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Estimation of Apolipoprotein A1, Haptoglobin and Alpha 2macroglobulin with some Biochemical Metabolic Markers in Nonalcoholic Fatty Liver Disease Iraqi Patients

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Abstract

"non-alcoholic fatty liver disease" starts with hepatic lipid accumulation and is a dangerous factor for disease development. Thus, we aimed to determine the serum levels of haptoglobin,alpha2 macroglobulin, apolipoprotein A1, gamma-glutamyl transferase, hemoglobin A1c, Total bilirubin, Triglyceride, cholesterol, low-density lipoprotein, high-density lipoprotein, very low-density lipoprotein, urea, and creatinine among patients with nonalcoholic fatty liver disease and healthy individuals. This study was carried out on 60 patients with NAFLD and 30 healthy subjects who were attending the Gastroenterology and Hepatology Teaching Hospital/Baghdad from August /2019 to March /2020. Patients data included age, sex, BMI, and abdominal ultrasound with other medical information. Serum samples were collected and then some biochemical tests were done by an Autoanalyzer, while serum apolipoprotein A1, haptoglobin, and Alpha 2maacroglobulin were measured by ELISA technique.

The study found that obesity (70%) and dyslipidemia (50%) are more common in NAFLD patients than another metabolic disease such as hypertension (20%) and diabetes mellitus (type 1 and 2) (3% and 30%) respectively. Also, the results showed a significant difference among the age group (p=0.006). NAFLD subjects had a highly significant elevation (p=0.000) in the mean ± SD of BMI, FBS, HbA1c, AST, ALP, cholesterol, triglyceride, LDL, VLDL, and Alpha 2 macroglobulin compared with the healthy control. Serum ALT and total bilirubin (mean ± SD) were significantly elevated (p=0.001) in NAFLD subjects (54.28±23.05IU/L and 14.47±9.65 µmole/l respectively) when compared with the mean± SD of healthy control. In addition, the results revealed a significant elevation (P=0.027) in the mean ± SD of serum albumin in NAFLD patients when compared with mean ± SD of healthy control and a significant elevation (P=0.002)in the mean ± SD of the LDL of the NAFLD patients as compared to the healthy control. However, the results showed a highly significant decrease (P=0.000) in the mean ± SD of serum HDL, Apolipoprotein, and haptoglobin. Furthermore, the present study observed that the optimal cut-off value was ≤67.13ng/ml for haptoglobin with a sensitivity and specificity of 80% and 93.33% respectively. In addition, the results revealed that the optimal cut-off value was >257ng/ml for Alpha 2Macroglobulin with a sensitivity and specificity of 73.33% and 86.67% respectively. The study found an optimal cut-off value of ≤86 ng/ml for Apolipoprotein A1 with a sensitivity and specificity of 85% and 90% respectively.

Keywords: Apolipoprotein A1(APO A1); Haptoglobin (Hpt.); Alpha 2macroglobin(A2M); nonalcoholic fatty liver disease (NAFLD); metabolic disease.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most public chronic hepatic diseases in the world and occurs in about 25% of individuals worldwide. The term NAFLD involves a common histological changing shift from simple steatosis to steatohepatitis (NASH) and fibrosis or cirrhosis-related NASH [1]. Patients with NAFLD are also more likely to be supplemented by insulin resistance, obesity, hyperglycemia,

hypertension, and dyslipidemia, so "non-alcoholic fatty liver disease" is thought to be a liver demonstration of the "metabolic syndrome" [2]. The prevalence of NAFLD" is rapidly rising in addition to obesity and diabetes, advancing to the largest common cause of liver disease for adults, teenagers, and infants in developing nations. So far, inadequate studies on proteins convoluted in NAFLD has been presented [4]. Despite the pathogenesis of "NAFLD" being not well understood, insulin resistance has long been reflected to play a key role in the progress of "nonalcoholic fatty liver disease" [5]. Hepatic unique proteins/molecules that can be used in commercial settings to classify liver fat are less likely to be identified. There is currently little awareness of the importance of transaminase levels in the diagnosis of liver disease [6,7]. Besides, a very common cause for the referral of patients to gastroenterology and/or hepatology clinics is the elevated aminotransferase levels in the liver function test. The diagnosis of "NAFLD" in most studies was dependent on abnormal levels of AST and ALT [5]. Although there are many markers of inflammation, hepatic markers that are special to classic hepatic enzymes such as ALT have been difficult to discover [6]. Albumin transfers fatty acids, hormones, and other chemicals, retains oncotic pressure and pH buffers, among other roles. Another research has shown that non-alcoholic fatty liver disease dysregulated cholesterol metabolism, which may lead to the seriousness of the disease [3]. In patients with NAFLD, some studies display alpha 2 macroglobulins engaging in hepatocyte-mediated fibrogenic response[9,10]. Apolipoprotein A1 is a protein encoded by the APOA1 gene in humans. It plays a significant role in lipid metabolism[6]. Haptoglobin (Hp) is an acute glycol-reactive plasma protein formed in the liver [12]. But is also expressed in the lungs, skin, spleen, kidney, and adipose tissue. Some evidence suggests that haptoglobin is one of many biomolecules released mainly from injured/dead cells, tissue matrix, infected immune cells and may help regenerating cells [13,14] into the bloodstream. Consequently, our study tried to recognize effective predictive indicators rather than specific diagnostic markers that may help reduce the occurrence of liver biopsy to estimate the development of nonalcoholic fatty liver disease

2. Materials & Methods

2.1. Patients & control

In this prospective study, a total of ninety blood samples were collected from two groups of participants: Group I included 60 cases of nonalcoholic fatty liver disease" patients in which the age ranged between (20-65) years. Group II was comprising of 30 healthy subjects aged (24-67) years who were negative for HCV and HBV by enzymes linked immunosorbent assay (ELISA). Participants were enrolled from the "Gastroenterology and Hepatology Teaching Hospital", who registered during the dates from August /2019 to March /2020. A special questionnaire form including descriptive information was designed and filled up for each patient. The questionnaire included age, sex, abdominal ultrasound result, and other medical information. The weight was determined on a scale in light clothes without shoes. Their height were also measured by a stadiometer. The Body Mass Index (BMI) was determined by weight (Kg) divided by square height (meters). Inclusion criteria were based on negative ELISA tests for HBV and HCV, patients with metabolic disorders (DM, hypertension, dyslipidemia, and obesity). An establishment of fatty liver disease was done by abdominal ultrasounds, biochemical tests, and clinical examination by the specialists. The exclusion criteria in this study include patients with no other causes of liver disease, autoimmune, HCC, or co-infection with hepatitis B virus and C and/or HIV, malignancies, and alcoholic fatty liver disease. This study was approved by the Ethics and Research Committee of the hospital under the supervision of the consultant, while approval for the sampling was obtained from patients and control.

2.2. Biological Samples

From each individual included in this study, 10 ml of blood sample was drawn by vein puncture using disposable syringes then centrifuged at 3500xg for 10 min and serum is separated from each anticoagulant-free blood sample by centrifugation and is divided into two aliquots; one is immediately used for biochemical tests by SIEMENS Autoanalyzer/ Dimension® Xpand® Plus Integrated Chemistry System /Germany and the other is placed into Eppendorf tubes and frozen at -20 C° until used for serum Apolipoprotein A1, serum Haptoglobin and serum Alpha 2 Macroglobulin measurement by ELISA technique(all commercial kits were supplied by Mybiosource / USA).

2.3. Statistical analysis

Using the available SPSS 24 statistical package (Statistical Packages for Social Sciences-Version 24), data analysis was carried out. Simple measurements of frequency, percentage, mean, standard deviation, and range were used to present the data. ANOVA was used to test the importance of the difference between various means (quantitative data). To assess the use of any parameter as a diagnostic or screening method for disease and the ability to determine the "cut-off value" that has optimal sensitivity and specificity for disease diagnosis, the Receiver Operating Characteristic "ROC" curve technique was used.

3. Results & Discussion

The characteristics of NAFLD patients are presented in Table (1). The present study examined 60 patients with NAFLD and showed that obesity (70%) and dyslipidemia (50%) is more common in NAFLD patients than other metabolic diseases such as hypertension (20%) which was diagnosed if patients are on antihypertensive therapy or their blood pressure is 140/90 mmHg and diabetes mellitus type 1 and 2 (3% and 30%) respectively. Also our study showed that only 20(33.3%) of NAFLD patient had no signs or symptom while other NAFLD patients have a weakness in all the body (43%), abdominal pain (40%) and extreme tiredness (36.7%) in addition to the other signs and symptoms, jaundice (23.3%), edema (6.7%) and loss of appetite (3.3%).

Table 1. Characteristics of NAFLD patients included in the study

Parameter	NAFLD group (n= 60) n (%)
Type of Metabolic disease	
Obesity	42(70%)
Dyslipidemia	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	4(6.7%)
No sign and symptom	20(33.3%)

This finding was higher than the prevalence of another study done by Younossi, et al which found that 51.34 % of patients were obese and diabetes types 2 was found in 22.51% of NAFLD patient, But this prevalence was less than the finding of a study in which the prevalence of dyslipidemia and hypertension were 69.16 % and 39.34% respectively in NAFLD patient[7]. The prevalence of our result varied widely due to differences in occupation, age, gender, lifestyle, and regions studied . Regarding the symptoms, although NAFLD patients are usually non-specific. AlKhater SA. found that clinically, only 42–59% of NAFLD patients present with abdominal pain [8] while Khoonsari M et al. found that fatigue is a common symptom and abdominal pain (37.4%) and loss of appetite (27.2%)[9]. These differences in percentages were attributed to different criteria of selection of patients in various studies.

Table (2) shows the baseline characteristics of the studied samples according to age and gender with the comparison of significance. This Table revealed that there were 31 male (51.6%) and 29(48.3 %) female patients with a mean age of (45.03±12.03) years in the NAFLD patients group. While there were 9 males (30%) and 21 females (70 %) as a healthy control group and the mean age was (37.40±12.45) years. There is a significant difference among age groups (p=0.006) and no significant difference among gender of these groups (P=0.051).

Table 2 . Baseline characteristics of the studied groups according to age and Gender

Variables		Patients	Healthy Control	P-value
Age (years)	Range	20-65	24-67	
	Mean ± SD	45.03±12.03	37.40±12.45	P=0.006 S (*)
Gender	Male No. (%)	31(51.7)	9(30.0)	
	Female No. (%)	29(48.3)	21(70.0)	P=0.051 NS (**)
Total No.		60	30	

*Significant difference between proportions using t-test for quality of means at 0.05
** non-Significant difference between proportions using pearson chi-square test at 0.05

This result is similar to the finding of [10]who observed no gender differences in the development of NAFLD but our study found the effect of age on the development of NAFLD which conflicts with other studies that found a greater prevalence of metabolic conditions in older individuals with a mean age of 41.2± 8.3 years [1,9]. While other studies confirmed these results joining age to an elevated risk of severe liver fibrosis, hepatocellular carcinoma, and type 2 diabetes [11].

Some demographic and biochemical parameters of NAFLD subjects and compared with healthy controls are shown in Table (3). Nonalcoholic fatty liver disease subjects had a highly significant increase (p=0.000) in the mean ± SD of BMI, FBS , HbA1c , AST,ALP, cholesterol , triglyceride , LDL, VLDL and Alpha 2 macroglobulin (31.53±4.32 kg/m^2, 115.09±25.46mg/dl ,6.48± 1.38 % , 38.23±13.49 IU/l ,111.05±31.14 IU/L, 205.43±51.19mg/dl , 136.24±52.81mg/dl, 35.01±18.37mg/dl and 315.79±104.59ng/ml , 18.65±3.41 mg.) respectively compared with the healthy control(22.89±3.05 kg/m^2 , 85.90±8.72 mg/dl , 4.48±0.36 % , 21.17±5.52 IU/l , 70.13±18.96 IU/L, 156.80±43.38mg/dl , 94.37±18.30 mg/dl, 90.35±39.99mg/dl , 18.65±3.41 mg/dl and 199.33±52.36 ng/ml) respectively . According to the World Health Organization body mass index greater than or equal to 25 is considered overweight, while obesity is defined as a body mass index greater than or equal to 30 [1]. The majority of NAFLD patients were overweight and obese (17/42) respectively. Serum ALT and total bilirubin were significantly elevated (p=0.001) in the mean ± SD of NAFLD (54.28±23.05IU/L and 14.47±9.65 µmole/l) when compared with the mean± SD of healthy control (33.47±19.05IU/L and 8.01±2.85µmole/l) respectively. Also the results revealed a significant raise (P=0.027) in the mean ± SD of the serum albumin (4.11±0.42 g/dl) in NAFLD patients when compared with mean ± SD of serum albumin in healthy control (3.89±0.45g/dl). However, the results showed a highly significant decrease (P=0.000)in the mean ± SD of the serum HDL, Apolipoprotein and haptoglobin (34.18±10.73mg/dl, 57.22±36.01ng/ml and52.56±34.74 ng/ml) of the NAFLD patients when compared with mean ± SD of the healthy

control(47.80±7.21mg/dl, 129.30±28.16 ng/ml and 125.83±36.54 ng/ml). While no significant differences was found between mean ± SD of serum urea and creatinine in NAFLD patients and healthy control (P=0.903 and P=0.110) respectively.

Table 3. Some demographic and biochemical parameters of the studied groups

Parameter	NAFLD Group(n=60) Mean ±SD	Control Group(n=30) Mean ±SD	t-test for Equality of Means (Sig. (2-tailed)
BMI(kg/m^2)	31.53±4.32	22.89±3.05	.000***
BMI :normal/overweight/ obese (N)	1/17/42	20/0/0	
F.B.S (mg/dl)	115.09±25.46	85.90±8.72	.000***
HbA1c (%)	6.48± 1.38	4.48±0.36	.000***
AST (IU/l)	38.23±13.49	21.17±5.52	.000***
ALT(IU/l)	54.28±23.05	33.47±19.05	.001***
ALP(IU/l)	111.05±31.14	70.13±18.96	.000***
Total bilirubin (µmole/l)	14.47±9.65	8.01±2.85	.001**
S. Albumin (g/dl)	4.11±0.42	3.89±0.45	.027*
S. Cholesterol (mg/dl)	205.43±51.19	156.80±43.38	.000***
Triglyceride(mg/dl)	175.05±91.87	94.37±18.30	.000***
HDL (mg/dl)	34.18±10.73	47.80±7.21	.000***
LDL(mg/dl)	136.24±52.81	90.35±39.99	.000***
VLDL(mg/dl)	35.01±18.37	18.65±3.41	.000***
B. urea (mg/dl)	27.17±6.61	26.97±8.99	.903*
S. creatinine(mg/dl)	0.84±0.18	0.78±0.14	.110*
Apolipoprotein A1(ng/ml)	57.22±36.01	129.30±28.16	.000***
Haptoglobin (ng/ml)	52.56±34.74	125.83±36.54	.000***
Alpha 2 macroglobin (ng/ml)	315.79±104.59	199.33±52.36	.000***

***highly significant (p<0.001) ** significant (p<0.05) * non significant (P>0.05)

This observation was similar to Tsuneto et al. who found that visceral fat accumulation due to obesity and increased BMI which has high metabolic activity and cause releases of free fatty acid (FFA) and adipokines such as leptin, tumor necrosis factor (TNF)-a, and adiponectin which lead to FFA released from visceral fat enter the liver across the portal vein, and increased FFA influx from the portal vein motivates triglyceride synthesis in the liver**.** Fatty liver is the state of triglyceride set down in the liver, and fatty liver is so a substitute marker of visceral fat accumulation and predicts the progress of fatty liver[15,16]. The research also shows that according to recent studies, elevated glucose and glycated hemoglobin associated with "NAFLD" show that impaired hepatic lipid and lipoprotein settle down and elevated oxidative stress in liver cells can rise the amassing of hepatic fat and trigger insulin resistance [12]. Therefore, an increase in the production of liver glucose and distribution to the peripheral circulation leads to serum glucose elevation. The HbA1c level was affected by the erythrocytes' lifespan and glucose permeability. Instead of the glucose transport rate, the trans-membrane glucose gradient is associated with HbA1c. In this study, HbA1c elevated in "NAFLD" may be identified as an increase in intracellular glucose in patients with" NAFLD" [21, 22]. Also, they showed highly significant elevation in AST and ALP in "NAFLD" patient this finding agrees with studies done by Sunitha S. et al. which found that the etiology of abnormal hepatic enzymes in type 2 diabetes mellitus(DM) may vary, but NAFLD was assumed to be the most common cause, so testing for AST, ALT, and ALP must therefore be performed to screen patients with type 2 DM for underlying "non-alcoholic fatty liver disease" [15]. Although significant elevation in ALT which was found according to a recent study the hepatic fat accumulation in NAFLD causes serum ALT to increase. Therefore increased ALT level was related to decreased insulin sensitivity, adiponectin, and glucose tolerance as well as elevated free fatty acids and triglycerides [16]. In the present study, highly significant elevations in VLDL and triglycerides were observed with significant elevations in low-density lipoprotein(LDL)and significant decreases in high-density lipoprotein (HDL) levels in patients with NAFLD due to liver overproduction of triglyceride-rich very-low-density lipoprotein (VLDL) particles in fasting conditions in patients with NAFLD or metabolic syndrome, then Insulin fails to inhibit both lipolysis and making of triglyceride-rich VLDL particles from the liver. Another consequence of hepatic insulin resistance contributes to the over-development of large triglyceride-rich VLDL particles in the liver and this is the key contributory factor underlying the elevation of serum triglycerides in "metabolic syndrome" and "NAFLD" patients. The increase in VLDL leads to a reduction in HDL cholesterol and also to the production of small dense LDL particles, which are considered to be highly atherogenic [21, 25]. Furthermore, the significant elevation in total bilirubin agree with studies which observed that fatty acid

metabolism perhaps leads to elevated total bilirubin in" NAFLD" patient through elevated de –novo- lipogenesis and peripheral fatty acids mostly derived from lipolysis of adipose tissue donate to the accumulation of hepatic fat in" NAFLD" [18] in which other studies found that several factors perhaps influence bilirubin–albumin binding affinity, including elevated FFA concentrations resulting in decreased binding affinity, thus significant elevation in serum albumin with total bilirubin was shown in our study. On the other hand, the decrease in the level of haptoglobins and apolipoprotein A1 in NAFLD may be correlated with the primary haptoglobin feature that binds free hemoglobin to the haptoglobin -Hb complex formation, as seen in other studies showing that the haptoglobin 2-2-Hb complex may also bind to Apolipoprotein A1 in HDL. The establishment of reactive oxygen species is generated to oxidize protein (i.e., glutathione-peroxidase and lecithin cholesterol acyltransferase, LCAT) and lipid components (cholesterol) of HDL-C as a result of binding Apolipoprotein A1 with the haptoglobins 2-2-Hb complex, causing HDL-C dysfunction and elevated total cholesterol in vivo [12, 27, 28]. Finally, the present study observed elevated alpha 2 macroglobulin in NAFLD due to increased free fatty acid in NAFLD patients induce a wide response on the hepatocyte production of fibrogenic cytokines such as alpha 2 macroglobulin as shown in the other studies [9, 29].

Table 4. Estimation of cutoff points, sensitivity, specificity, and AUROC of the parameters in NAFLD and Healthy control

Variable(s)	AUC	P-value	95% CI	Cut-off value	Sensitivity (%)	Specificity (%)
Haptoglobin (ng/ml)	.914	.000	.836 - .963	≤67.13	80	93.33
Alpha2Macroglobin (ng/ml)	.803	.000	.710 - .897	>257	73.33	86.67
Apolipoprotein A1(ng/ml)	.932	.000	.014-.123	≤86	85	90

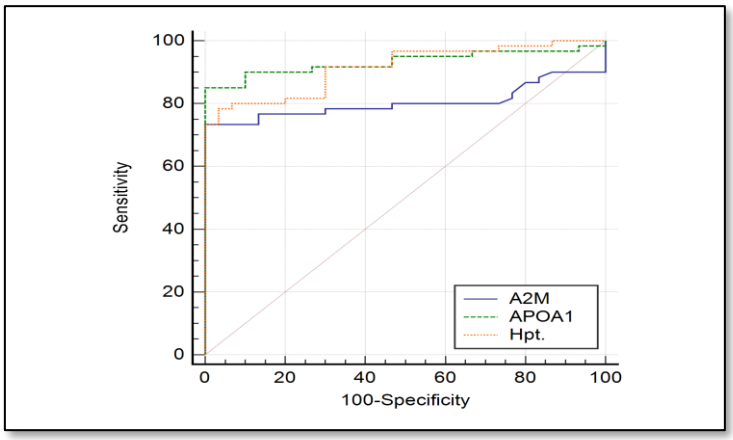

Fig. 1. ROC curves estimation for studied parameters in NAFLD and Healthy control group

Recently, ROC analysis has been recommended to estimate the power of serum assays to discover advanced liver disease, table (4) shows that AUC for haptoglobin was 0.914, and P-value equal to 0.000.The optimal cut-off value was ≤67.13ng/ml with sensitivity and specificity 80% and 93.33% respectively, 95% confidence interval equal 0.836 - 0.963. While observed AUC for Alpha 2 Macroglobulin as 0.803 and P equal to 0.000. The optimal cut-off value was >257ng/ml with sensitivity and specificity 73.33%, 86.67% respectively, 95% confidence interval equal to 0.710 - 0.897. Also, the results revealed that AUC for Apolipoprotein A1 was 0.932 and P equal to 0.000. The optimal cut-off value was ≤86 ng/ml with sensitivity and specificity of 85% and 90% respectively, 95% confidence interval equal to 0.014-0.123 as in Figure (1).
Decreased haptoglobin ≤67.13 ng/ml in NAFLD patients may be considered a sensitive test and specific test for diagnosis of NAFLD patients but with fibrosis. This finding was less than another study done by Shalably et al. which shows haptoglobin at 116 U/ ml with AUC 0.786, sensitivity 80%, specificity 65% serves as a novel diagnostic biomarker for the detection of hepatocellular carcinoma and cirrhosis [30]. Also, Apolipoprotein A1 levels ≤86 ng/ml was a good marker for the diagnosis of NAFLD but this result was less than the finding of another study done by Shukla A. et al. which found that Apolipoprotein A1 level ≤140 mg/dL correlated well with advanced fibrosis in NAFLD patient [31]. So, this result due to differences in a population study. Besides, elevated alpha 2 macroglobulins>257 ng/ml was a highly specific and sensitive marker for the diagnosis of NAFLD but this result was higher than an optimal cut of value with less sensitivity and high specificity compared with a study done by Shukla, et al which found that the optimal cut-off value was 193.5 ng/ml with sensitivity and specificity 80% and 46.7% respectively. Also, other studies observed direct involvement of both in the hepatic mechanisms of insulin resistance and fibrogenesis[31,32].

4. Conclusions:

Overweight and obesity may be associated with NAFLD especially in patients with metabolic disorders. Besides that our study found that haptoglobin and Apolipoprotein are the more sensitive and specific tests for diagnosis of NAFLD patients. While Alpha2 macroglobulin is a less sensitive and specific test for the diagnosis of NAFLD patients. In addition, older age and metabolic factors such as obesity, dyslipidemia, hypertension or type 2 diabetes are risk factors for NAFLD.

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