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BIOCHEMICAL STUDIES ON UROKINASE PLASMINOGEN ACTIVATOR (UPA) AS FIBROTIC MARKER IN HEPATITIS C PATIENTS

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ABSTRACT

Introduction: Biopsy is standard procedure in the evaluation of liver diseases, but it is an invasive method subject to sampling error. **Aim:** Non-invasive methods for the assessment of liver fibrosis are clinically important where hepatitis C virus (HCV) is common in Egypt. Our aim was to Validate and compare the performance of upA as simple blood marker of liver fibrosis in HCV patients in addition to GSH and NO. **Patients and methods:** A total of 75 Egyptian patients with clinically and laboratory confirmed hepatitis C patients (HCV) and liver fibrosis were included in the present study. They were recruited from the Internal Medicine Department, EL Ahrar Hospital, Zagazig, Egypt that approved the present study. **Result:** NO values were significantly increased gradually according to the progression of fibrosis degree (F1, F2, F3, and F4). While, there was no significant difference between F3 and F4 stages. Also, GST activity was extremely significant elevated gradually with the degree of liver fibrosis till F4. Meanwhile, GST activity was reduced in F4 compared to other fibrotic degrees. On a hand, uPA levels were significantly increased in F1, F2, F3 and F4 compared to healthy control individuals (F0). **Conclusion:** using of Urokinase-type plasminogen activator (uPA) a simple and non-invasive biochemical marker in addition to glutathione s-transferase and nitric oxide for the assessment of different stages of hepatic fibrosis as alternatives to liver biopsy which is invasive, expensive, painful and in some settings impossible to do in patients with chronic HCV infection.

INTRODUCTION

HCV and its long-term resultant consequences, is a major endemic medical health problem in Egypt.[1] In the Nile Delta and Upper Egypt, infection rates can be much higher at around 26% and 28%, respectively. With incidence rates between 2 and 6 per 1000 every year, this leads to an estimated 170,000 new cases every year to add to the 11.5 million patients suffering from the disease.[2] Chronic hepatitis C virus infection is important globally as a cause of liver- related morbidity and mortality with hepatic fibrosis, cirrhosis and hepatocellular carcinoma as the major clinical sequelae.[3] Liver fibrosis is a significant health problem, with a worldwide mortality attributable to cirrhosis and primary liver cancer of around 1.5 million deaths per year.[4] In patients with chronic viral hepatitis, precise definition of the hepatic fibrosis stage is the most important parameter to assess the risk of disease progression and to decide for an immediate and appropriate antiviral therapy. In these patients liver biopsy represents the gold standard for valuating the presence, type and stage of liver fibrosis.[5] This procedure, however, is invasive, stressful for patients, costly and difficult to standardize. Therefore, in recent years there has been an increasing interest in the possibility of identifying and describing liver fibrosis by using non invasive, surrogate markers measurable in peripheral blood.[6,7]

Urokinase-type plasminogen activator (uPA) is a serine protease, and together with its membrane associated receptor uPAR, is part of the uPA/uPAR system, which is an important component of the fibrinolytic system. In addition, it plays an important role in cancer progression and metastasis. In addition, expression pattern, secretion and function of uPA members have been observed in a broad range of human malignancies.[8,9]

Oxidative stress is a state of imbalance between the production and dismutation, or detoxification, of reactive oxygen species (ROS) by cellular mechanisms that can significantly affect signal transduction, gene expression and functional responses of involved cells or cause cell damage. Evidence of oxidative stress has been detected in almost all the clinical and experimental conditions of chronic liver diseases with different etiology and fibrosis progression rate and oxidative stress has been proposed as a major pro-fibrogenic mechanism.[10,11] Cellular glutathione (GSH) and related enzymes such as glutathione peroxidase (GSH-Px), glutathione S-transferase (GST) and glutathione reductase (GR) are among the principal protective mechanisms against endogenous and exogenous toxic substances and free radicals-mediated damage in liver tissue as well as in other tissues.[12,13]

Nitric oxide (NO) is a molecule that plays an important and complex role in immune responses. In addition to the microbicide function, which has been extensively described, this radical is notable for its capacity to modulate activation and direction of the innate and adaptive immune responses. Furthermore, different concentrations of NO can modulate the immune response in different ways, which can be beneficial or harmful, depending on the model studied. The consequences of the production of NO in viral infections are no less complex. On the one hand, NO is important in the resolution of some viral infections; on the other hand, it could cause or potentiate deleterious effects on the host. Thus, advances in our knowledge of the role of NO in immunomodulation and in the pathogenesis of viral diseases could contribute to the development of vaccines and therapeutic strategies.[14,15]

Aim of the work

The aim of this work is to evaluate the accuracy of simple and more-safe biochemical marker urokinase plasminoge activator in addition to glutathione s-transferase and nitric oxide for the assessment of hepatic fibrosis as alternatives to liver biopsy in Egyptian patients with chronic HCV infection.

Liver biopsy has been the gold standard for measurement of fibrosis. The area under the receiver operating characteristic curve (AUROC) for liver biopsy is 0.97.[16] Obtaining the correct fibrosis stage is critical because it controls clinical decisions on HCV treatment, although this is likely to evolve in the setting of new direct-acting antiviral regimens with high efficacy and minimal side-effects, as well as prediction of HCV-related complications. There are three main scoring systems, Metavir, Ishak and Knodell, which are used to score fibrosis. Liver biopsy is limited in its practicality due to its invasive nature, expense, pain, risks of bleeding, variations in the histologic grading, and sampling variability. Much of the current HCV research is focused on efforts to find accurate non-invasive imaging techniques and serum markers that will stage fibrosis.[7]

SUBJECTS AND METHODS

Subjects and Patients

A total of 75 Egyptian patients with clinically and laboratory confirmed chronic hepatitis C (CHC) and liver fibrosis were included in the present study. They were recruited from the Internal Medicine Department, EL Ahrar Hospital, Zagazig, Egypt that approved the present study. An informed consent was obtained from each individual participated in the present study and all were fully informed concerning the nature of the disease and the diagnostic

procedures involved. The HCV infection and liver fibrosis were diagnosed based on biochemical, serologic and histological criteria. In addition to 15 samples from healthy volunteers (negative control) were used.

Liver biopsy

Needle liver biopsy specimens (n = 75) were taken from the patients and examined by a pathologist unaware of the laboratory results. Biopsies were processed for diagnostic purposes, fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4 µm thick and routinely stained with hematoxyline and eosin stain. Fibrosis was assessed according to the Metavir scoring system on a five-point scale (F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = few septa, F3 = numerous septa without cirrhosis and F4 = cirrhosis). Activity grading by the Metavir system (based on the intensity of periportal and lobular necro-inflammation) was scored as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity and A3 = severe activity. The presence of stage F2, F3 or F4 was termed 'significant fibrosis', whereas the term 'advanced fibrosis' was reserved for stage F3 or F4. The presence of stage F4 was termed 'liver cirrhosis'.[17]

Blood samples

Blood samples were collected from all healthy and patients by vein-puncture within 2 weeks of liver biopsy. Sera were separated from the blood samples and tested fresh for the tested biochemical marker urokinase plasminogen activator according to the method of Finckh et al.[18] in addition to glutathione s-transferase activity by using Biodiagnostic kit method (Biodiagnostic company, Dokki, Giza, Egypt), according to the method of Habig et al.[19] and nitric oxide level using Biodiagnostic kit method (Biodiagnostic company, Dokki, Giza, Egypt), according to the method of Montgomery and Dymock.[20]

Statistical Analysis

All statistical analyses were done by a statistical software package (Statistical Package for Social Sciences (SPSS 15.0) for Microsoft Windows, SPSS Inc.). Descriptive results were expressed as mean ± SD and range or number (percentage) of patients with a condition. Differences in continuous variables were assessed using Student's t-test or analysis of variance (ANOVA) and X^2 test for categorical variables.[21]

RESULTS

Diagnosis of samples

The present study involved 75 patients with clinically and laboratory confirmed chronic hepatitis C and liver fibrosis in addition to 15 negative controls (healthy volunteers). Positive patients with liver fibrosis were divided into different degrees according to METAVIR system as: 22 patients were categorized as F1 by (29%), 26 were F2 by (35%), 9 were F3 by (12%) and 18 patients were F4 by (24%).

Biochemical Parameters in all studied groups

The mean NO, GST and uPA levels in healthy control individuals (F0) were found to be 17.91 ± 1.15 (µmol/l), 27.24 ± 3.2(U/L) and 65.19 ± 9.41 (pg/ml) respectively. NO values were significantly increased gradually according to the progression of fibrosis degree to be 26.16 ± 2.8, 55.41 ± 7.8, 93.30 ± 4.6, 93.34 ± 10.23 in F1, F2, F3 and F4 by 46.06%, 209.55%, 420.94% and 421.16%; respectively; ($p<0.001$), compared to healthy control individuals (F0). While, there was no significant difference between F3 and F4 stages, (Fig.1). Also, GST activity was extremely significant elevated gradually with the degree of liver fibrosis till F4, to be 169.43 ± 20.69, 394.43 ± 51.95, 565.44 ± 47.90, and 224.88 ± 22.76 in F1, F2, F3, and F4 by 521.98%, 1347.98%, 1975.77%, and 725.55; respectively; ($p<0.001$), compared to healthy control individuals (F0). Meanwhile, GST activity was reduced in F4 compared to other fibrotic degrees (F1, F2, and F3), (Fig. 2). On a hand, uPA levels were significantly increased to be 181.41 ± 15.27, 321.34 ± 72.60, 577.50 ± 37.21 and 690.04 ± 24.52 in F1, F2, F3 and F4 by 178.28%, 392.93%, 785.87% and 958.51%; respectively; ($p<0.001$), compared to healthy control individuals (F0), as shown in (Fig. 3).

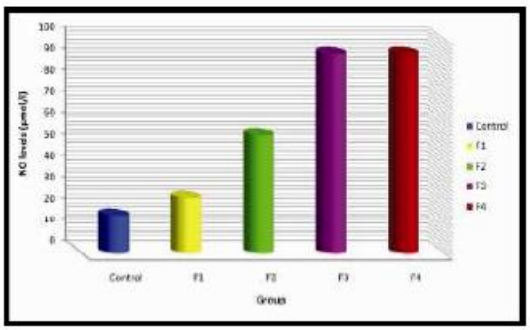


Fig. 1: No levels in all studied groups.

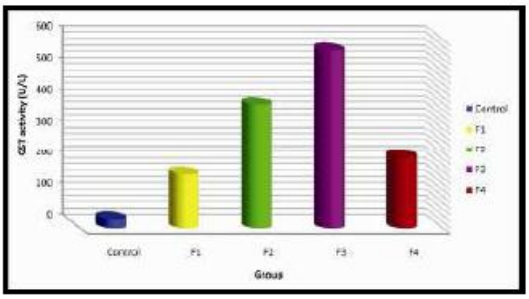


Fig. 2: GST activity in all studied groups.

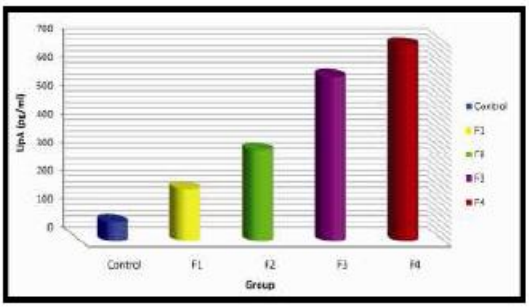


Fig. 3: uPA levels in all studied groups.

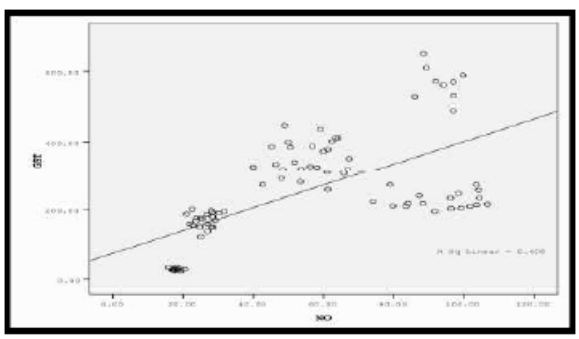
Correlations between different studied parameters among studied groups

There were significant positive correlations between NO, GST and uPA to each other according to the degree of liver fibrosis.

Table 2: Pearson's correlations analysis between different studied parameters in patients studied groups.

Parameter		NO	GST	MMP-8
NO	**r**	------	**0.639****	**0.898****
GST	**r**	**0.639****	--------	**0.465****
uPA	**r**	**0.947****	**0.528****	-------

**Correlation is significant at p<0.001.



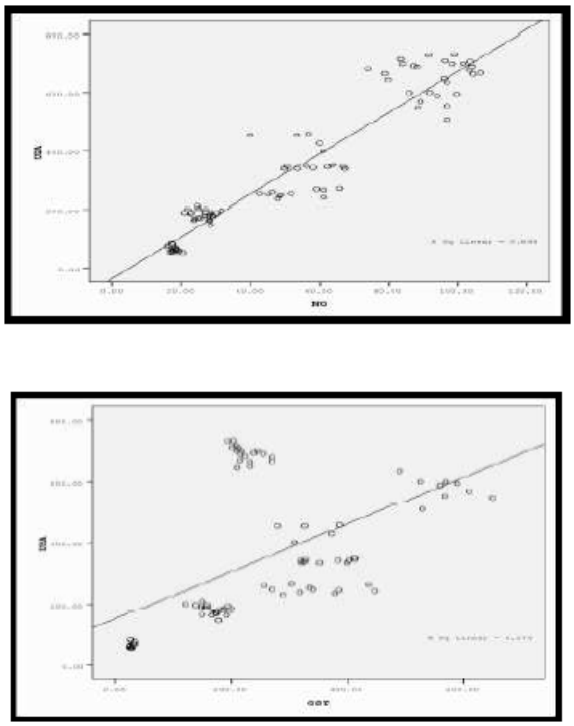


Fig. 4: Correlations between different parameters in all studied groups.

The laboratory data of liver fibrosis and non liver fibrosis, advanced liver fibrosis and liver cirrhosis

In the present study, patients with significant fibrosis were associated with higher mean NO, GST and uPA than those of non significant liver fibrosis with extremely significant difference ($p<0.0001$; table 2). Patients with advanced liver fibrosis were associated with higher mean NO, GST and uPA than those of non advanced liver fibrosis with extremely significant difference ($p<0.0001$; table 3). Also, patients with liver cirrhosis were associated with higher mean NO, GST and uPA than those of non liver cirrhosis with extremely significant difference ($p<0.0001$; table 4).

Table 2: Levels of liver fibrosis markers in significant liver fibrosis and non significant liver fibrosis.

Marker	Significant N=53	Non significant N=37	P value
NO(µmol/l)	56.9±35.5	17.9±1.2	< 0.0001
GST (U/l)	343.8±123.4	111.1±72.5	< 0.0001
uPA(pg/ml)	458.4±228.4	63.9±7.8	< 0.0001

Table 3: Levels of liver fibrosis markers in s advanced liver fibrosis and non advanced liver fibrosis.

Marker	Advanced N=27	Non advanced N=63	P value
NO(µmol/l)	82.1 ±28.6	23.2± 13.9	< 0.0001
GST (U/l)	338.4±166.8	209.8 ±134.3	< 0.0001
uPA(pg/ml)	652.5±61.1	143.6 ±134.1	< 0.0001

Table 4: Levels of liver cirrhosis markers in liver cirrhosis and non liver cirrhosis.

Marker	cirrhosis N=18	Non cirrhosis N=72	P value
NO(µmol/l)	93.3± 10.2	27.2±22.1	< 0.0001
GST (U/l)	224.8± 22.7	254.3±173	< 0.0001
uPA(pg/ml)	690.0±24.5	197.8±١٩١	< 0.0001

DISCUSSION

Clinical management of chronic hepatitis C is dependent on the extent of liver fibrosis. Liver biopsy, the gold standard, is still recommended in the majority of patients.[22] However, it is an invasive procedure responsible for severe complications in about 0.5% of cases. Sample variability is another limitation. The biopsy specimen appears to be poorly reliable when its length is inferior to 15 mm.[23,24] However, liver biopsy is invasive, requires an experienced gastroenterologist, examination is required by a professional histopathologist, adds expense and is associated with complications and mortality patients with chronic hepatitis C.[25,26] Moreover, liver fibrosis is evaluated by histological scores, which have inter-observer variability especially among non-expert pathologists.[27] Biomarkers are being developed as alternatives to liver biopsy for predicting liver fibrosis in patients with chronic hepatitis C.[28] A simple, reproducible, low-cost and non-invasive tool that can follow the evolution of the disease overtime would be beneficial for the testing physician and is desired by the patients.[23] In the present study, five biomarkers: nitric oxide (NO), glutathione s-transferase (GST), urokinase plasminogen activator (uPA), matrix metalloproteinase-8 (MMP-8) and Galectin-3 (Gal-3) were measured using standard methodologies in 75 patients with clinically and laboratory confirmed chronic hepatitis C and liver fibrosis in addition to 15 negative controls (healthy volunteers). The mean NO, GST and uPA levels in healthy control individuals (F0) were found to be 17.91 ± 1.15 (µmol/l), 27.24 ± 3.2(U/L), 65.19 ± 9.41 (pg/ml) respectively.

Nitric oxide (NO) values were significantly increased gradually according to the progression of fibrosis degree to be 26.16 ± 2.8, 55.41 ± 7.8, 93.30 ± 4.6, 93.34 ± 10.23 in F1, F2, F3 and

F4 by 46.06%, 209.55%, 420.94% and 421.16%; respectively; ($p<0.001$), compared to healthy control individuals (F0). While, there was no significant difference between F3 and F4 stages. Nitric oxide (NO) is a free radical produced during L-arginine metabolism. In addition to its physiological activities in vascular and neuronal functions, its role in the immune system as a microbicide and tumor-killing mediator has been well described, as well as its release by activated macrophages. NO is produced by a variety of immune and non-immune cells and is involved in the regulation of several immune functions. Furthermore, different concentrations of NO can modulate the immune response in different ways, which can be beneficial or harmful, depending on the model studied. On the one hand, NO is important in the resolution of some viral infections; on the other hand, it could cause or potentiate deleterious effects on the host.[14,15]

Hepatic fibrosis in patients chronically infected with hepatitis B and C also appears to be correlated with increased expression of iNOS. Although the molecular mechanisms have not been well elucidated, it was shown that fibrosis levels were correlated positively with iNOS expression, as well as that of TGF-b, which is an oxidative stress inducer and profibrogenic cytokine (29). Reactions of NO with cysteines in important virus enzymes, such as reverse transcriptase and protease, have been extensively described. Generally, the modification of proteins by NO affects viral replication or infectivity, with a positive modification of the host. Proteases are enzymes that are important for the breaking of viral polyproteins and that act, usually after formation of the virion, by making the virion able to infect new cells.[15] There is a controversy regarding the production of NO in chronic HCV patients with studies reporting an increase[30,31], a decrease[32], or no change[33,34] in its level.

During this study, Glutathione s-transferase (GST) activity was extremely significant elevated gradually with the degree of liver fibrosis till F4, to be 169.43 ± 20.69, 394.43 ± 51.95, 565.44 ± 47.90, and 224.88 ± 22.76 in F1, F2, F3, and F4 by 521.98%, 1347.98%, 1975.77%, and 725.55; respectively; ($p<0.001$), compared to healthy control individuals (F0). Meanwhile, GST activity was reduced in F4 compared to other fibrotic degrees (F1, F2, and F3). Glutathione and related enzymes such as glutathione peroxidase, glutathione S-transferase (GST) and glutathione reductase (GR) are among the principal protective mechanisms against endogenous and exogenous toxic substances and free radicals-mediated damage in liver tissue as well as in other tissues.[12,13]

A decreased GSH level in chronic liver diseases has been reported in many reports.[35,36] GST is a sensitive marker in the diagnosis of alcoholic liver disease[37] as well a reliable marker in monitoring the response to chronic liver disease treatment.[38] In both clinical and experimental HCC, reduced global activity of GST has been observed within tumors[12] and the specific isoforms GSTπ1 and GSTα1[39,40] have been shown to be over expressed and have been used as biomarkers in experimental models of HCC.

In the present study, Urokinase plasminogen activator (uPA) levels were significantly increased to be 181.41 ± 15.27, 321.34 ± 72.60, 577.50 ± 37.21 and 690.04 ± 24.52 in F1, F2, F3, and F4 by 178.28%, 392.93%, 785.87% and 958.51%; respectively; ($p<0.001$), compared to healthy control individuals (F0). Urokinase plasminogen activator (uPA) is a serine protease, and together with its membrane associated receptor uPAR, is part of the uPA/uPAR system, which is an important component of the fibrinolytic system. In addition, it plays an important role in cancer progression and metastasis. In addition, expression pattern, secretion, and function of uPA members have been observed in a broad range of human malignancies.[8,9] A number of investigations has indicated that uPA, uPAR and PAI-1 are elevated in hepatocellular carcinoma (HCC) in comparison to normal liver tissues.[41,42] Moreover, the uPA system has been suggested to be operative in HCC and cirrhotic liver, more than fibrolamellar HCC.[43] Further, uPAR- and PAI-1-elevated levels were correlated to poor survival in HCC patients.[42] In comparison to liver cirrhosis, higher levels of uPA and uPAR were found in malignancy and tuberculosis of the liver.[44,45] **Berres et al.,**[46] reported that soluble urokinase plasminogen activator receptor (suPAR) is associated with progressive liver fibrosis in hepatitis C Infection, as they assessed suPAR serum levels in 146 chronically HCV infected patients by enzyme-linked immunosorbent assay and correlated them with biopsy-proven histologic stage of liver fibrosis and noninvasive liver fibrosis markers (aspartate transaminase to platelets ratio index score, transient elastography). Their study revealed that suPAR serum levels were strongly associated with the histologic stage of liver fibrosis.

From this study we conclude that: It was shown that fibrosis levels were correlated positively with Nitric oxide (NO) concentration. Glutathione s-transferase (GST) activity was a reliable monitoring the response to chronic liver disease treatment. Addition of (NO) and (GST) to (uPA) gives a significant improvement in detection of different stages of fibrosis in patients with HCV. Therefore, combination of multiple markers may be more valuable in the

diagnosis of liver fibrosis. **Finally,** There were significant positive correlations between NO level, activity of GST with uPA to each other according to the degree of liver fibrosis.

We can recommended that, using of Urokinase plasminogen activator (uPA) a simple and non-invasive biochemical marker for the assessment of different stages of hepatic fibrosis as alternatives to liver biopsy which is invasive, expensive, painful and in some settings impossible to do in patients with chronic HCV infection in addition of glutathione s-transferase activity and level of nitric oxide.

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