Serum Leptin, Ferritin and Uric Acid as Predictors of Fibrosis and Sustained Virological Response in Chronic Hepatitis C Patients

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Abstract: Hyperleptinemia, hyperferritinemia and hyperuricemia have been implicated in hepatic inflammation and fibrosis and assumed to be predictors of poor response to antiviral therapy. The aim of study is to evaluate the role of serum leptin, ferritin and uric acid in progression of chronic hepatitis C (CHC) disease and their role in detection of virological response to combined antiviral therapy (pegylated interferon PEG-IFN-α/ribavirin RBV). Study design is a prospective study; conducted on 40 patients with CHC infection eligible for treatment with combined antiviral therapy before the combined antiviral therapy introduced and 30 healthy volunteers as controls. Patients with chronic liver disease due to other causes were excluded from the study. All patients and controls were subjected to through clinical evaluation, abdominal ultrasonography, complete blood picture (CBC), liver enzymes (AST/ALT), total bilirubin and leptin, ferritin and uric acid investigations. Plasma HCV-RNA viral load by real-time polymerase chain reaction (PCR) and liver biopsy using Metavir score were done for HCV patients only. We compared level of serum leptin, ferritin and uric acid level between patients and with controls. We estimated serum leptin and ferritin sensitivity, specificity and accuracy in both responders and non-responders to treatment. Study findings were as the follow: serum leptin and ferritin higher concentrations were predictive factors to failure of combined antiviral treatment and correlated with inflammatory activity and advanced liver fibrosis. Serum uric acid level was not significantly associated with inflammatory activity, stage of hepatic fibrosis and interferon treatment failure. In Conclusion CHC is associated with higher leptin and ferritin levels. High serum leptin and ferritin levels are negative predictors of response to combined antiviral treatment in CHC. Serum leptin had higher sensitivity, specificity and accuracy than serum ferritin; so leptin considered better than ferritin in prediction of response to antiviral therapy in chronic HCV patients.

1. Introduction

Chronic hepatitis C is a leading cause of liver-related morbidity and mortality in Egypt and throughout the world. Despite progress in treating chronic hepatitis C with IFN or with IFN and ribavirin, most patients do not experience a sustained virological response with receipt of these therapies[1].

Iron is essential for life, but both severe iron deficiency and iron overload pose dangerous health risks and potentially fatal[2]. It found that body iron level may affect the course of HCV infection and the response to IFN therapy. Some studies have suggested that excess iron in the liver may predispose patient to persistent viral infection and could have a negative effect on the response to IFN therapy[3]. Also, other studies have shown that patients with lower serum levels of ferritin, lower transferrin saturation values and lower levels of iron in the liver have an improved response to IFN therapy[4]. Several studies have demonstrated that reduction of iron via therapeutic phlebotomy leads to improvements in serum levels of aminotransferases in patients with chronic hepatitis C and improves responses to standard IFN therapy[5,6].

Leptin is the product of the obese (ob) gene, which expressed by adipose tissue, liver and other organs. It plays an important role in the regulation and metabolism of body fat and may induce insulin resistance, increase fatty acid concentrations in the liver and enhance lipid peroxidation[7]. Leptin may act as an immunomodulator, inducing the release of cytokines, such as tumor necrosis factor (TNF)-α, interferon (INF)-γ, interleukin (IL)-18 and tumor growth factor (TGF)-β1, thus promoting liver steatosis and fibrosis[8]. Several studies reported that high serum leptin concentrations in patients with CHC correlated with more severe steatosis, lower viremia and a lower antiviral response[9].

In a retrospective study high serum uric acid was found to be a predictive of poor response to interferon therapy[10]. In chronic HCV patients under

haemodialysis high serum uric acid was found to be associated with more oxidative stress, which is harmful to liver[11].

Relatively few data have been published on serum ferritin, leptin and uric acid levels as putative risk factors for liver diseases; especially HCV infection in Egypt population. Understanding the role of these indicators as a risk factor for developing liver diseases is important to prevent morbidity, developing new treatment strategies to prevent HC progression and improve response to IFN therapy. The present study aim is to investigate the relation of serum leptin, ferritin and uric acid to HCV infection, the grade of liver fibrosis and the response to combined IFN therapy.

2. Subjects and Methods

A sample size at least of 29 from the positive group (patients) and 29 from the negative group (control) achieve 81% power to detect a difference at a significance level of 0.05. In this prospective study design conducted from June 2012 till June 2013 we includes 40 patients (33 males and 7 females) with chronic CHC infection eligible for treatment with antiviral combined therapy (PEG-IFN-α/RBV) before receiving the therapy and 30 controls (healthy volunteers including 25 males and 5 females) age and sex matched with CHC patients according to the sample size calculated above. The sample technique was convenient sample were patients and controls (relatives of the patients) selected during the 3 months from the beginning of the study from Qena Interferone Therapy Center for Chronic Hepatitis C Patients, then all the selected patients who receive the combined antiviral therapy for 3 months were followed after 6 months of stop of the therapy. Exclusion criteria include patients with other causes of chronic liver disease, decompensated liver disease, history of heart failure, diabetes, thyroid diseases, abnormal renal function, cancer, previous treatment with antiviral agents and use of drugs known to induce liver steatosis within the last 6 months.

All patients and controls were subjected to clinical assessment, abdominal ultrasonography and laboratory investigations (CBC, liver enzymes AST/ALT, total bilirubin and leptin, ferritin and uric acid). Seven ml of venous blood were drawn from every individual after an overnight fast; 2 ml on EDTA anticoagulant for CBC (using Sysmex KX21; Sysmex corporation), 1.8 ml on sodium citrate for prothrombin time; concentration and INR (using Siemens coagulometer, Germany) and the other 3 ml in a plain vacutainer left to clot, centrifuged and serum was separated and aliquoted in Eppindorf tubes and stored at -80°C till used for estimation of liver enzymes (AST/ALT), total bilirubin, uric acid (using

Cobas C311, Roche diagnostics), ferritin (using Architect i2000, Abbott) and leptin (done by ELISA kit from Labor Diagnostika Nord GmbH & Co. KG (LDN) were done.

Plasma HCV-RNA concentration (viral load) determined by real-time polymerase chain reaction (PCR) and liver biopsy done for CHC patients only. In liver biopsy; liver specimens for histological evaluation were fixed by formalin and embedded in paraffin. Stained sections were evaluated according to METAVIR scoring system that includes semi-quantitative assessment of liver disease inflammation grads and fibrosis stages[12]. Liver biopsy inflammation grades divided into 4 categories: no activity A0, mild activity A1, moderate activity A2 and severe activity A3. Liver biopsy fibrosis stages classified to 4 stages: no fibrosis F0, mild fibrosis F1, moderate fibrosis F2 and advanced fibrosis F3. Virological response to antiviral combination therapy defined as HCV RNA below the limit of detection in a sensitive assay ≥ 24 weeks after treatment completion was assessed in CHC patients by qualitative PCR for HCV-RNA after 6 months of stop of the therapy[2].

The study protocol had been approved by the Ethical Committee in Qena Faculty of Medicine and both patients and controls gave informed verbal consent to participate in the present study.

Statistical analysis of data was done using SPSS (statistical program for social science version 17). Results with continuous variables are expressed as means and ± standard error of the mean (mean ± SD) and were compared using t-tests. P value of < 0.05 and or 0.001 is considered significant. Correlation was done between ferritin, Leptin and uric acid level and liver inflammatory grades and fibrosis stage. Sensitivity, specificity and accuracy of both leptin and ferritin were estimated with a cut of value to identify which is the better screening test in prediction of response to antiviral therapy in chronic HCV patients.

3. Results

This is study conducted on 40 patients (33 males and 7 females), aged 22-55 years with chronic CHC infection eligible for treatment with antiviral combined therapy before PEG-IFN-α/RBV combined antiviral therapy is given and 30 healthy volunteers (25 males and 5 females) aged 22 - 58 years; were recruited for participation as controls.

The investigated laboratory parameters of the studied groups were represented in table l. Patients with CHC infection had higher baseline serum leptin and ferritin levels than control (P = 0.0001 & 0.003 respectively) while no significant difference in uric acid levels (figure1a, b & c).

Liver biopsy showed that according to the stage of liver fibrosis; patients were divided into 8 cases

20% with stage F0, 12 cases 30% with stage F1, 12 cases 30% with stage F2 and 8 cases 20% with stage F3. Patients were grouped according to necro-inflammatory activity into 1 case 2.5% with grade A0, 15 cases 37.5% with grade A1, 19 cases 47.5% with grade A2 and 5 cases 12.5% with grade A3 (table 2). There were positive correlations between baseline serum leptin and ferritin levels and liver inflammation grades and fibrosis stage as shown in tables 3 & 4 and figures 2a, 2b & 3a, 3b. However, no correlation was detected between serum uric acid levels and the grades of liver inflammation and fibrosis stages (tables 3 & 4).

Twenty two (55%) of patients achieved sustained virological response (SVR) after 6 months (24 weeks) of stop of the therapy which is assessed by negative qualitative HCV-RNA after stop of the therapy, while 18 patients (45%) did not achieved SVR . The laboratory data of the responders and non-responders were shown in table (5). The baseline serum leptin and ferritin levels were significantly higher in non-responder patients (p value <0.0001 for each) while uric acid level showed no significant difference (table 5, figures 4a, b & c).

The sensitivity, specificity & accuracy of serum leptin was 77.8, 90.9, 85.0 respectively with the best cut off value at 22 (AUC=0.927) and for ferritin levels was 72.2, 81.8, 77.5 respectively with the best cut off value at 303.9 (AUC=0.843) in prediction of response to antiviral combination treatment (table 6 & figure 5).

Table 1: Baseline laboratory parameters of the studied CHC patients and control group

| Parameter	Patients No (40)	Control No (30)	P value
AST (IU/L)	37.72±21.75	18.37±8.59	0.0001*
ALT (IU/L)	28.25±17.82	21.23±8.53	0.0001*
Prothrombin conc. (%)	90.15±8.65	89.90±7.81	0.814
Bilirubin (mg/dl)	0.95±0.45	0.83±0.20	0.002*
Hemoglobin (g/dl)	13.82±1.49	13.50±1.76	0.163
Platelets (x109/L)	225.08±78.67	266.07±48.14	0.001*
Leptin (ng/ml)	19.59±16.31	6.37±2.62	0.0001*
Ferritin (ng/ml)	282.17±214.56	79.24±68.03	0.003*
Uric acid (mg/dl)	4.652±4.39	4.49±1.10	0.194

*P value: significant t-test

Figure (1a): Comparison between baseline leptin in patients and control groups (p < 0.0001)
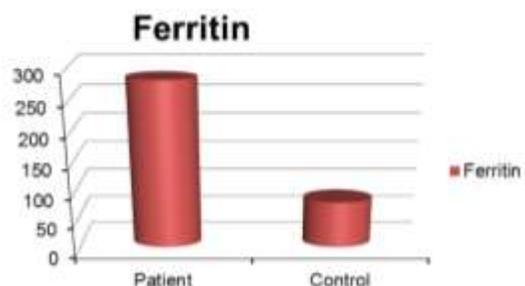
Figure (1b): Comparison between baseline ferritin in patients and control groups (p = 0.003)
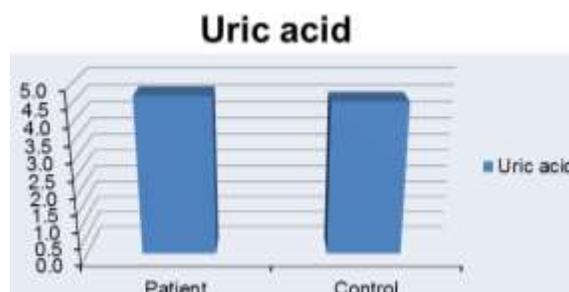
Figure (1c): Comparison between baseline uric acid in patients and control groups (p = 0.194)

**Table (2): Liver Biopsy-Fibrosis Stages and Inflammation Grades in CHC patient**

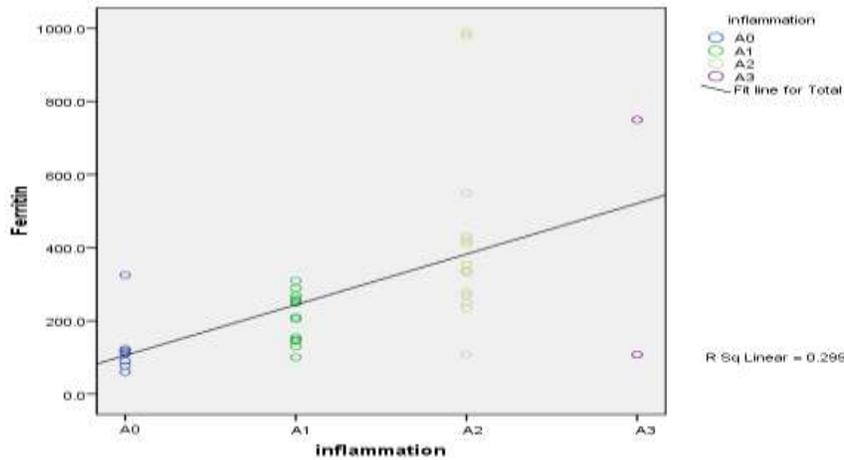| Liver Biopsy-Fibrosis Stage	Patients No (40)	%
No fibrosis (F0)	8	20
Mild fibrosis (F1)	12	30
Moderate fibrosis (F2)	12	30
Advanced fibrosis (F3)	8	20
Liver Biopsy-Inflammation Grade		
No activity (A0)	1	2.5
Mild activity (A1)	15	37.5
Moderate activity (A2)	19	47.5
Severe activity (A3)	5	12.5

Table (3): Correlations between serum leptin, ferritin, uric acid and liver biopsy inflammation grades

Correlations	r value liver biopsy inflammation grades	p value
Leptin	+ 0.867	<0.0001*
Ferritin	+ 0.630	<0.0001*
Uric acid	+ 0.135	0.407

*P value: significant
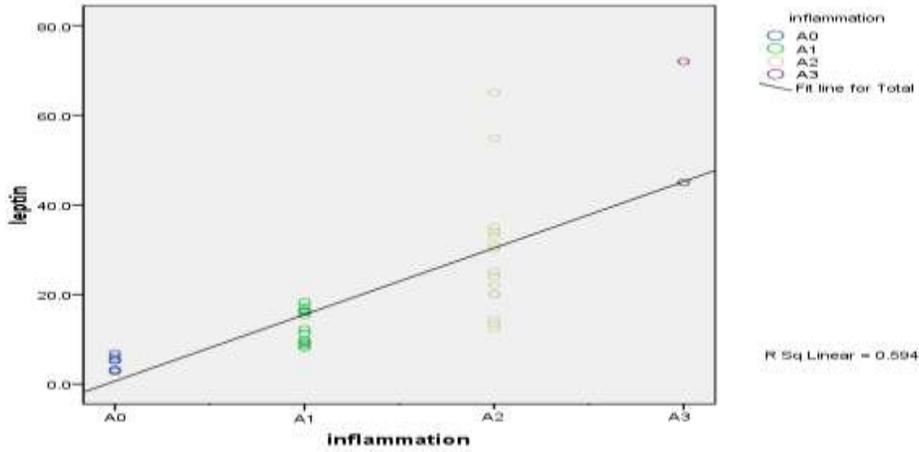
Figure (2a): Correlation between serum leptin and liver biopsy inflammation grades

Figure (2b): Correlation between serum ferritin and liver biopsy inflammation grades

Table (4): Correlations between serum leptin, ferritin, uric acid and liver biopsy fibrosis stages

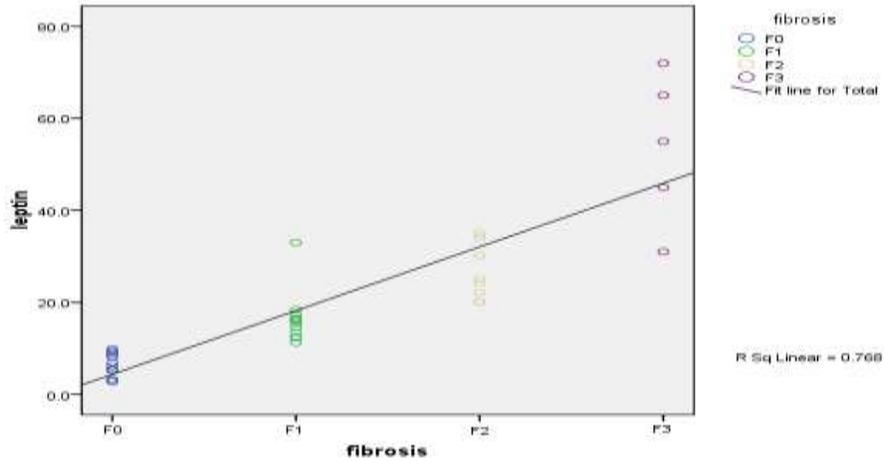| Correlations	r value liver biopsy fibrosis stage	p value
Leptin	+ 0.938	<0.0001*
Ferritin	+ 0.719	<0.0001*
Uric acid	+ 0.062	0.706

*P value: significant

Figure (3a): Correlation between serum leptin and liver biopsy fibrosis stages
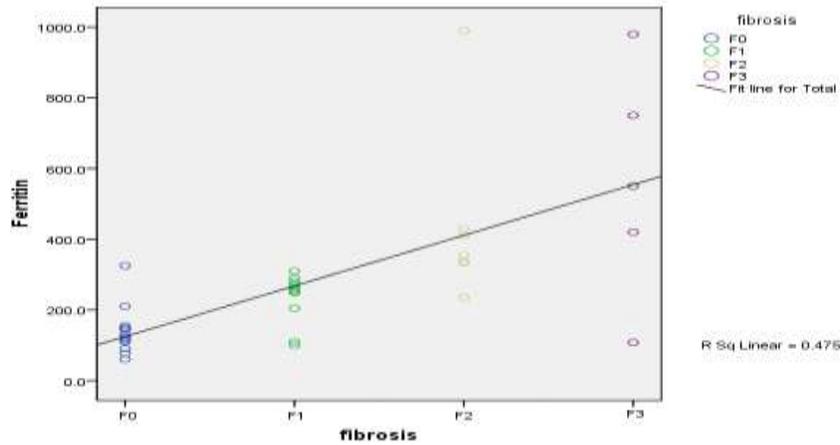

Figure (3b): Correlation between serum ferritin and liver biopsy fibrosis stages

Table (5): laboratory investigations of the responders and non-responders in CHC patients'

| Parameter	Responders		Non-responders		P value
	NO = 22	55%	NO = 18	45%	
AST (IU/L)	32.2±19.69		44.4±22.79		0.077
ALT (IU/L)	20.6±14.37		37.5±17.52		0.002*
Proth. conc. (%)	93.6±5.77		85.8±9.73		0.003*
Bilirubin (mg/dl)	1.01±0.44		0.872±0.45		0.322
Hemoglobin (g/dl)	14.18±1.27		13.39±1.665		0.098
Platelets (x109/L)	258.8±1.66		183.8±70.85		0.002*
Leptin (ng/ml)	10.02±5.16		31.28±17.74		<0.0001*
Ferritin (ng/ml)	177.02±81.12		410.69±256.30		<0.0001*
Uric acid (mg/dl)	3.81±0.84		5.67±6.44		0.188

*P value: significant t-test
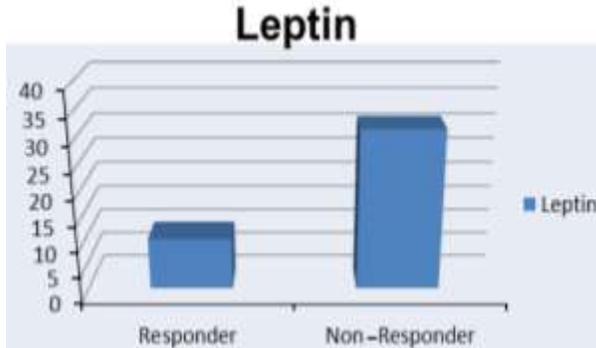
Figure (4a): Comparison between serum leptin in responders and non-responders of the CHC patients' group (p value < 0.0001)
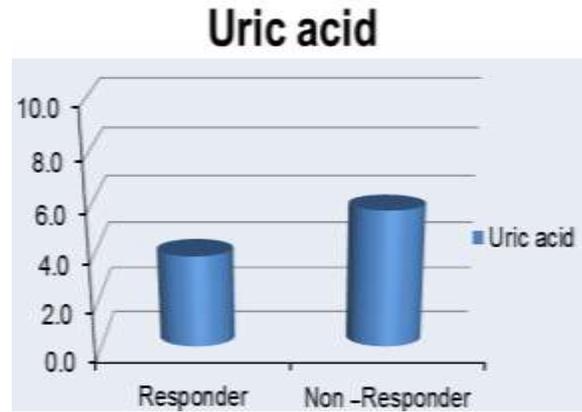

Figure (4a): Comparison between serum ferritin in responders and non-responders of the CHC patients' group (p value < 0.0001)

Figure (4c): Comparison between serum uric acid in responders and non-responders of the CHC patients' group (p = 0.188)
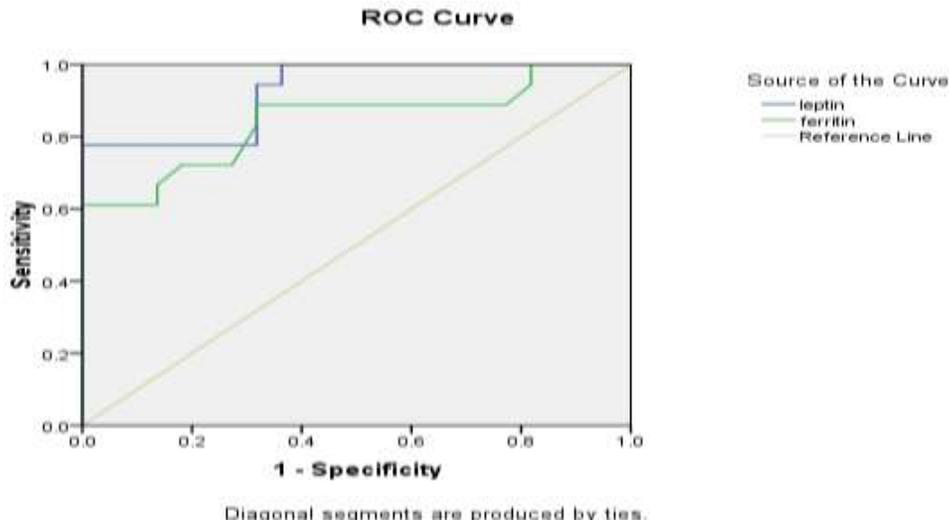
Parameter	Sensitivity	Specificity	Accuracy	Cut off value	AUC*	Odds ratio
Leptin	77.8	90.9	85.0	22.0	0.927	1.352
Ferritin	72.2	81.8	77.5	303.9	0.843	1.014

AUC* Area under the curve

Figure (5) ROC curve and table (6) represents the best sensitivity and specificity of serum leptin and ferritin in prediction (screening) of response to antiviral combination therapy.

ROC curve: receiver operator curve represents the positivity criterion of screening test (sensitivity, specificity, false negative and false positive) in relation to stander (reference) test

4. Discussion:

Leptin is a circulating 16-kDa non-glycosylated protein secreted from the adipocytes of white fat into the blood. Serum leptin levels have been found to be higher in patients with chronic hepatitis C (CHC) and particularly in those with more severe fibrosis or cirrhosis[13]; however, the precise mechanisms underlying the profibrogenic action of leptin has not been elucidated[14]. HCV-related steatosis is not always virally related and other factors may coexist as obesity and alcohol consumption[15].

In this study, leptin levels in patients with chronic HCV infection were increased as compared with controls (table 1). Liu and his colleagues, 2005[16] found that the extents of leptin elevation are closely related with the severity of abnormal fat metabolism and hepatic steatosis in chronic HCV-infected patients, as they found that the serum leptin levels were correlated with body fat, body mass index (BMI) and Apo B, but not with liver function and serum HCV RNA levels. Although many studies demonstrated profibrogenic role of leptin[17,18] mainly through direct influence on human hepatic stellate cells[19], others did not reveal any role of leptin in determining severity of steatosis and fibrosis in patients with chronic hepatitis C[20].

The mechanisms; by which chronic HCV infection causes elevated serum leptin levels, are not completely known, but inflammation and abnormal fat metabolism in patients with chronic HCV infection might be involved[16]. The virus can increase glutathione turnover by eliciting free radical–mediated lipid peroxidation, which would affect iron metabolism within the hepatocyte and promote fat droplet deposition[21]. Also, an increase in the concentration of monounsaturated fatty acids has been found in the liver of chronic hepatitis C patients, but not in non-HCV-related liver disease. Thus, HCV may affect lipid metabolism and cause triglyceride accumulation resulting in hepatocyte steatosis[22]. On the other hand, leptin could induce the release of cytokines such as tumor necrosis factor-α, interferon-γ, interferon-18 and tumor growth factor-β$_1$ and these could mediate liver steatosis and fibrosis [23, 24].

In the current study, serum leptin levels were correlated with both liver biopsy inflammatory grades and fibrosis stage (tables 3&4 and figures 2a&3a respectively). Also, serum leptin levels were significantly decreased in responders to antiviral therapy than in non-responders (table 5). According to Widjaja et al, 2001; bound leptin was higher in chronic hepatitis C patients than in controls and its concentration was decreased in sustained responders to antiviral therapy compared to non-responders[25]. Hourigan et al, 1999; suggested that the increase in BMI through the presence of steatosis leads to higher staging of liver fibrosis and decrease liver function in HCV patients[26].

In the current study, serum ferritin level were found to be significantly higher in CHC patients at baseline than in the healthy control group (p = 0.003); see table 1. This could be due to infection with HCV leads to accumulation of iron in the liver and increased serum ferritin level. The mechanisms would be explained partially by the down-regulation of hepcidin which is a key regulator of iron homeostasis[27]. Another possible mechanism is the increased levels of transferrin receptor 2 in chronic HCV infection[28]. Also, the release of ferritin from hepatocytes as a result of hepatocyte necrosis leads to increased serum ferritin[29]. Beside these mechanisms serum ferritin is also elevated in any inflammatory conditions[30].

Our results were similar to the results of Bonkovsky, 2002 who found that 30 - 40 % of HCV patients show increased serum transferrin, iron saturation and serum ferritin or increased hepatic iron concentration[3]. Also, we have found positive correlations between serum ferritin and both of liver inflammatory grades and fibrosis stages P < 0.0001 for each (tables 3, 4). We found also a significant reduction in serum ferritin levels in responders to antiviral therapy than in non-responders. These findings comes in line with other researches in which elevated iron indices found to be correlated with progression of liver disease and decreased response to antiviral therapy[31,32]. The correlation between high serum ferritin and hepatic fibrosis could be explained by excess iron increase the formation of reactive oxygen species, which include hydroxyl radicals, may cause activation and proliferation of hepatic stellate cells and up regulate synthesis of smooth muscle actin and collagen, thus contributing to hepatic fibrosis[33]. Also iron deposition in hepatocytes enhances HCV replication, thus facilitating the viral infection in the liver[34].

Lange et al, 2012; found that serum ferritin was independently associated with advanced liver fibrosis and the presence of steatosis, but not with necro-inflammatory activity[2]. Serum ferritin was found to be one of the strongest predictors of treatment response, with an odd ratio for treatment failure comparable to the IL28B genotype, although ferritin level appeared to be somewhat less pronounced in HCV genotype 1 patients than in patients infected with HCV genotype 3. Serum ferritin is an independent predictor of treatment failure in patients

infected with HCV genotype 3 in whom the utility of IL28B genotyping is limited, even though a substantial number of patients cannot be cured with PEG-IFN-a and ribavirin alone[35,36]. This indicates that serum ferritin is a promising test to be included in the panel of predictors of response to IFN-a-based therapy[37,38]. Van Thiel et al, 1994 suggested that hepatic iron content predicts a response to IFN therapy. They showed that hepatic iron content of non-responders was almost twice that of responders[39].

Some researchers found that serum ALT levels significantly improved in 32 iron-depleted patients with chronic HCV infection[32], but with no significant reduction in serum HCV RNA levels[33]. Thus, the authors concluded that long-term maintenance of iron depletion is a safe and effective alternative to IFN treatment and could be particularly indicated for those patients who do not respond to antiviral therapy or cannot tolerate such drugs[40]. Also long-term phlebotomy with a low-iron diet therapy was found to reduce the risk of progression of chronic HCV infection to hepatocellular carcinoma[39].

Increased hepatic iron concentration is predictive of a poor response to IFN monotherapy[41]. Piperno and colleagues, 1996 found that iron depletion by phlebotomy did not improve the response to IFN-a in both native and non-responder patients[42]. On the other hand, Tsai and colleagues, 1997 observed that IFN retreatment preceded by iron depletion rescued 15% of previously non-responder patients[43]. Fargion and colleagues, 2002 studied 114 previously untreated patients with chronic HCV infection who received IFN alone or phlebotomy followed by IFN therapy and found that iron removal improved the rate of response to IFN[4]. Similarly, the reduction of necro-inflammatory activity and the improvement of the response to IFN were observed by Carlo et al, 2003 in the group of patients who underwent phlebotomy before antiviral therapy[44]. Also, the combination treatment was effective in previous IFN non-responders "60% of sustained virological response in the combination group compared with 13% in the IFN-alone group"[45].

Musallam observed a significant increase in both serum ferritin levels and transient elastography values as a measurement of hepatic stiffness in patients with thalassemia major as a predictive of fibrosis[46]. Furthermore; increased serum ferritin levels in patients waiting for liver transplantation increases the frequency of liver related mortality. Also, the addition of serum ferritin to MELD score increased the prediction of one year mortality by 7.5%[47].

Uric acid is recognized as a marker of oxidative stress which harms the liver, as it produce xanthine oxidase enzyme which is involved in producing radical-oxygen species. On contrary to this, uric acid also acts as an "antioxidant", a free radical scavenger and a chelator of transitional metal ions which are converted to poorly reactive forms[48].

Serum uric acid level has been studied in relation to liver disease. Petta reported that hyperuricaemia in chronic hepatitis C and in non-alcoholic fatty liver disease patients was independently associated with severity of steatosis[49,50]. Also, Mastoi et al, 2010 found that uric acid increased significantly in HCV patients. In our study, we didn't find increase in uric acid level in hepatitis C patients than the control group. We also didn't find any significant correlation between uric acid and both liver fibrosis stages and inflammation grades[51]. In this study serum uric acid level was lower in responders than in non-responders to antiviral therapy but not statistically significant. Ahmed et al, 2013 found that uric acid level in serum had no impact on the response to antiviral therapy[52]. In contrary to our results, Pellicano et al, 2008 found that serum uric acid level > or = 5.8 mg/dl is predictive of poor response to HCV treatment[53].

In current study, we found that the sensitivity and specificity of serum leptin level at a cut off value of 22.2 in prediction of response to combined antiviral treatment in HCV patients were much better than serum ferritin level at a cut off value of 303.9 (77.8, 90.9 for leptin & 72.0, 81.8 for ferritin respectively). Contrary to our results, Elshimi et al, 2013 found that at a cut off value of 4.65 for leptin and 81.5 for ferritin, the sensitivity and specificity for each were 74.4, 18.6 and 76.7, 18.6 respectively[54]. These contradictions in the results may be attributed to the low cut off value they encounter in their study.

Conclusions and Recommendations:

The results concluded that serum leptin and ferritin were found to be higher in chronic HCV patients than in control and were predictors and correlated with the progression of liver fibrosis and inflammation grades. hyperleptinemia and hyperferritinemia were found in non-responders to combined antiviral treatment, so estimation of serum leptin and/or ferritin may aid in assessing the efficacy of the antiviral treatment. Lowering of leptin and ferritin may help improving the response to antiviral therapy. Serum leptin is better than serum ferritin in prediction of the response to antiviral therapy in chronic HCV patients. Serum uric acid level has no relation with liver fibrosis and inflammation stages of chronic HCV infection or prediction of the response to antiviral treatment.

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