Advantages of Alpha-2-Macroglobulin levels to evaluate liver fibrosis in CHC patients.

Abstract: For the diagnosis and staging of hepatic fibrosis in patients with chronic hepatitis C, Liver biopsy is considered the gold standard. For its invasiveness and limitations, there are new non-invasive scores to replace liver biopsy. **The aim** is to evaluate the diagnostic power of one of the acute phase proteins called α-2-macroglobulin (A2M) in differentiating the phases of liver fibrosis. **Materials & methods**: 120 HCV infected Patients were classified into patients with non-significant fibrosis (F0-F1, n=66) and those with significant fibrosis (F2-F4, n=54). Blood samples were collected and α-2-Macroglobulin levels were assayed using an enzyme-linked immunosorbent assay. HCV RNA and HCV antibodies, liver function tests and platelet counts; besides, liver biopsies were evaluated, and the numerical value of APRI was re-evaluated. **Results:** A2M can efficiently differentiate patients with non-significant (F0, F1) from those with significant fibrosis (F2 –F4). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and area under ROC curve (AUC) of such test were 87, 94, 92, 89 and 0.93, respectively. The combination of the results of A2M to those of APRI Result in a modified MDA function of {0.038 * (A2M) + 0.068 * (APRI) – 9.19}. This MDA function greatly enhances its positive predictive value (PPV), negative predictive value (NPV) and area under ROC curve (AUC). **Conclusions:** The level of A2M can help in

assessing hepatic fibrosis in HCV Egyptian patients, especially when combined with that of APRI.

Keywords: Alpha-2-macroglobulin, APRI, Liver fibrosis, Hepatitis C virus.

1 Introduction

HCV is considered a major health problem worldwide [1]. Evaluation of fibrosis was only by using what called gold standard liver biopsy, the gold standard for assessment of degree of fibrosis, up till now. However, it is an invasive technique and has many limitations as it need experts to perform. Due to this limitations, many studies focused on development of non-invasive markers as an alternative to diagnose liver diseases to reduce the need of liver biopsy [2] [3].

Unfortunately, these scores were not very accurate when applied on different genotypes as they behave differently. In addition, the noninvasive alternatives for liver biopsies should be cheap, simple, and easy to perform, safe, and reproducible [4].

The human α-2-macroglobulin (A2M) is a serum glycoprotein synthesized (primarily by hepatocytes) in the liver [5]. A2M is a protease inhibitor implicated in many processes via a unique trapping mechanism. In addition, the A2M family are the major liver products associated with the inflammatory response [6].

Therefore, A2M levels were tested for their abilities to discriminate the stages of liver fibrosis and/or enhancing the diagnostic power of ARRI.

2 Methods

2.1 Patients

This study was conducted on 120 CHC attending the Egyptian liver institute and Hospital (ELRIAH), Mansoura, Egypt. The patients were chosen from adult males and females. All patients were negative for other causes of chronic liver diseases including viral hepatitis A and B. The positivity for the presence of HCV was confirmed in all patients using HCV RNA by quantitative polymerase chain reaction RT-PCR.

2.1.1 Patients' consent

Informed written consent from each patient and local ethical committee approval were obtained before starting the data collection. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

2.1.2 Exclusion criteria

Patients with decompensated liver disease (ascites, jaundice, bleeding, or encephalopathy), evidence of coexistent liver disease, HBV, HIV, autoimmune disease and liver transplantation. Patients, who had received any previous courses of antiviral treatment or immunosuppressive therapy, were also excluded.

2.2-Samples and blood markers:

Patients go through history taking, clinical examination, routine laboratory liver functions: AST, ALT, Albumin Using (Cobas Integra 400, Roch, Switzerland), and Hematological studies including Complete blood picture (hemoglobin, Red blood cells, Platelets and White blood cells) were done using D-cell 60 automated Hematology analyzer (Sysmex Xi 1800 incorporation, Japan), international normalized ratio (INR) was performed using (Sysmex® CA-1500, Japan) auto analyzer.

2-3. Human Alpha-2-Macroglobulin (A2M).

A2M was evaluated by solid-phase enzyme-linked immunosorbent assay (Boster Biological Technology Co., Ltd., Catalog No; K6610A) using 96-well microplates in accordance with the manufacturer's instruction. A standard curve was constructed using multiple dilutions of

the recombinant protein. The color development was stopped, and the optical density was measured at 450 nm and a reference filter of 620 nm (Stat Fax, U.S.A). Standard curve was obtained by plotting the optical densities against the corresponding standards, concentrations.

2.4. Liver biopsy

Needle liver biopsy specimens (n = 120) were obtained with an 16 G. Tissue specimens obtained by liver biopsy were fixed immediately in 10% formalin solution and sent to the pathologist at the same day and were routinely stained with haematoxylin–eosin stain. The liver biopsies had to measure at least 15 mm and/or contain five portal tracks, except for cirrhosis, for which no limitation was required. Subjected to single pathologists who was blinded to patients' clinical and laboratory features. The stage of fibrosis and grade of inflammatory activity in liver were determined according to the METAVIR scoring system [7]. F0, no fibrosis; F1, portal fibrosis alone; F2, portal fibrosis with rare septa; F3, portal fibrosis with many septa; and F4, cirrhosis. In this study, the stages of liver fibrosis were classified into three groups; namely, significant fibrosis (F2- F4), and non-significant fibrosis (F0-F1).

2.5-Statistical analysis

Statistical analysis was performed by Medcalc software version 15.0 (Medcalc 15.0, Mariakerke and Belgum). Continuous variables were expressed as mean ±standard deviation (SD). Comparisons of fibrotic Markers as well as routine laboratory tests and fibrosis stages were analyzed by Mann-Whitney U-test using a two-sided P-value. A value of $P < 0.05$ was considered statically significant. All significant fibrogenic biomarkers with a high area under the receiver-operating characteristic curve (AUC) and a high significance in univariant analysis were included in the stepwise linear regression analysis to produce a model to identify significant fibrosis. For formulation of the modified predictive score, optimal cut-off values were chosen to maximize the sum of sensitivity and specificity (Youden index). The diagnostic power of the developed model was measured by estimating its AUCs ROC curve was done to determine the cut-off point, AUC, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

2.6 Formula of the selected Score:

1- APRI: [AST (upper limit of normal)/ platelets x 10^9/L) X 100] [8]

3 Results

3.1 Patient's characteristics

The main baseline characteristics of CHC patients with hepatic fibrosis regards to the main classifications of hepatic fibrosis are outlined in table1. The results showed significant increases in the activities of ALT and AST in sera of patients with significant fibrosis (F2-F4), compared with those of non-significant (F0-F1, $p<0.0001$). Also, the results showed significant decrease of serum albumin levels ($p<0.0007$) and platelets count ($p<0.0181$) in case of patients with significant fibrosis compared with those with non-significant. In addition, the results showed no significant difference in ALP activity or total bilirubin levels in sera of patients with significant fibrosis compared with those with non-significant fibrosis (Table1).

3.2 Performance characteristics and AUCs for candidate biomarker A2M, versus APRI as non-invasive score in hepatic fibrosis:

At cutoff point >218.6 mg/dl, A2M was able to differentiate patients with significant from those with non-significant fibrosis with sensitivity of 87%, specificity 93.9%, PPV 92% and

NPV 89% with an area under curve of 0.93 ($p<0.0001$), (Table 2). On contrary, the performance characteristics of APRI in discriminating patients with significant from non-significant fibrosis gave a sensitivity of 49.1%, specificity of 78.79%, PPV 60.5% and NPV 63.6% at its original cutoff value (AUC=0.663, Figure 1)

3.3 Combination of A2M with APRI.

The results of the ability of A2M to enhance the diagnostic power of APRI was showed in Table 3. In fact, after combining the individual values of APRI with those of A2M, an MDA function was established. MDA= (0.038 * (A2M) + 0.068 * (APRI) – 9.19). A cutoff value of >75.19 AUC value was 0.94, and the sensitivity was 87%, specificity was 94% with PPV of 93% and NPV of 90% to discriminate patients with significant from those with non-significant fibrosis.

Thus, The combination of the individual values of A2M with those of APRI exaggerate the diagnostic power of the latter in discriminating patients with significant fibrosis from those with non-significant fibrosis.

Table 1: Biochemical investigations in patients with non-significant (F0–F1), significant (F2–F4).s

	Significant fibrosis (F2-F4) (n= 54)	Non-significant fibrosis (F0, F1) (n=66)	P-value
Age(years)	51.79 ± 11.05	36.57± 10.31	<0.0001
ALT(U/L)	49.88 ± 24.7	30.54 ± 14.03	<0.0001
AST(U/L)	37.51 ± 26.51	27.47 ±10.72	0.009
ALP(U/L)	72.93 ± 23.66	75.32 ± 24.28	0.4
Alb(g/dl)	4.41 ± 0.299	4.55 ± 0.31	0.004
T.bil(mg/dl)	0.57 ± 0.18	0.6 ± 0.19	0.54
Plt ($\times 10^9$/l)	218.07 ± 53.49	248.99 ± 58.18	0.002
I.N.R.	1.09 ± 0.09	1.07 ± 0.04	0.802
A2M(mg/dl)	280.01 ± 69.48	150.59 ± 47.55	<0.0001

Variables were expressed as mean ± SD. Reference values: aspartate aminotransferase (AST) up to 40 U/l; alanine aminotransferase (ALT) up to 40 U/l; albumin 3.8–5.5 g/dl; bilirubin up to 1 mg/dl; platelet count 150–450 × 10^9/l, INR, international normalized ratio A2M Alpha-2-macroglobulin.

[a]P-value for comparison between minimal and significant fibrosis in the estimation group

Table 2: Diagnostic values of Alpha-2-macroglobulin (A2M) versus those of the other non-invasive score (APRI) with optimal cut-off for discriminating stages of liver fibrosis.

Significant fibrosis versus non- significant fibrosis							
Parameter	**Cut off**	**AUC**	**Sp**	**Sn**	**PPV**	**NPV**	**P**
A2M	>218.6	0.93	94	87	92.2	89	<0.0001
APRI	>1.5	0.66	78	49	60.5	63	0.0011

AUC: area under the receiver-operating characteristic curve; PPV: positive predictive value; NPV: negative predictive value

P>005 is considered non-significant; P<0.05 is considered significant and P<0.001 is considered very significant.

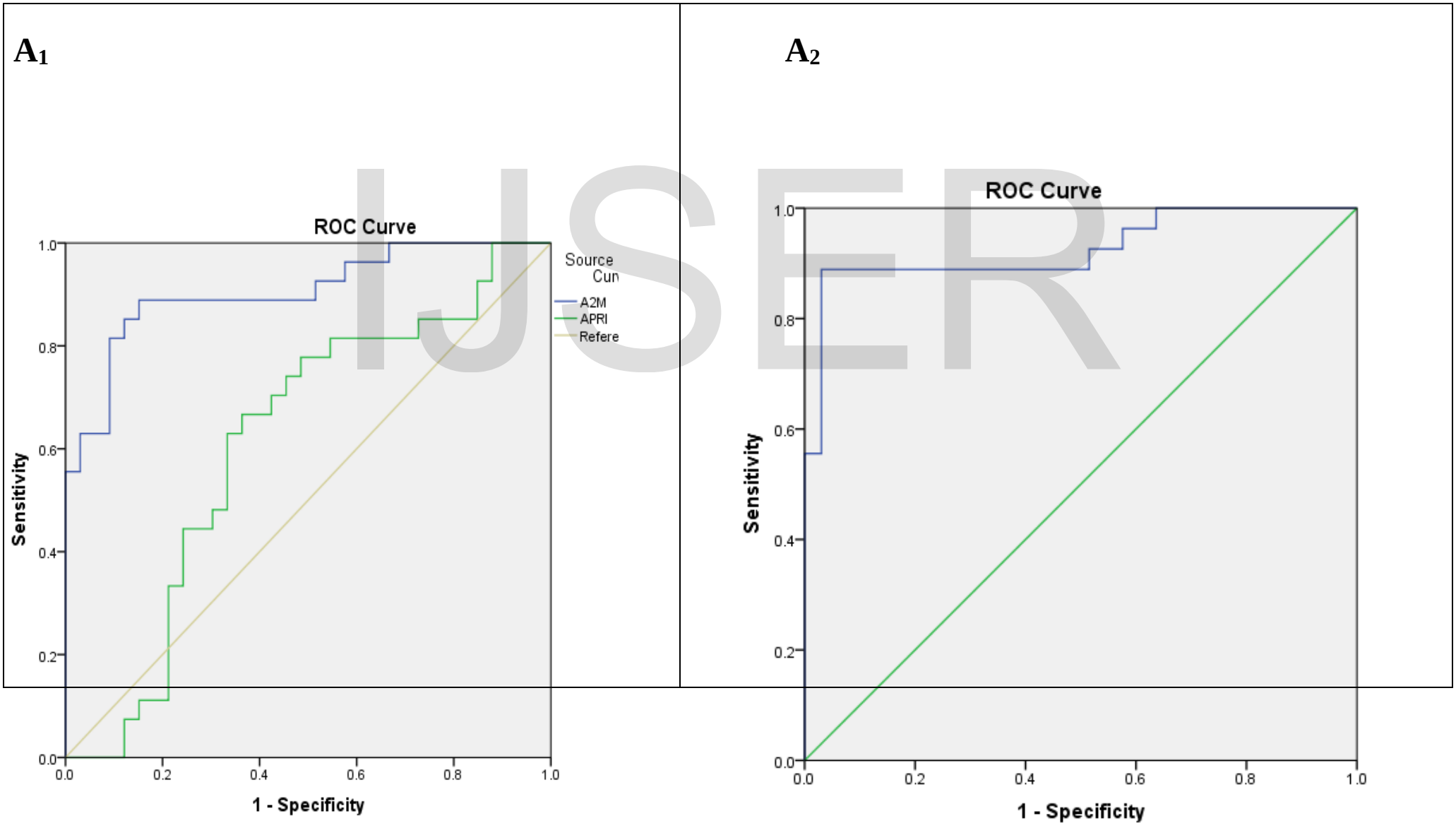
Table 3: Combination between A2M and the other non-invasive scores (APRI) Score in non-significant versus significant one.

Significant fibrosis versus non- significant fibrosis							
Logistic regression of Alpha-2-Macroglobulin (A2M) combination with APRI							
With APRI	**0.038 * (A2M) + 0.068 * (APRI) – 9.19**						
Parameter	**Cut off**	**AUC**	**Sp**	**Sn**	**PPV**	**NPV**	**P**
Modified APRI	> 75.19	0.94	94	87	93	90	<0.0001

AUC: area under the receiver-operating characteristic curve; PPV: positive predictive value; NPV: negative predictive value

P>005 is considered non-significant; P<0.05 is considered significant and P<0.001 is considered very significant.

Figure1: A1 **ROC curve of the selected non-invasive score (APRI) Versus the ROC curve of A2M, A2 is the ROC Curve of the newly combined APRI with A2M for discriminating between non-significant via significant fibrosis**



4 Discussion:

This study showed that A2M can strongly differentiate between patients with significant fibrosis from those patients with non-significant fibrosis with AUC of 0.93, ($p<0.0001$) with sensitivity and specificity of 87% and 94%, respectively.

Since A2M is a protease inhibitor; One can suggest that elevation in its level can help to inhibit the catabolism and/or the degradation of matrix proteins, a result that confirm the positive relationship of its level with the accumulation of extracellular matrix ECM and exaggeration of the severity of the disease, which is really the case in the present study [22]. Thus, its role in enhancing the fibrotic processes in the liver must be included [14], especially in cirrhotic patients. The elevated level in A2M in sera of patients with significant fibrosis could be explained when it was found that the A2M was mainly detectable in HSC. Beside the presence of A2M in the activated HSC can add more to its involvement in the mechanism of ECM over production and illustrate its ability to differentiate the end stage of liver diseases from the earlier ones[23]. The elevated levels of A2M gene, which was demonstrated in the cultured Ito cells, with subsequent increase in its secretion, confirm its role in regulation of collagen metabolism, synthesis and deposition with a resultant increase in hepatic fibrosis. [19], and cirrhosis [15-17]. Since HCV infection can participate in Ito cells stimulation, one can expect A2M elevation with subsequent ECM accumulation as a result of the inhibition of their degradation. Taking together, the advantages of A2M in assessing hepatic fibrosis will be one of our expectations.

The latter expectation can add more to superiority of A2M over that of APRI in diagnosing hepatic fibrosis. This is because APRI is a widely used algorithm for prediction of advanced fibrosis with AUC of 0.8, a sensitivity of 41% and specificity of 95 % as original study demonstrated [24].

APRI is consists of a two reliable markers of hepatic fibrosis and cirrhosis. One of them is Aspartate aminotransferase (AST) that is being a more reliable and durable marker for the degree of necroinflammatory activity in patients with cirrhosis [20]. The second is platelets count, which typically begins to decrease once bridging fibrosis is present and often falls below the lower limit of the normal range in liver cirrhosis. Thus, it was expected for APRI to achieve high diagnostic accuracy in identifying patients with significant fibrosis, in case of HCV patients with genotypes other than 4 [10]. Unfortunately, APRI gave lower sensitivity, specificity and AUC, which were 49%, 78% and 0.66, respectively when applied for patients of the present study [9].

Therefore, for enhancement of the diagnostic accuracy of APRI, the individual results of A2M were added those of APRI. Surprisingly, the logistic regression of the diagnostic power of APRI was increased. In this regard, the AUC was 0.94 in differentiating patients with non-significant fibrosis from those with significant fibrosis. Further, the NPV, PPV, sensitivity and the specificity were 90%, 93%, 87% and 94%, respectively.

5 Conclusion

the established MDA score can not only include a direct marker of liver fibrosis but also contain an indirect marker to precisely predict the sensitivity of hepatic fibrosis in patients with chronic hepatitis C. this is actually the conclusion of this study.

6 REFERENCES:

[1] **Lavanchy D.** Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect 2011; 17:107–115.

[2] **Yeshua H, Oren R**. Noninvasive assessment of liver fibrosis. Ann Transplant 2008; 13:5–11.

[3] **Poynard T, Lebray P, Inqiliz P, Varaut A, Nqo Y, Norha P, et al**. Prevalence of liver fibrosis and risk factors in a general population using non-invasive biomarkers (Fibro Test). BMC Gastroenterology 2010; 10:40.

[4] **Cross T, Antoniades C, Harrison P**. Non-invasive markers for the prediction of fibrosis in chronic hepatitis C infection. Hepatology Res 2008; 38:762–769.

[5] **Kan CC, Solomon E, Belt KT, Chain AC, Hiorns LR, Fey G**. Nucleotide sequence of cDNA encoding human alpha 2-macroglobulin and assignment of the chromosomal locus. Proc. Natl Acad. Sci. USA 82(8), 2282–2286 (1985).

[6] **Kushner I**. The phenomenon of the acute phase response. Ann. NY Acad. Sci. 389, 39–48 (1982).

[7] **Baranova A, La LP, Birerdinc A and Z. M. Younossi**. Non-invasive marker for hepatic fibrosis. BMC Gastroenterology 2011; 11: article 91

[8] **Wai CT, Greenson JK, Fontana RJ, et al**. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology 2003; 38:518–26.

[9] **Hamad I Al Ashgar, Mohammed Q. Khan, Mohammed Al-Ahdal,Sahar Al Thawadi, Ahmad Salem Helmy,1,3 Ahmed Al Qahtani, andFaisal M. Sana** Hepatitis C Genotype 4: Genotypic Diversity, Epidemiological Profile, and Clinical Relevance of Subtypes; Saudi J Gastroenterol. 2013 Jan-Feb; 19(1): 28–33

[10] **Claudia Della Corte, Anna Alisi, and Valerio Nobili**. indirect markers of fibrosis in chronic liver diseases: Is aspartate transaminase-to-platelet ratio (APRI) a useful test?. Hepat Mon. 2011 Jul 1; 11(7): 558–559

[11] **Karsdal MA, Henriksen K, Leeming DJ, Mitchell P, Duffin K, Barascuk N, et al.** Biochemical markers and the and the FDA Critical Path: how biomarkers may contribute to the understanding of pathophysiology and provide unique and necessary tools for drug development. Biomarkers 2009; 140:357-64.

[12] **Fattovich G, Llovet JM.** Risk factors for hepatocellular carcinoma in HCV cirrhosis: what we know and what is missing. J Hepatol 2006; 44: 1013-1016

[13] **Simona Bota, Roxana Sirli, Ioan Sporea, et al**. A new scoring system for prediction of fibrosis in chronic hepatitis C. Hepat Mon. 2011;11(7):548-555

[14]. **Hall PK, Roberts** : Physical and chemical properties of human plasma alpha 2 macroglobulin. Biochem J 171:27-38, 1978.

[15]. **Murray-Lyon IM, Michin Clarke HG, McPherson K, Wil- liams R**: Quantitative immunoelectrophoresis of serum pro- teins in cryptogenic cirrhosis, alcoholic cirrhosis and active chronic hepatitis. Clin Chim Acta 39:215-220, 1972

[16]. **Nalpas B, Boigne JM, Zafrani ES, Zimmermann R, Berthe- lot P**: Perturbations de dix prot6ines plasmatiques au cours des h6patopathies alcooliques. Gastroenterol Clin Biol 4:646-654, 1980

[17]. **Skrede S, Blomhoff JP, Elgjo K, Gjone E**: Serum proteins in diseases of the liver. Scand J Clin Lab Invest 35:399-406, 1975

[18]. **Munck Petersen C, Christiansen BS, Heickendorff L, Inger- slev J**: Synthesis and secretion of ct2-macroglobulin by hu- man hepatocytes in culture. Eur J Clin Invest 18:543-548, 1988

[19]. **Andus T, Bauer J, Gerok W**: Effects of cytokines on the liver. Hepatology 12:364-375, 1991

[20]. **Thapa BR, Anuj W**. Liver Function Tests and their Interpretation. Indian J Pediatr. 2007;74:663–671.

[21]. **Cheung BM, Ong KL, Wong LY**. Elevated serum alkaline phosphatase and peripheral arterial disease in the United States National Health and Nutrition Examination Survey 1999-2004. Int J Cardiol 2009; 135: 156-161.

[22]. **Nikolaus Fortelny, Georgina S. Butler, Christopher M. Overall1, and Paul Pavlidis**. Protease-inhibitor interaction predictions: Lessons on the complexity of protein-protein interactions 2017. The American Society for Biochemistry and Molecular Biology 2017.

[23]. **Andus, T., Ramadori, G., Heinrich, P. C., Knittel, T., and Meyer zum Buschenfelde,**. Cultured Ito cells of rat liver express the a2-macroglobulin gene. Eur. J. Biochem. 168,641-646.

[24] **Wai CT, Greenson JL, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS.** A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology 2003; 38: 518-26.

EL-Shahat A Toson

Chemistry Departments (Biochemistry Division), Faculty of Science, Damietta University, Damietta, Egypt, eatoson@yahoo.com.

Gamal E Shiha

Internal Medicine Departments, Faculty of Medicine, Mansoura University, Mansoura, Egyptian Liver Research Institute and Hospital (ELRIAH) Mansoura, Egypt, G_Shiha@hotmail.com.

Adel E Abed

Chemistry Departments (Biochemistry Division), Faculty of Science, Damietta University, Damietta, Egypt, Adelabed1989@gmail.com.