Correlation Between Some Biochemical Markers of Fibrosis in Nonalcoholic Fatty Liver Disease Iraqi Patients Diagnosis by Fibroscan

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Abstract

Introduction: nonalcoholic fatty liver disease starts with liver fat accumulation which is a dangerous factor for disease progression. This study aimed to determine serum of galectin 3 binding protein(G3BP), serum retinol 4 binding protein(R4BP), Haptoglobin(Hp), alpha-2-macroglobin(A2M), apolipoprotein A1(ApoA1), gamma-glutamyl transferase(GGT), hemoglobin A1c(HbA1c), Total bilirubin, Triglyceride(TG), cholesterol, low-density lipoprotein(LDL), high-density lipoprotein (HDL), very low-density lipoprotein(VLDL), Albumin, urea, and creatinine among patients with NAFLD and healthy individuals. Method: Sixty Iraqi patients with NAFLD and thirty Iraqi healthy control were attending Gastroenterology and Hepatology Teaching Hospital/Baghdad during the period from August /2019 to March /2020. Then taken an age, gender, body mass index, and abdominal ultrasound result with other medical information. All the biochemical tests are done by Autoanalyzer, while serum G3BP, R4BP, Hp, A2M, and Apo A1 are measured by the ELISA technique. Results: The present study observed a highly significant increased (P = 0.000) in the HbA1c, Total bilirubin, G3BP, and A2M. A highly significant elevated (P = 0.001) in triglyceride, LDL, and VLDL. Besides, a highly significant decrease (P = 0.000) in the HDL, Apo A1, and Haptoglobin. But, the recent study found a decrease significantly in serum albumin with the progression of fibrosis (p= 0.013). Also, retinol 4 binding protein in NAFLD patients raised significantly (P=0.027) with stages of fibrosis when compared with healthy control. In addition, the present study observed that positive correlations were observed with significance at P<0.01 between stages of fibrosis with cholesterol (mg/dl) and GGT (IU/L) (r = 0.428 with P = 0.001 and r =0.790 with P = 0.000 , respectively).While highly significant and negative correlations were found between HDL (mg/dl) (r=0.376 with P=0.003); Haptoglobin (ng/ml) (r=0.840 with P=0.000); Apo A1 (ng/ml) (r=0. 865 with P=0.000). But, the positive significant correlation between Alpha 2 macroglobin and stages of fibrosis in NAFLD patients. Finally, no significant correlation at P>0.05 notably between Triglyceride, R4BP, and G3BP with stages of fibrosis.Conclusion: We conclude that total cholesterol, A2M, and GGT were positively correlated with the fibrosis stage. Also, showed HDL, Hpt. , and Apo A1were negatively correlated with fibrosis in NAFLD patients. Finally, we observed that older age, obesity, dyslipidemia, hypertension, and type 2 diabetes are risk factors for nonalcoholic fatty liver disease.

Keywords: Galectin 3 binding protein(G3BP), Retinol 4 binding protein(R4BP), nonalcoholic fatty liver disease (NAFLD), Haptoglobin, Alpha2macroglobin, Apolipoprotein A1.

1. Introduction

"NAFLD" is one of the most community chronic hepatic diseases and its occurrence about 25 % of the world-wide (1). The term "NAFLD" includes a common liver histological change from simple steatosis to steatohepatitis (NASH) and NASH related to fibrosis or cirrhosis(2). Frequently, NAFLD patients are more associated with insulin resistance, obesity, hyperglycemia, hypertension, and dyslipidemia. So, it is believed to be a liver demonstration of metabolic syndrome(3,4). G3BP is a member of a cysteine-rich domain superfamily macrophage scavenger receptor and is involved in inflammatory distress and immune response(5,6). RBP4 is known as a unique biomarker for obesity and insulin resistance(6). Newly identified RBP4 refers to the lipocalin family and is the exact transporter protein for retinol (vitamin A) in the blood. So, it is prominently expressed on the hepatic cell and adipose tissue (7). Albumin is carriages fatty acids, hormones, and other compounds, maintains oncotic pressure, and buffers pH. Other studies found that dysregulated cholesterol metabolism in NAFLD which perhaps contributes to disease severity(8). Apolipoprotein A1 is the principal protein part of plasma HDL particles and is a protein encoded by the APOA1 gene in humans. Also, it

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plays a significant role in lipid metabolism(9). Haptoglobin is an acute plasma reactive glycol-protein that is produced in the liver (10). But, the expression is also seen in the lung, skin, spleen, kidney, and adipose tissue. Some evidence indicates that haptoglobin is one of several biomolecules that are primarily released into the bloodstream from damaged / dead cells, tissue matrix, infected immune cells and probably regenerating cells(11,12). Alpha-2-macroglobulin is a large plasma protein establish in the blood and is synthesized chiefly by the liver and locally via macrophages, fibroblasts, and adrenocortical cells(13). In patients with NAFLD, some studied display A2M engaging in hepatocyte-mediated fibrogenic response(9,10). The elevated aminotransferase levels in the liver function test are a very common cause for referral of patients to gastroenterology and/or hepatology clinics. Diagnosis of NAFLD was dependent on the abnormal concentration of AST and ALT in most of the studies(14). While there are many inflammation markers, it was difficult to discover hepatic markers that are unique to classic hepatic enzymes such as ALT(15). Unusual serum gamma glutamate transferase levels were independently correlated with severe liver fibrosis in patients with nonalcoholic fatty liver disease in a recent study (16). This study aimed to found the correlation between apolipoprotein A1, haptoglobin, galectin3 binding protein, retinol4binding protein, GGT, and Alpha 2macroglobin with some biochemical metabolic markers and stages of fibrosis in NAFLD patients.

2. Subjects, Materials and Methods

Patients and control

Sixty subjects are diagnosed with NAFLD who review to Gastroenterology and Hepatology Teaching Hospital in Baghdad are enrolled in this study during the period from August /2019 to March /2020. The age ranged from 20-65years with excluded other causes of liver disease like autoimmune, hepatocellular carcinoma, or coinfection with hepatitis B and C virus and/or HIV, malignancies, and alcoholic fatty liver disease. In addition to thirty healthy control without Hepatitis C and B virus or any other disease who were in the age range 24-67 years.

Initially, NAFLD patients are first diagnosed by using abdominal ultrasounds, biochemical tests, and clinical examination by specialists. Weight was measured with a scale. Their heights were also measured by a stadiometer. The BMI was calculated according to weight (Kg) divided by the square of height (meters). Inclusion criteria were based on a negative enzyme-linked immunosorbent assay (ELISA) test for hepatitis C and B virus, patients with metabolic disorders (DM, hypertension, dyslipidemia, and obesity). The exclusion criteria in this study include patients with no other causes of liver disease, autoimmune, hepatocellular carcinoma, or co-infection with hepatitis B and C virus and/or human immunodeficiency virus, malignancies, and alcoholic fatty live diseaee.

Biological Samples

From each individual that was included in this study, 10 ml of the blood sample was drawn by vein puncture using disposable syringes then was centrifuged at 3500xg for 10 min and serum was separated from each anticoagulant-free blood sample by centrifugation and was divided into two aliquots; one was immediately used for biochemical tests by SIEMENS Autoanalyzer/ Dimension® Xpand® Plus Integrated Chemistry System /Germany and the other was placed into Eppendorf tubes and frozen at -20 C° for three months until used for apolipoprotein A1, haptoglobin, galectin3 binding protein, retinol4binding protein, gamma-glutamyl transferase, and Alpha 2macroglobin measured by ELISA technique(all commercial kits were used supplier from My BioSource / USA).

Statistical analysis

Data were analyzed by using the statistical package of SPSS-25 (StatisticaliPackages for Social Sciences-version 25). Data were obtained in simple measurements of frequency, percentage, mean, standard deviation, and range. The significance of different means (quantitative data) was tested by using the ANOVA test for differences among more than two independent means. Also, the significance of differences of different percentages (qualitative data) was tested by using the Chi-square test (χ2-test). Statistical significance was considered whenever the P-value was equal to or less than 0.05. Finally, Pearson correlation was calculated for the correlation between variables with its t-test for testing the significance of correlation.

3. Results and Discussion

Characteristics of the NAFLD patients are shown in Table 1. This study found that the percentage of obesity (70%) and dyslipidemia (50%) are more common in NAFLD patients than other metabolic diseases such as hypertension (20%) which is diagnosed based on if patients are on antihypertensive therapy or their blood pressure is more than 140/90 mmHg and diabetes mellitus type 1 and 2 (3% and 30%) respectively. Although in NAFLD patients are usually Asymptomatic. But, the present study showed that only 20(33.3%) of NAFLD patient have no sign or symptom while other NAFLD patient has a weakness (43%), abdominal pain (40%) and extreme tiredness (36.7%).In addition to the others sign and symptom jaundice (23.3%), edema (6.7%), and loss of appetite (3.3%).

Table 1: Characteristic of NAFLD patients.	
Parameter	**NAFLD group (n= 60) n (%)**
Type of Metabolic disease Obesity Dyslipidemia	42(70%) 30(50%)
Hypertension	12(20%)
DM type 1	2(3%)
DM type 2	18(30%)
No other metabolic disease	14(23.3%)
Sign & symptom	
Extreme tiredness	22(36.7%)
jaundice	14 (23.3%)
weakness	26(43.3%)
Abdominal pain	24(40%)
Loss of appetite	2(3.3%)
Odema No sign and symptom	4(6.7%) 20(33.3%)

This finding was higher than the finding of other studies done by Younossi ZM, et al which found that 51.34% was obese and diabetes type 2 was 22.51% of NAFLD patients. But the present study observed a percentage less than the percentage of these study which the prevalence of dyslipidemia and hypertension 69.16 % and 39.34% respectively in NAFLD patients (17). Another study done by AlKhater SA. showed that clinically, only 42–59% of NAFLD patients present with abdominal pain (18) while Khoonsari M et al. found that fatigue is the common symptom and abdominal pain (37.4%) and loss of appetite (27.2%)(19). These differences in percentages were attributed to different criteria of selection of patients in various studies.

The data demonstrated by table I I and figure 1 showed highly significant differences (P=0.000) between male patients which have a high percentage (16.7%) at the age range(30-39 and 40-49 years) and female patients which have a high percentage (23.3% and 10%) at the age range (50-59 and 40-49 years) respectively in compared with other age range in both genders. Also, the male patients have more percentage (51.7%) than female patients(48.3%).

Table 2: Distribution of the NAFLD patients according to age and gender.					
			Gender		Total
			Female	Male	
Age (years)	20-29	Count	0	6	6
		% of Total	0.0%	10.0%	10.0%
	30-39	Count	4	10	14
		% of Total	6.7%	16.7%	23.3%
	40-49	Count	6	10	16
		% of Total	10.0%	16.7%	26.7%
	50-59	Count	14	0	14
		% of Total	23.3%	0.0%	23.3%
	60-69	Count	5	5	10
		% of Total	8.3%	8.3%	16.7%
Total		Count	29	31	60
		% of Total	48.3%	51.7%	100.0%
P-value			0.000*		
*Significant difference between proportions using Pearson Chi-square test at 0.05					

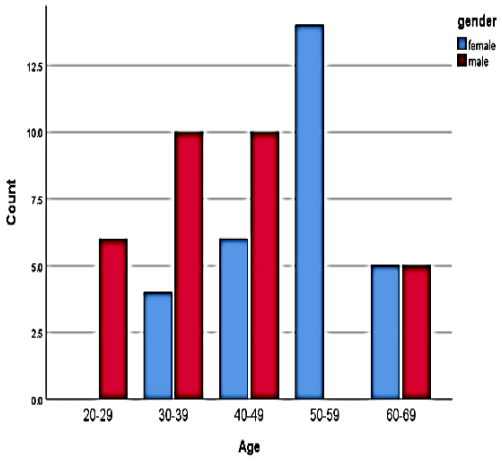

Figure 1: Distribution of the NAFLD patients according to age and gender.

The recent study has smaller than the result of other study done by Miptah, et al. (20) which found that (62.9 %)of the NAFLD patients are male while (45.8%) are female while the percentage of age at range (50-59 and 40-49 years) are (72.4% and 62.7%) respectively. While other studies found that the percentage of males (55.5%) and females (44.5 %) of the NAFLD patients(21). These differences in results due to differences in occupation, lifestyle, and regions.

In table 3 showed patients with fibrosis were diagnosed by fibroscan and classified according to METAVIR classification from F01-6, F1 6.1-7, F2 7-9, F3 9.1-10.3, and F4 ≥10.4 and BMI which appearing that the percentage of normal weight (F1 (1.7%) F3 , but the overweight (2 (3.3%) F0, 6 (10%) F1 and F2, 1(1.7%) F3 and 2(3.3%) F4), while the obese class I (10 (16.7%) F0, 11 (18.3%) F1, 6(10%) F2 ,4(6.7%) F3 vs F4 0(0%)) . Also the percentage of obese class II

(3(5%) F1 and 4(6.7%) F2). Finally the percentage of obese class III (2(3.3%)F2 and F4) with a significant difference between stage of fibrosis and BMI (P=0.004).

Table 3: A Comparison study between the body mass index and stage of fibrosis in studied groups.

Stage of fibrosis		Body mass index (kg/m2)					Total
		18.5-24.9 normal weight	25-29.9 Overweight	30-34.9 obese classI	35-39.9 Obese classs II	>40 Obese class III	
F0	Count	0	2	10	0	0	12
	% of Total	0.0%	3.3%	16.7%	0.0%	0.0%	20.0%
F1	Count	0	6	11	3	0	20
	% of Total	0.0%	10.0%	18.3%	5.0%	0.0%	33.3%
F2	Count	0	6	6	4	2	18
	% of Total	0.0%	10.0%	10.0%	6.7%	3.3%	30.0%
F3	Count	1	1	4	0	0	6
	% of Total	1.7%	1.7%	6.7%	0.0%	0.0%	10.0%
F4	Count	0	2	0	0	2	4
	% of Total	0.0%	3.3%	0.0%	0.0%	3.3%	6.7%
Total	Count	1	17	31	7	4	60
	% of Total	1.7%	28.3%	51.7%	11.7%	6.7%	100.0%
P-value		0.004*					

*Significant difference between proportions using Pearson Chi- test at 0.05

The above result lesser than the percentage of other studies done by Schmitz *et al.*, which found that the percentage of fibrosis with NAFLD in obese patients (28%) had a no fibrosis (F0), patients (43%) had a stage 1 fibrosis (F1), patients (21%) a stage 2 fibrosis (F2) and patients (8%) a stage 3 fibrosis (F3). None of our patients showed manifest cirrhosis (F4)(22). So, that differences may be related to the number of studied groups and lifestyle in our country.

Comparison between means concentration of biochemical parameters in studied groups with stages of fibrosis showed in Table IV and fig. 2 . The NAFLD patient observed highly significant elevated (P = 0.000) in the HbA1c, Total bilirubin, G3BP, and Alpha2macroglobin. Also, highly significant elevated (P = 0.001) for Triglyceride, LDL, and VLDL. While observed a highly significant decrease (P = 0.000) for HDL, Apolipoprotein A1, and Haptoglobin But, we found decreased significantly (p= 0.013) in the serum albumin Finally, R4BP raised significantly (P=0.027) with stages of fibrosis in the liver when compared with healthy control.

Table 4: Comparison between means concentration of biochemical parameters in studied groups according to the stage of fibrosis.

ANOVA test P-value	Control N=(30) mean±SD	F4 N=(4) mean±SD	F3 N=(6) mean± SD	F2 N=(18) mean±SD	F1 N=(20) mean±SD	F0 N=(12) mean±SD	Variables
.000**	4.47±0.37	8.05±0.06	6.80±2.48	6.66±1.79	6.0±.29	5.97±0.72	HbA1c (%)
.000**	21.17±5.51	58.88±15.51	40.17±19.87	43.00±14.32	34.90±7.12	28.75±3.65	AST (IU/l)
.000**	33.47±19.63	98±19.01	65±12.66	53.22±20.40	50.05±23.03	43±16.43	ALT (IU/l)
.000**	8.47±2.036	35.40±5.77	24.39±13.31	10.41±3.22	8.45±2.99	8.77±3.12	T.B. (µmole/l)
.000**	3.97±.34	2.95±.58	2.92±.19	3.01±.61	3.36±.37	3.64±.43	Alp (g/dl)
.000**	156.80±43.36	254.5±17.08	227.33±42.34	215.22±48.45	202.5±55.41	168.33±37.64	Ch. (mg/dl)
.001**	94.37±18.30	227±73.69	223.67±122.89	173.56±96.36	164.65±86.75	153±81.08	TG (mg/dl)
.000**	47.80±7.22	20.25±4.19	27.67±9.21	34.56±9.21	35.2±10.78	39.83±10.45	HDL (mg/dl)
.001**	90.35±39.99	188.85±23.06	154.96±59.69	145.96±39.20	134.37±57.08	97.9±47.2	LDL (mg/dl)
.001**	18.65±3.41	45.4±14.74	44.73±24.58	34.71±19.27	32.93±17.35	30.60±16.22	VLDL (mg/dl)
.000**	22.50±10.10	200±.00	200±.00	68.05±28.92	37.60±9.33	33.67±6.23	GGT (IU/l)
.017*	23.53±9.73	39.88±7.63	31.16±12.12	31.99±11.65	29.72±11.65	28.72±4.79	RBP4 (ng/ml)
.000**	11.30±1.69	17.31±3.68	15.63±3.63	14.75±1.69	14.67±1.99	13.29±3.52	G3BP (ng/ml)
.000**	129.3±28.16	26.25±4.27	27.83±2.14	34.56±15.53	59.1±.75	113.08±37.42	ApoA1 (ng/ml)
.000**	192.66±48.39	386.50±41.33	362.17±19.62	356.72±66.02	318.42±99.69	261±119.67	A2M (ng/ml)
.000**	125.83±36.54	25.97±2.57	30.59±2.80	34.71±2.74	51.87±23.51	100.35±44.36	Hpt(ng/ml)

**highly significant (p<0.001) ; * significant (p<0.05) ; T.B. (total bilirubin) ; ch. (cholesterol); TG(triglyceride).

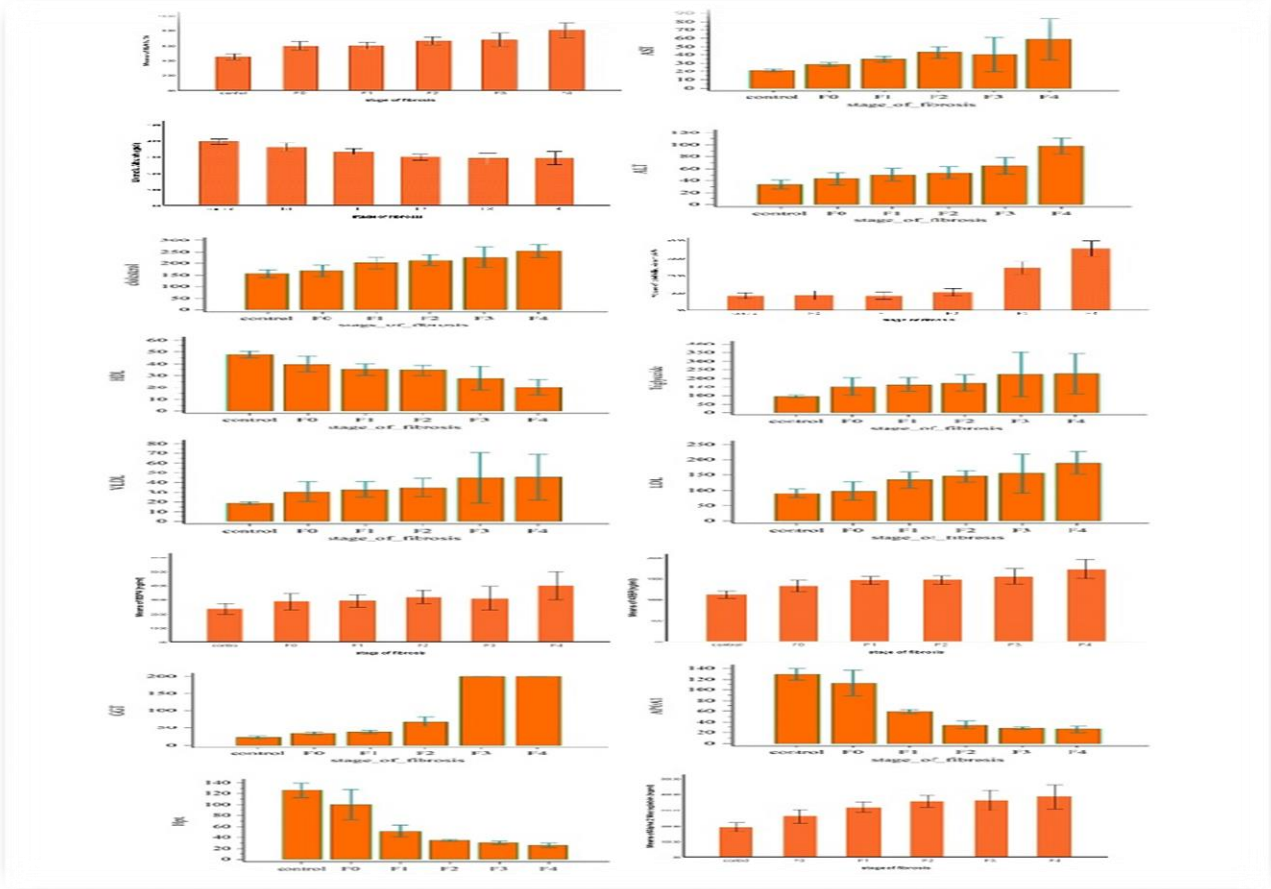

Figure 2 : Comparison between means concentration of biochemical parameters in studied groups according to the stage of fibrosis

The marked findings for the fibrosis stages progression in NAFLD patients agree with studies done by Tanaka K, et al. which observed prevalence and hepatic fibrosis of NAFLD elevated according to elevated of blood glucose, up to 8.0% HbA1c(23). Also, this study proved that a consequence of chronic hepatic injury from the buildup of lipids in the extracellular matrix leads to liver fibrosis which results architectural changes in the hepatic parenchyma then causes liver dysfunction and portal hypertension. So, indirect markers influenced by hepatic injury, portal hypertension, or alter hepatic function were all potential markers for predicting liver fibrosis stages such as AST, ALT, and GGT reflect hepatic injury while cholesterol, haptoglobin, and alpha2 macroglobulin reveal changed liver function by architectural change (24). So, the present study observed that progressive fibrosis in patients with NAFLD can be a diagnosis based on efficient noninvasive clinical laboratory data to remove the need for the invasive biopsy as shown by Hossain N, et al. which found that increase levels of ALT($P < .0001$), AST($P < .0001$), and serum triglycerides ($P = .0154$), but decrease levels of HDL ($P < .0001$) was independent analysts of moderate to severe fibrosis diagnosis in NAFLD patient(25). Recently, some studies found that circulating VLDL increased as a consequence of early changes in the hepatic synthetic function when NAFLD developed to sever fibrosis (26). Also, this study observed that elevated serum LDL with the progression of fibrosis NAFLD patients as shown in other studies done by Lucero D, et al. which observed a significant relationship between small density LDL were a distinct subclass of LDL and fibrosis in NAFLD patient (26). Bilirubin is perhaps associated with NAFLD via fatty acid metabolism. Moreover, free fatty acids perhaps interfere with bilirubin metabolism at any fibrosis stages, from blood transport to its conjugation by the hepatocyte as shown in our study(27). Although no studies explain the role of cholesterol in the development and progression of liver fibrosis. But , some studies in a mouse model show dietary cholesterol augments liver fibrosis because free fatty acid induce hepatic fibrosis gene in the liver (28). Serum albumin is one of the indicators for liver synthetic function, so the present study shows the albumin levels continued to decrease as the liver lost more function as shown in other studies (29,30). Moreover, recent data show that increased serum RBP4 levels are specifically related to obesity and type 2 diabetic-associated as shown in the present study (31).Low Apo A1 level and increased GGT level related well with advanced fibrosis in NAFLD patient as shown by other studies done by Shukla A. , etal. (16). Also.the present study agrees with a study done by Molina, et al. which found that elevated A2M during liver fibrosis development (32). In the present study, results showed that regarding to Spearman's correlation coefficients, positive correlations were accounted with significance at P<0.01 between stages of fibrosis in NAFLD).patient for cholesterol (mg/dl) and GGT

(IU/L) (r = 0.433 with P = 0.001 and r =0.841 with P = 0.000 , respectively) .While highly significant and negative correlations were accounted between HDL (mg/dl) (r=0.376with P=0.003); Haptoglobin(ng/ml) (r=0.639 with P=0.000); Apolipoprotein A1 (ng/ml) (r=0. 753 with P=0.000) with stages of fibrosis. Also, positive significant correlation(r= 0.278 with p=0.032) observed in A2M (ng/ml). But, no significant correlation at P>0.05 notably between Triglyceride , RBP4 , G3BP with stages of fibrosis in NAFLD patients as shown in (Table V).

Table 5: Pearson Correlation Coefficients among studied parameters in NAFLD patients.

parameters	Stage of fibrosis	
	r	p
Total Cholesterol(mg/dl)	.433**	.001
Triglyceride(mg/dl)	.236	.069
HDL(mg/dl)	-.429**	.001
GGT (IU/l)	.841**	.000
RBP4(ng/ml)	.015	.907
G3BP(ng/ml)	.242	.062
Haptoglobin(ng/ml)	-.639**	.000
Alpha2Maroglobin(ng/ml)	.278*	.032
ApolipoproteinA1(ng/ml)	-.753**	.000

**Correlation is significant at the 0.01.* correlation is significant at the 0.05

When comparing the performance of different serum markers for liver fibrosis yield stage of fibrosis in NAFLD was highly positively correlated with total cholesterol and GGT but not with triglycerides as shown in other studies (33,34). While the present study approves that HDL cholesterol concentration was negatively correlated with fibrosis score which constitutes one of the criteria for the diagnosis of metabolic syndrome. Also, their findings agree with the study done by Shukla A., et al. which found that negative Apo A1 correlated well with advanced fibrosis because it has been shown to have a protective effect against fatty liver disease due to its antioxidant properties, as found in a recent study on rabbits which induced by a high -fat diet (16,35). Also, other studies observed that Apo A1 is the major protein component of HDL, is synthesized in the liver. Like other liver synthesized proteins, the concentrations of Apo A1 decrease with the progression of liver fibrosis and decrease even further with the onset of liver cirrhosis (36). Furthermore, this study agrees with the study found that haptoglobin negatively correlated with fibrosis may be explained by the different roles of hepatocyte growth factor and acute phase response. As observed in experimental fibrosis, Transduction with hepatocyte growth factor gene suppresses the increase of transforming growth factor and reduces the synthesis of haptoglobin (37). Finally, A2M was a positive correlation with the stage of fibrosis agree with studies that found that a A2M positively correlated with hepatic-necro-inflammation and fibrosis(24,38).

Conclusions: We conclude that total cholesterol, A2M, and GGT were positively correlated with the fibrosis stage. Also, showed HDL, Hpt. , and Apo A1were negatively correlated with fibrosis in NAFLD patients. Finally, we observed that older age, obesity, dyslipidemia, hypertension, and type 2 diabetes are risk factors for nonalcoholic fatty liver disease.

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