001	0.468***	0.000
serum albumin(g/dl)	0.305*	0.018	-0.605***	0.000	-0.567***	0.000
AST(U/L)	-0.285*	0.016	0.339**	0.004	0.610***	0.000
ALT(U/L)	-0.106	0.378	0.038	0.755	0.334**	0.005
AST/ALT ratio(AAR)	-0.144	0.232	0.406***	0.001	0.392***	0.001
AST/platelet ratio (APRI)	-0.293*	0.014.	0.359**	0.003	0.586***	0.000
Prothrombin Time(sec.)	-0.420**	0.002	0.566***	0.000	0.473***	0.001
relative SERPINA4 expression levels	0.328*	0.036	-0.242	0.138	-0.297	0.067

Figures

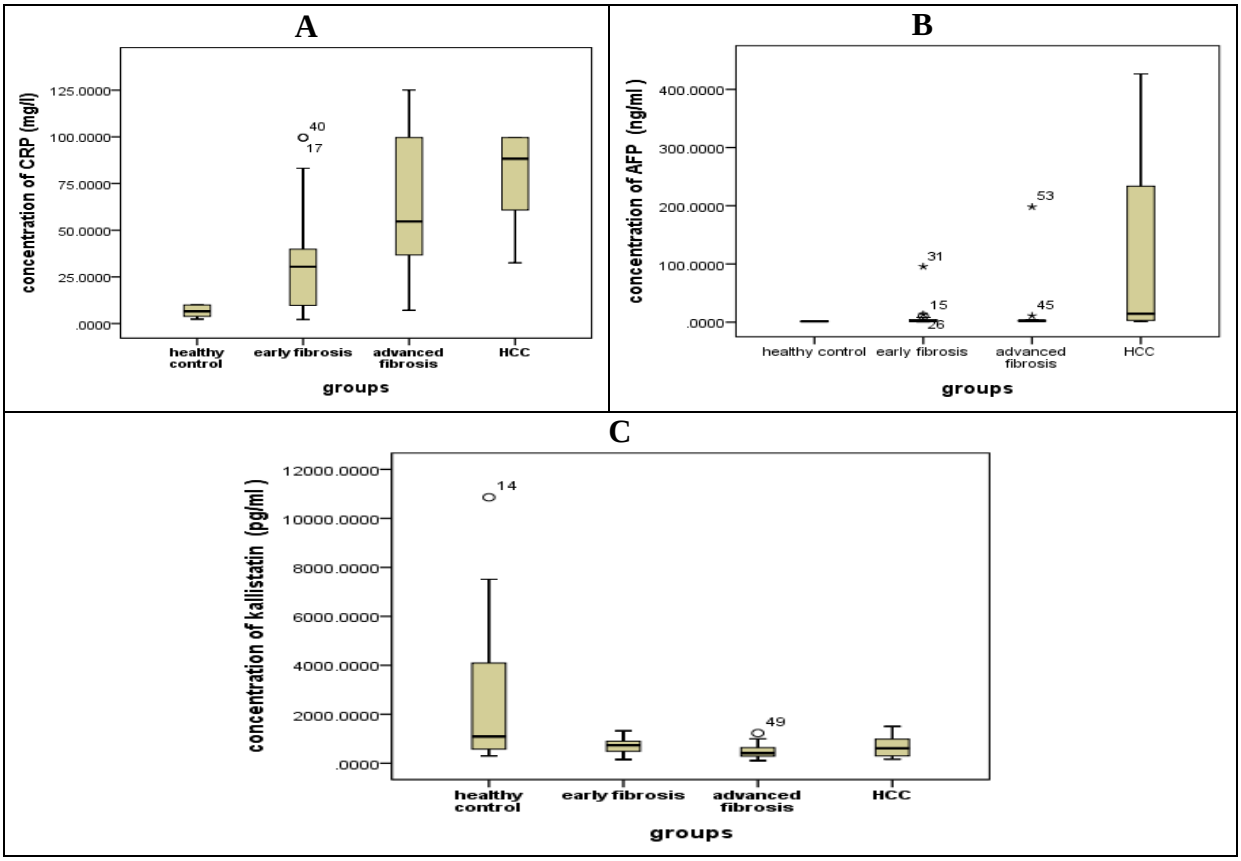

Figure 1: Serum levels of different biomarkers in the studied groups.

The box represents the interquartile range. The whiskers indicate the highest and lowest values and the line across the box indicates the median value.

Overall significance of differences among 4 groups was determined by Kruskal–Wallis: for CRP, P<0.001; for AFP, P<0.001; and for kallistatin, P<0.015.

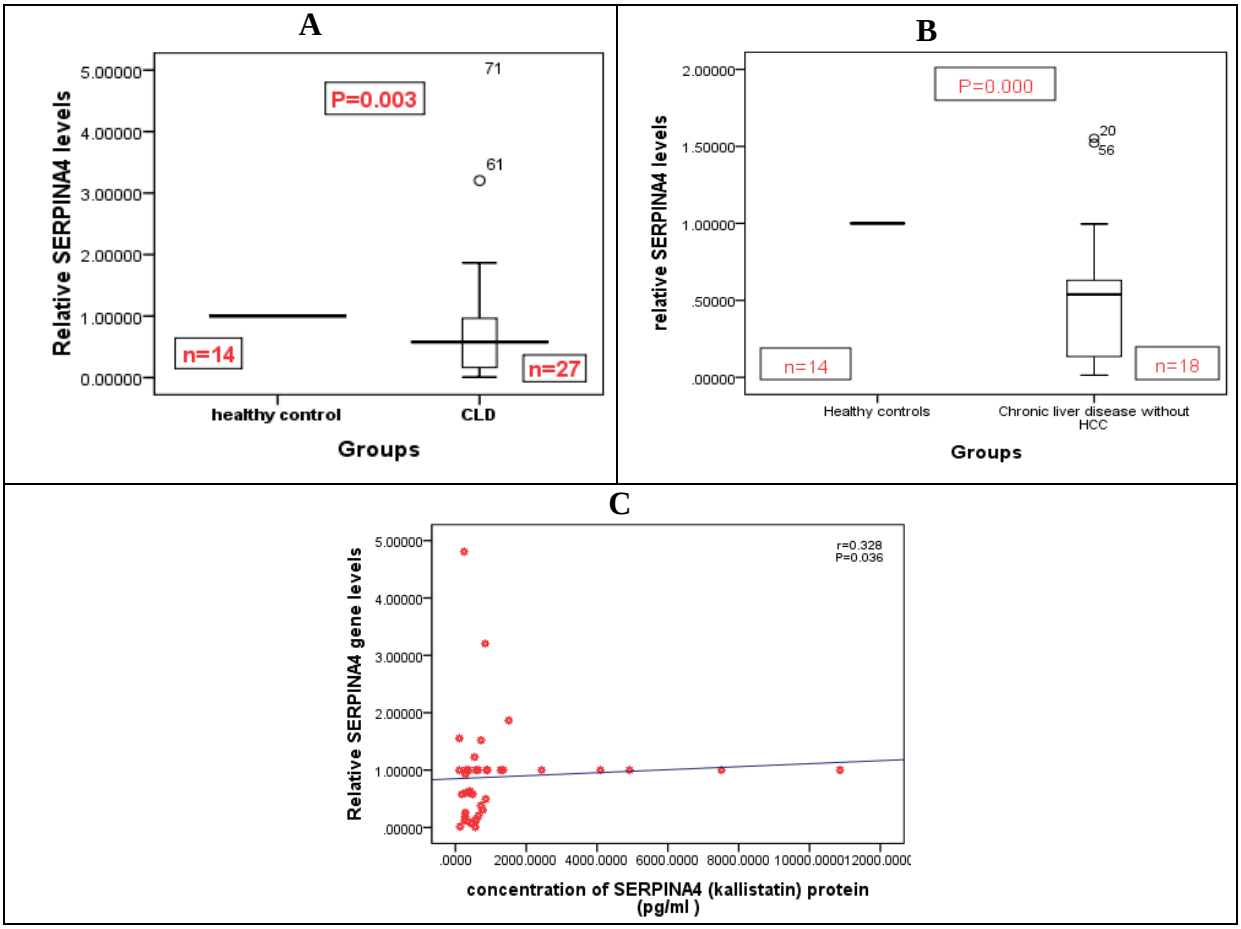

Figure 2: Comparison between relative SERPINA4 expression levels in different studied groups by RT-PCR.

Relative SERPINA4 expression levels in CLD and its clinical significance. **A**) SERPINA4 expression levels were significantly down-regulated in CLD patients as compared with the normal control. GAPDH was used as an internal control. **B**) SERPINA4 expression levels were significantly down-regulated in CLD patients without HCC as compared with the normal control. GAPDH was used as an internal control. **C)** Correlation between expression levels of SERPINA4 by real-time PCR and its protein levels by ELISA in the same cases.

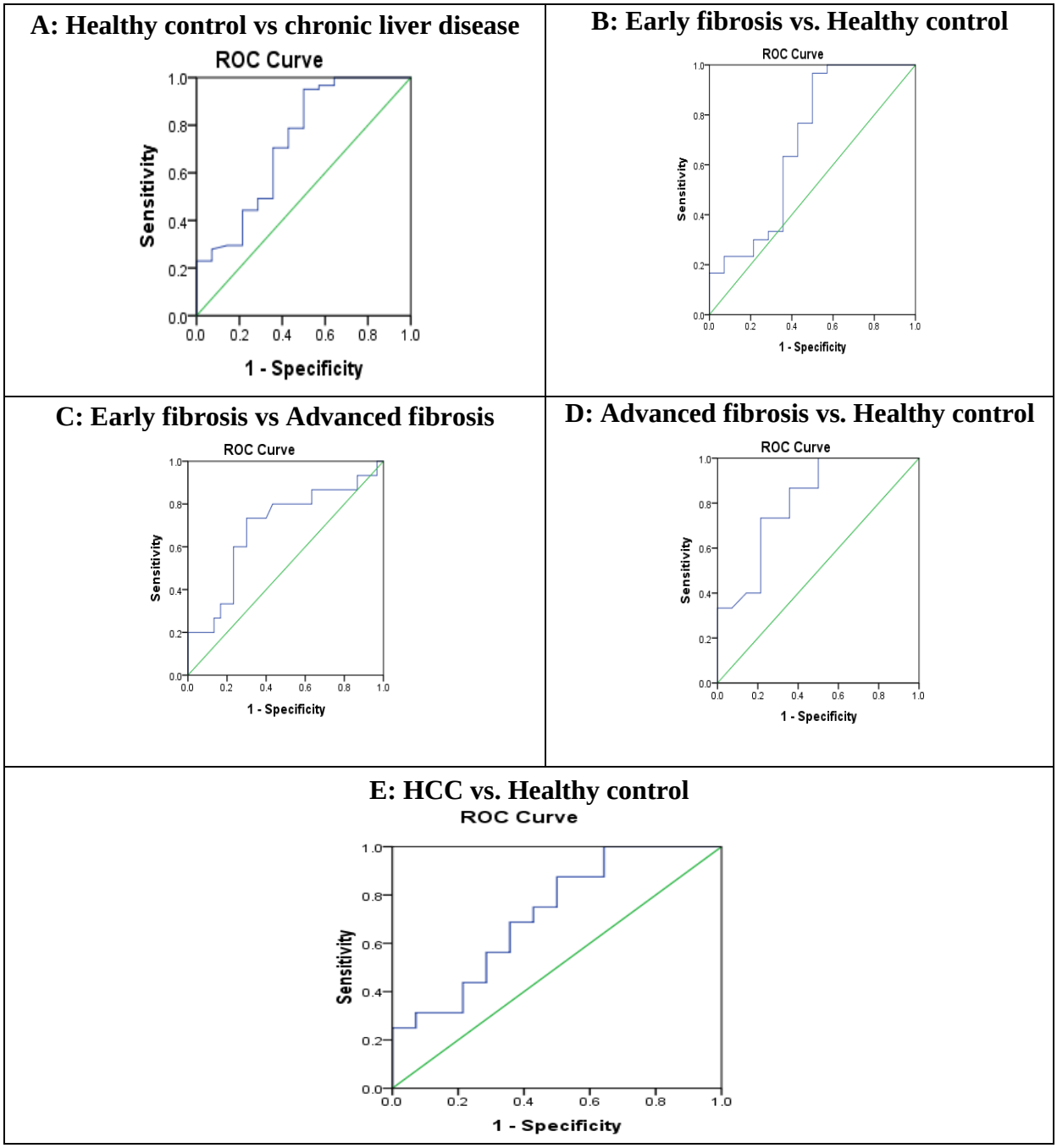

Figure 3: Diagnostic value of kallistatin for CLD. A, ROC curve analysis of kallistatin for discrimination between chronic liver disease patients and healthy control subjects (AUC: 0.725 (95% CI: 0.561-0.890), P = 0.009. The cutoff value 1253.5 (pg/ml) has sensitivity 95.1% and specificity 50%. B, ROC curve analysis of kallistatin for discrimination between patients with early fibrosis and healthy control subjects (AUC: 0.688 (95% CI: 0.496-0.880), P = 0.047). the cutoff value 1224.89 (pg/ml) has sensitivity 96.7% and specificity 50%. C, ROC curve analysis of kallistatin for discrimination between early fibrosis and advanced fibrosis patients (AUC: 0.686 (95% CI: 0.513-0.858), P = 0.044. the cutoff value 586.49 (pg/ml) has sensitivity 73.3% and specificity 70%. D, ROC curve analysis of kallistatin for

discrimination between advanced fibrosis patients and healthy control subjects (AUC: 0.807 (95% CI: 0.647-0.967), P = 0.005. The cutoff value 577.88 (pg/ml) has sensitivity 73.3% and specificity 78.6%. E, ROC curve analysis of kallistatin for discrimination between HCC patients and HC subjects (AUC: 0.719 (95% CI: 0.534-0.904), P = 0.042. The cutoff value1246.96 (pg/ml) has sensitivity 87.5% and specificity 50%.

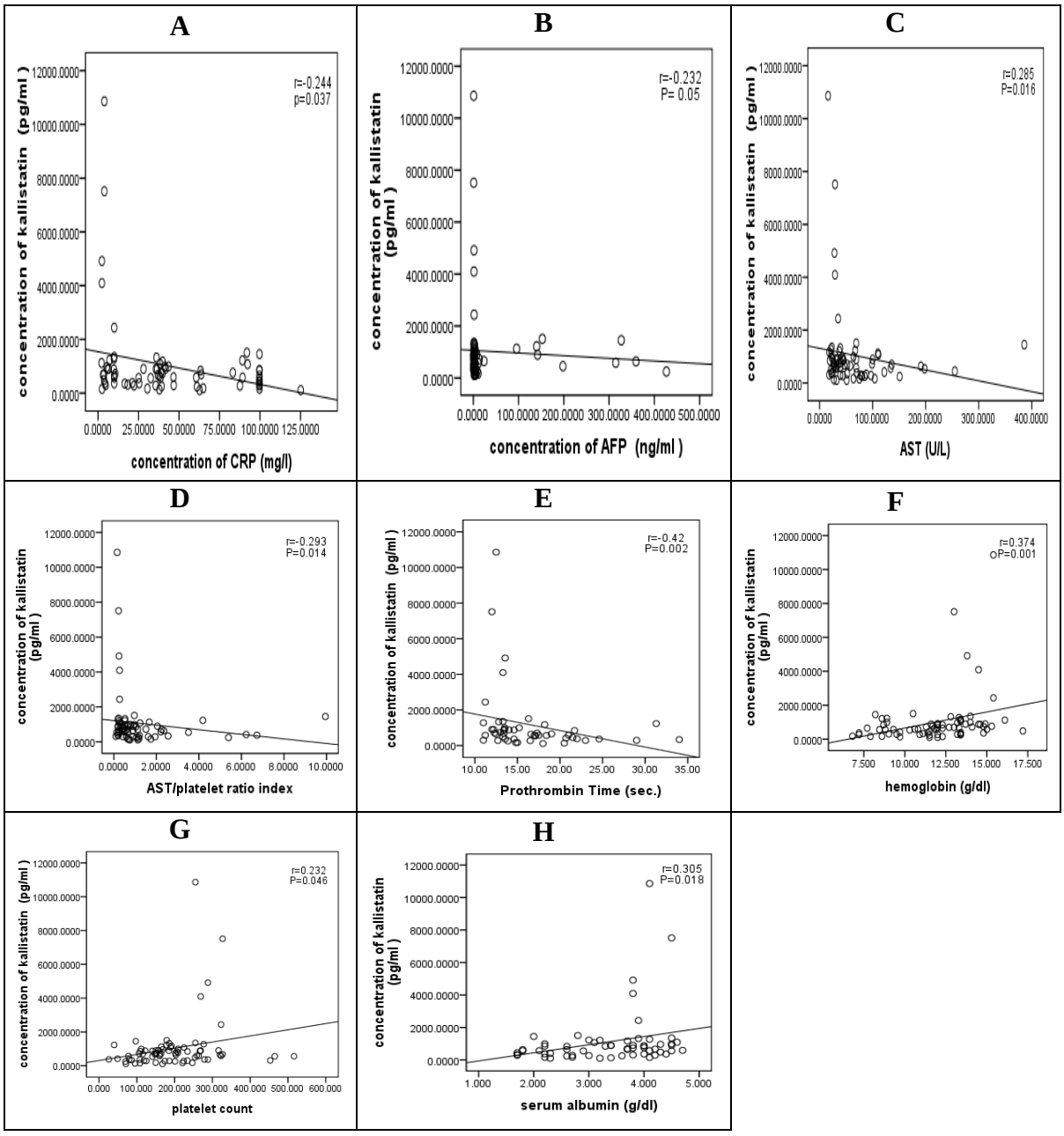

Figure 4: Relationship between kallistatin levels and AFP or other liver function test parameters. Relationships between kallistatin levels and CRP (A), AFP (B), AST (C), AST /platelets ratio index (D), prothrombin time (E), hemoglobin (F), platelet count (G), or serum albumin (H) are shown. Spearman's rank correlation coefficient used to determine these correlations.

DISCUSSION

Early detection of the progression of fibrosis is a crucial step for preventing further complications of chronic liver diseases. Currently, liver biopsy is still the gold standard method for assessing hepatic fibrosis. However the risk of clinical complications, poor acceptance and sampling errors are some of its remarkable limitations.[18]

Recently, several new non-invasive biomarkers have been evaluated as potential alternatives to liver biopsy. However, identifying the hepatic fibrosis at an early stage remains an open challenge, due to the poor diagnostic accuracy of many circulating biomarkers and algorithms in the early and mild stages of liver fibrosis when compared to advanced fibrosis.[19,20] Hepatic fibrosis is a silent disease affecting people all over the world as well as Egyptian population. The slow and asymptomatic progression of the disease in the majority of cases is considered as one of the major obstacles to adopt specific biomarkers for early detection of fibrosis. This study aimed to demonstrate the utility of kallistatin as a potential biomarker for the early detection of hepatic fibrosis and liver cirrhosis.

Results from the present study showed that RBCs count was significantly decreased in advanced liver fibrosis and HCC than in both healthy subject and early liver fibrosis patients. The main mechanism especially in those with portal hypertension is blood loss in variceal or other gastrointestinal bleeding. Also, therapy used for the treatment of hepatitis C may be associated with the development of anemia.[21]

In the current study, a statistically significant decrease in the mean of platelet counts was observed in patients with CHC than control subjects. As expected, mean platelet count in the advanced fibrosis group was extremely decreased compared to those in early fibrosis group and the control subjects and these suggested that platelet count might be helpful in the assessment of chronic liver diseases.

Chronic hepatitis C has been reported as one of the several causes that induce thrombocytopenia, even in chronic non-cirrhotic patients.[22,23]

Thrombocytopenia in chronic liver disease may be explained by suppression of platelet production by the bone marrow as a result of viral infection, alcohol consumption, iron overload, and medications. Splenic sequestration of platelets due to hypersplenism may be another cause of the reduction in the platelet numbers.[21,24]

Also decreased activity of thrombopoietin (hematopoietic growth factor) as well as high levels of platelet-associated immunoglobulins (PAIgG), which are responsible for the high rate of platelets destruction in CLD patients, is other causes of thrombocytopenia.[24-26]

Lu et al., declared that thrombocytopenia was a valid surrogate of cirrhosis and acceptable marker for the identification of individuals at high-risk for HCC, especially in areas that had a high prevalence of HCV.[27] Furthermore, He et al., reported that hemoglobin values and platelet counts were significantly low in HCV-infected patients In comparison to the controls.[28] Mean platelet count in HCC patients was significantly higher than that in advanced fibrosis in the current study. One patient (6.25%) of HCC group actually has thrombocytosis with platelet count >400000 mm^3 while five cases (31.25%) have thrombocytopenia with platelet count <150000 mm^3. All hepatocellular carcinoma cases have a cirrhotic background and this preneoplastic state is the strongest predisposing factor.[29] However, the incidence of liver decompensation in patients with HCC is variable. Signs of liver failure and portal hypertension take a leading position in HCC patients with decompensated cirrhosis while “toxic syndrome” coincides more frequently with the absence of decompensation.[30-32]

Thus, depending on the pattern and the occurrence of cirrhosis, thrombocytopaenia may not be a dominant feature. Also, High platelet count may predominate from other mechanisms.

High platelet count and even thrombocytosis are common in several malignant diseases[33] including those of the ovary[34] and liver.[35]

The link between platelets and angiogenesis may be responsible for high platelet count in patients with malignancies. VEGF, which is a platelet-derived cytokine appeared in many malignant diseases, promotes the aggregation of platelet by endothelial cells. Aggregated platelets lead to the release of many potential angiogenesis regulators such as platelet-derived endothelial cell growth factor (PD-ECGF)[36,37] VEGF-A[38], VEGF-C.[39] The previous findings suggest the active and causative role of platelets in tumor angiogenesis.

The current study is in good agreement with Saadeh et al. who found that differences in white blood cell count between cirrhotic and non-cirrhotic patients were not significant.[40] However, it does not support other results which showed a significant association between WBCs and CLD. He et al. detected that WBCs count was significantly lower in HCV-

infected patients as compared to controls.[28] Also, Friedman who categorized persistent leukocytosis as one of the paraneoplastic syndromes associated with HCC.[41] The AST and ALT are abundant hepatic enzymes that catalyze the transfer of amino groups to form the hepatic metabolites pyruvate and oxaloacetate, respectively. The ALT is found in the cytosol of the liver, whereas two AST isoenzymes are located in the cytosol and mitochondria, respectively. Both the ALT and AST are released from damaged hepatocytes into the blood after hepatocellular injury or death.

We observed that ALT levels were significantly higher in early liver fibrosis and HCC patients than in healthy subjects. Also, ALT levels decreased in advanced liver fibrosis patients than early fibrosis group. This is in agreement with Motawi et al., who reported a significant increase in ALT between HCC and early fibrosis group. However they didn't find any significant difference between levels of ALT in early fibrosis group and advanced fibrosis group.[42] Also, Ahmed and his colleagues reported that ALT levels deceased in advanced fibrosis than in HCC patients.[43] In all studied group, there were a statistically significant increase in the median of AST in relation to the development of chronic liver disease. This is in agreement with Fouad et al.[44], who reported a high level of AST in cirrhotic patients in comparison with non-cirrhotic cases. The same finding was also detected by Green and his coworkers.[45]

Multiple studies had reported that APRI is of a great value and has high accuracy in the prediction of advanced fibrosis.[6] In our study the median level of APRI score showed a statistical difference between different stages of liver fibrosis and healthy control. This matches well with earlier researchers' observations.[44, 46-48] However, no statistically significant different was found between APRI in patients suffering from the advanced fibrosis and patients with HCC. This may be due to the increase in the platelets count of certain patients with HCC patients than in patients with advanced fibrosis.

In patients with advanced liver fibrosis and HCC a significant increase in AAR score was shown in both early liver fibrosis patients and healthy subjects, but no significance difference was observed between patients with early fibrosis and healthy control. Ahmed et al. and Abd-Elghany et al. in their studies documented that there was no statistically significant difference between different stages of liver fibrosis and AAR.[43,49] Whereas Demerdash et al. mentioned that there was a significant association between AAR levels and development of chronic liver disease.[50] In HCC group, the AAR levels were showed a slightly decrease than in advanced

liver fibrosis group. This can explained by the low level of ALT detected in advanced liver fibrosis patients than that in HCC.

Kallistatin is expressed in several organs and tissues but it is primarily produced in the liver. In our study, the expression levels of SERPINA4 were detected in peripheral blood samples of 18 chronic liver disease patients without HCC, 9 HCC patients and 14 healthy controls by using quantitative real time PCR to confirm the results obtained by ELISA. The results revealed that the median level of SERPINA4 significantly decreased in patients who have chronic liver disease as compared to control, suggesting a potential link between its down-regulated levels and development of liver disease. Also, kallistatin levels were determined in serum of healthy, early liver fibrosis, advanced liver fibrosis and HCC. The median level of kallistatin showed a significant decrease in early fibrosis patients as compared to control. Kallistatin levels were also lower in advanced liver fibrosis patients than both early liver fibrosis patient and healthy subjects. In hepatocellular carcinoma patients, levels of kallistatin were higher than advanced fibrosis patients but lower than both early liver fibrosis and healthy controls. This substantiates previous findings by Badola and his coworkers[51] who mentioned that SERPINS A1, A3, A4 and A11 shared distinct banding patterns with high correlation, and this reflects that they are predominantly expressed in the liver. Also, they declared that SERPINA4 was highly expressed in the normal human tissue, but its expression decreased in cases with liver fibrosis. Furthermore, Chao et al.[12] studied the expression of kallistatin in different types of cells included blood cells and hepatocytes. They confirmed that kallistatin which produced in blood cells and hepatic cells was significantly reduced in plasma samples of patients with liver disease.

This finding is in a good agreement with that obtained by Cheng et al.[17] who found that kallistatin level reduced significantly in the serum of early liver fibrosis patients; however, there was a marked reduction in kallistatin level of cirrhotic patients.

In the experimental model, Diao et al. reported that the secreted Kallistatin could protect the liver against CCl4 induced damage.[52] They observed a significant decrease in the serum level of peroxidation marker accompanied with enhancement in the activities of the hepatic anti-oxidative defense system in kallistatin gene-transferred mice. Also, Huang et al., declared that liver injuries induced by CCl4 in animal model were attenuated by administration of kallistatin as CCl4-induced liver fibrosis treatment.[53] Kallistatin attenuated the pathological progression of fibrosis with concomitant fewer and smaller fibrotic nodules.

Also in our study, levels of kallistatin in HCC patients were increased than its levels in advanced fibrosis patients this may be due to the antitumor activity of kallistatin as it plays a key role in inhibiting tumor growth and metastasis. Lu et al. mentioned that administration of recombinant kallistatin protein attenuates tumor growth and intramural neo-vascularization in grafted hepatocarcinoma mice and reduces VEGF expression in HCC cells.[54]

CONCLUSION

Our findings support that kallistatin may be an efficient biomarker in early detection of fibrosis. Also, it indicated that combination of kallistatin with AAR and APRI could improve the sensitivity and specificity for chronic liver disease diagnosis and this can be used as a practical mean of clinical differential diagnosis for early stage of liver fibrosis. However, our study is limited because the small sample size, all participants in this study are from the Egyptian population and we examined chronic liver disease induced by HCV-infection with no information if our findings would be valid for other patients with different etiologies. Thus, further investigation should be carried out in order to validate these findings.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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