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Original Research Article

Noninvasive Markers for Detection of Nonalcoholic Fatty Liver Disease in Diabetic Egyptian Patients

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Nonalcoholic fatty liver disease (NAFLD), which is closely associated with obesity, has become the most common chronic liver disease in children and adolescents. The prevalence of pediatric NAFLD is 3% to 10% in normal-weight subjects and reaches a value of 80% in obese individuals. *Patients and Methods:* The present study included 200 diabetic patients developed to nonalcoholic fatty liver, recruited from Gastroenterology Outpatients Clinic in El Sahel Teaching Hospital. The study participants were divided into two groups: 100 healthy controls and 200diabetic patients with NAFLD (100 adults and 100 children). *BMI:* was calculated according to the following equation: weight (Kg) / height² (m²). Study population underwent fasting blood sampling to assess blood glucose (FBG), total cholesterol, HDL-cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin, INR, alpha-fetoprotein (AFP) and creatinine by biochemical laboratory methods. Interleukin 6(IL6), Tumor necrosis factor (TNF-α) and f Monocyte Chemoattractant Protein-1 (MCP-1) were analyzed by ELIZA. *Results:* This study showed highest sensitivity (88%) at MCP-1 level 309 ng/ml and highest specificity when compared with IL6 and TNF- α respectively (80%, 80% and 72%, 65%). The cutoff value of MCP-1equal 309 ng/ml above which the patient will develop to NALFD.

Keywords: Nonalcoholic fatty liver disease, Interleukin 6, Tumor necrosis factor, Monocyte Chemoattractant Protein-1

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), which is closely associated with obesity, has become the most common chronic liver disease in children and adolescents[(1,2)]. The prevalence of pediatric NAFLD is 3% to 10% in normal-weight subjects and reaches a value of 80% in obese individuals [(3,4)]. These data are alarming because, even if the long-term course of pediatric NAFLD is not yet known, some evidence exists of a possible course toward cirrhosis and liver failure, resulting in an increased need for liver transplantation[(5,6)].

The progression of NAFLD toward liver fibrosis and cirrhosis strongly depend on the presence of a necroinflammatory and fibrogenic milieu defined as nonalcoholic steatohepatitis (NASH)[(7,8)]. Liver biopsy has been the gold standard for describing liver histology and deciding on treatment option [(9,10,11)], this technique is a valuable method but has some major side effects, the most common are bleeding in the liver and pain around the biopsy area, others include sampling from a tiny fraction, lack of manpower to undertake several biopsies, mortality rates, sampling error, and subjective estimation of fibrosis among pathologists[(12,13,14)] even more importantly, liver biopsy is not suitable for repeated short-term assessment during follow-up[(15)]. So today, a wide variety of noninvasive methods are also available. They are safe, easy, and currently being validated for diagnosis of NAFLD. They include various imaging tests, biochemical and hematological marker. Many noninvasive markers of NAFLD have been proposed so far[(16)].

Although all available markers have suboptimal diagnostic accuracy, they may reduce the need for liver biopsy when used alone or in combination[(17)]. These findings are more relevant for the pediatric setting, in which liver biopsy is perceived as more risky than in adults. Our study will show, is interleukin 6 a predictor of the degree of fatty liver in a population with NAFLD. If our data are confirmed by subsequent studies, interleukin 6 may allow a simple and efficient screening of patient at risk of progressive liver disease. Other Studies

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shows that there's a relation between NAFLD and Tumor necrosis factor Alpha (TNF- α) which is secreted by macrophages in adipose tissue of obese models, by hepatocytes as a response to chronic inflammatory activity.

TNF is a cell signaling protein involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. It is produced chiefly by activated macrophages, although it can be produced by many other cell types. Obesity translates an expansion in adipose tissue. That expansion may occur in inert subcutaneous tissue. The excessive accumulation of fat in adipocytes promotes an increase in oxidative stress, which deregulates adipocytokines production and promotes low-grade inflammatory state in the adipose tissue, through the release of interleukin (IL)-6 and monocyte chemotactic protein (MCP)-1 among others. Subsequently, there is activation of macrophages, M1, and lymphocytes, Th1, promoting further release of proinflammatory cytokines, tumor necrosis factor (TNF-α).

Tumor necrosis factor-α (TNF-α) has been considered to be a key player in the progression from simple fatty liver to nonalcoholic steatohepatitis (NASH). Some studies indicate role of Monocyte Chemoattractant Protein-1 known as CCL2 secreted by adipocytes and consider a member of CC chemokine family and plays a pivotal role in inflammatory process. So in the current study, we will evaluate interleukin 6, TNF-Alpha and MCP-1 in diabetic patient aiming to determine the diagnostic performance of IL6, TNF-α and MCP-1in detection of NALFD as a low-cost, and easily available markers suitable for everyday clinical practice.

PATIENTS AND METHODS

The present study included 200 diabetic patients developed to nonalcoholic fatty liver, recruited from Gastroenterology Outpatients Clinic in El Sahel Teaching Hospital. The study participants were divided into two groups: 100 healthy controls and 200diabetic patients with NAFLD (100 adults and 100 children). Informed consent was obtained from all participants before enrollment in this study. The study was carried out in accordance with the principles of the Declaration of Helsinki, and its appendices, and local and national laws.

Selection Criteria

To be eligible for the study, patients had to fulfill the following criteria: no history of current or past excessive alcohol intake, negative tests for hepatitis B surface antigen and to hepatitis C virus antibody, absence of history and findings consistent with cirrhosis and other chronic liver diseases. All subjects were subjected to complete clinical examination, anthropometric measurements and body mass index was calculated for all subjects, laboratory tests and a liver US scan.

BMI: was calculated according to the following equation:
weight (Kg) / height² (m²)

Laboratory investigations

Study population underwent fasting blood sampling to assess blood glucose (FBG), total cholesterol, HDL-cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total & direct bilirubin, INR, alpha-fetoprotein (AFP) and creatinine by biochemical laboratory methods. IL6, TNF-α andMCP-1were be done by ELIZA according to manufacturer's instructions.

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 23 software (SPSS Inc, Chicago, ILL Company). Categorical data were presented as number and percentages while quantitative data were expressed as mean, standard deviation and median. Comparison of continuous data between two groups was made by using unpaired t-test for parametric data and Mann-Whitney test for non-parametric data. Comparison of continuous data between more than two groups was made by using one-way ANOVA for parametric data and Kruskal-Wallis test for nonparametric data.

Chi-square test was used for comparison between categorical data. Receiving operating characteristic (ROC) analysis curves and the corresponding area under the curve were calculated for providing the accuracy of prediction markers. ROC curve was used for estimation of sensitivity (i.e., true positive rate), specificity (i.e., true negative rate), positive predictive value (PPV), negative predictive value (NPV) and cutoff values showing the best equilibrium between sensitivity and specificity were evaluated. The accepted level of significance in this work was stated at 0.05 ($p < 0.05$ was considered significant).

RESULTS

This study was conducted on 300 participants, one hundred were healthy individuals and two hundred were diabetics. The diabetic cases were divided into 100 adults and hundred children. Table 1 showed a comparison between all studied groups regarding gender and BMI distribution. Regarding gender, male to female ratio in control and diabetic group was (1.5:1, 2.1:1) respectively. BMI of NAFLD group patients was higher than the control 32.4±11.3 and 23±8.5 respectively. Table 4 showed that there was a significant difference between diabetic patients and control regarding MCP-1 level ($P<0.001$).

The level of MCP-1 is higher in diabetic patients group than in the healthy group. Table 5 showed that there was no significant difference between diabetic patients and control regarding TNF-α ($P=0.057$). Table 6 showed that there was no significant difference between diabetic patients and control regarding IL6 ($P=0.66$). Table 7 showed highest sensitivity (88%) at MCP-1 level 309 ng/ml and highest specificity was 91% more than IL6 and TNF- α respectively (80%,80% and 72%, 65%). The cutoff value of MCP-1equal 309 ng/ml above which the patient will develop to NALFD.

DISCUSSION

Non-alcoholic fatty liver disease (NAFLD) is emerging as the most common cause of chronic liver disease worldwide [(18)], probably related to the increasing incidence of obesity and type-2 diabetes [(19)]. It has been recognized that NAFLD represents an important burden of disease for patients with type-2 diabetes mellitus. Individuals with type-2 diabetes not only have a high prevalence of NAFLD, up to 70% [(20)], but also seem to have an increased severity of disease[(21–23)]. NAFLD is strongly associated with obesity, insulin resistance/type 2 diabetes mellitus and the metabolic syndrome. Obesity, particularly central obesity, is highly predictive of hepatic steatosis and disease progression[(24,25)]. In overweight subjects, the prevalence of steatosis is at least two times more frequent than in lean subjects[(26)], being directly proportional to the increase of body mass index (BMI)[(27)].

Table 1: Demographic data of all studied groups

Variable	Healthy subjects (control) N= 100	Diabetic patients N=200
Gender Male n=146	60	137
Female n=124	40	63
Male: Female Ratio	1.5:1	2.1:1
BMI	23±8.5	±32.4±11.3

Table 2: Biochemical parameters in healthy subjects and NAFLD patients.

Variables	Healthy subjects	Children Patients	Adult Patients	P value
AFP	6.860 ± 0.45	5.40 ± 0.7	9.54 ± 0.5	0.880
Alb	3.850± 0.05	4.828 ± 0.04	3.700 ± 0.06	0.105
ALT	30.0 ± 1.34	104.78 ± 2.1	136.44 ± 1.38	0.01 S
AST	32.40 ± 2.03	80.76 ± 2.7	146.380 ± 3.2	0.001 S
BMI	28.850 ± 1.80	30.98 ± 1.3	35.420 ± 1.09	0.03
Creatinine	0.99 ± 0.04	1.134 ± 0.16	1.080 ± 0.04	0.746
D.Bil	0.150 ± 0.01	0.260 ± 0.02	0.222 ± 0.03	0.293
Glucose	101.85 **±** 3.1	199.24 ± 12.4	204.40 ± 13.09	0.192
Cholesterol	165.3± 19.08	201 ± 18.09	223.4± 22.7	0.001
Triglyceride	108.7± 17.11	177± 29.33	197± 30,35	0.01
Hb	11.80 ± 0.39	11.586 ± 0.4	10.294 ± 0.18	0.009
IL-6	109.65 ± 4.60	129.88 ± 1.4	134.96 ± 3.2	0.09
TNF-α	102.3± 0.19	119.5± 1.8	130.4**±** 7.1	0.045
MCP-1	209± 21.4	321± 31.**2**	385**±** 47.3	0.001 S
T. bilirubin	0.745 ± 0.045	1.078 ± 0.06	0.974 ± 0.08	0.276

Data were expressed as mean ± SEM, Significance level at $p < 0.05$

Table 3: Comparison between obese and non-obese diabetic patients

Variables	Obese Patients	Non-obese Patients	P value
AFP	10.9 ± 0.21	8.48 ± 0.56	0.76
Alb	3.7± 0.34	4.9 ± 0.11	0.51
ALT	85.0 ± 7.11	37.09 ± 6.1	0.001S
AST	76.40 ± 17.22	40.76 ± 8.3	0.001 S
BMI	35.59 ± 3.60	29.3 ± 4.1	0.02
Creatinine	1.2 ± .09	1.04 ± 0.03	0.68
D.Bil	0.350 ± 0.04	0.110 ± 0.09	0.43
Glucose	187 **±** 12.8	145.22 ± 19.1	0.20
cholesterol	174.2± 14.06	199.4 ±22.2	0.001S
Triglycerid	165.3± 11.3	189.3±17.8	0.03
Hb	11.20 ± 0.39	11.6 ± 0.4	0.09
IL-6	110.22 ± 9.80	122.41± 9.9	0.08
TNF-α	107.2± 9.9	120.3± 11.3	0.056
MCP-1	211± 23.5	334±6 ؛30	0.001 S
T. bilirubin	0.65 ± 0.09	0.97 ± 0.05	0.211

Data were expressed as mean ± SEM, Significance level at $p < 0.05$

Table 4: Mean ±SD of MCP-1 (pg/ml)

	Healthy subjects(Control)	Diabetic Patients
N	100	200
Mean ± SD	305.9±57.1	407.3±72.7
P value	<0.001	

Table 5: Mean ± SD of TNF-α

	Healthy subjects(Control)	Diabetic Patients
N	100	200
Mean ± SD	116.3±44.9	199.28±33.9
P value	0.057	

Table 6: Mean ± SD of IL6

	Healthy subjects (Control)	Diabetic Patients
N	100	200
Mean ± SD	143.5±18.4	176±22.8
P value	0.66	

Table 7: Sensitivity and specificity of diagnostic values of MCP-1, IL6, and TNF-α for detection of NAFLD in diabetic cases.

Test	Cut-off value	AUC	sensitivity	Specificity
MCP-1	> 309 ng/ml	0.87	88%	91%
IL6	> 127 ng/ml	0.76	80%	80%
TNF-α	> 118 ng/ml	0.67	72%	65%

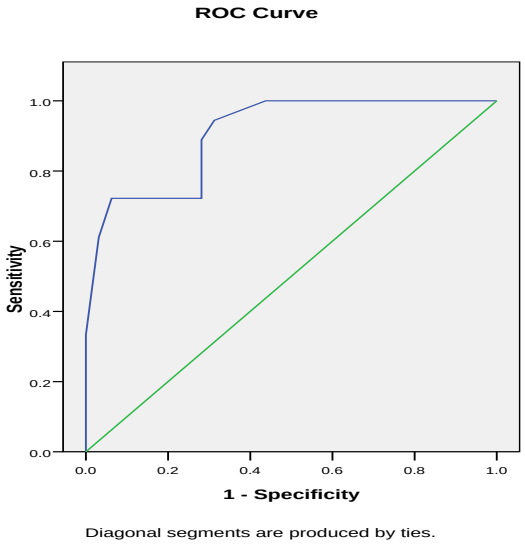

Fig 1: Receiving operating characteristic curve of MCP-1

This comes in agreement with our study, as BMI was significantly higher in diabetic patients with NAFLD in comparison with non-diabetics without NAFLD (control group) also in agreement with other studies who found Patients with NAFLD had larger waist circumferences, higher BMI and were more frequently obese than those without steatosis [28]. Again, the following studies report predictors of NAFLD using routine clinical parameters in cohorts of obese children. Sartorio *et al* [29] reported a multivariate analysis of 267 obese children and found that BMI *Z*-score, ALT, uric acid, glucose and insulin were useful predictors of NAFLD. Neither of these studies used a histological diagnosis of NAFLD.

In our study, ALT, AST were significantly higher in diabetic patients with NAFLD in comparison with non-diabetics without NAFLD and in obese patients compared to non-obese patients. Also, in agreement with Sartorio *et al*[29]) regarding ALT, and with Leite et al [28] who found that patients with NAFLD had higher ALT levels than those without NAFLD, although when dichotomized at the upper limit of normal, no difference was observed between subjects with and without liver steatosis. Otherwise, when ALT was dichotomized at 39 U/L, a significantly higher prevalence of high normal range ALT was observed in patients with NAFLD. Nevertheless, even at this cut-off value, ALT did not show a good predictive performance for being proposed as a screening test for ultrasonographic

NAFLD detection. Furthermore, in our study**,** the other liver function tests did not show any differences between diabetic patients with NAFLD and non-diabetics without NAFLD (control) as well as in the study of Leite et al [(28)], as they found no differences in the other liver function tests between patients with and without liver steatosis.

The development of non-invasive biomarkers of disease has become a major focus of interest in nonalcoholic fatty liver disease (NAFLD). The large prevalence of the disease and the invasive nature of the investigation means that screening with liver biopsy is impractical. In addition to screening, the differentiation of those with simple steatosis vs steatohepatitis and fibrosis is clinically important as the prognosis of each differs. Serum biomarkers may be a combination of simple markers derived from large data sets or direct markers of disease activity. Serum markers of inflammation, apoptosis and oxidative stress in addition to fibrosis have been extensively studied in patients with NAFLD [(30)]. Biomarkers of NAS and fibrosis have also been reported by a few pediatric studies as Sartorio *et al*. These studies are relatively limited by the size of the cohorts involved and are mostly validation of adult biomarkers. Neither of these studies used a histological diagnosis of NAFLD[(29)].

Little is known about the evolvement of NAFLD, a relative benign condition to NASH, an irreversible inflamed stage of liver disease. Inflammatory cytokines have been proposed to play a central role in the progression of NAFLD[(31)]. Cytokines are central mediators of hepatic inflammation, cells apoptosis and cells regeneration. TNF-α and IL-6 have been found to be the initial cytokines chemokines that being produced after hepatic insults [(31,32)]. TNF-α has been known to interact between fat accumulation and hepatic inflammation[(32)].

Coulon *et al* [(32)] suggested that TNF-α was significantly higher in NASH patients compared to simple steatosis patients. This highlighted the potential role of TNF-α in NASH. Furthermore, TNF-α has also been previously known to be an important cytokine that regulates insulin resistance by affecting insulin receptor substrate-1(IRS-1) and insulin receptor kinase (IRK) in the insulin signal transduction pathway [(33)]. Nevertheless, the involvement of TNF-α in insulin resistance is debatable. However, a recent study by Aparicio-Vergara *et al* [(34)] demonstrated a disassociation of TNF-α induced hepatic inflammation and insulin resistance.

The results of circulating levels of adipokines as (TNF-α)or IL-6 as predictor of disease are inconsistent however and may not be sensitive or specific enough to act as robust biomarkers in isolation[(30)]. This somehow agreed with our results since although TNF -α was higher in diabetic patients with NAFLD than in healthy subjects (control group), yet this difference was insignificant. TNF -α gave only 72% sensitivity and 65% specificity for detection of NAFLD in diabetic patients.

IL-6 is a pro-inflammatory cytokine that has been studied extensively for its wide range of biological function. IL-6 has been proposed to have direct and indirect deleterious role such as induction of inflammation, hepatoprotector, regulators of acute phase response and insulin signaling[(35)]. Wieckowska *et al*[(36)] demonstrated a markedly increased hepatic IL-6 expression assayed with immunohistochemistry in patients with NASH as compared to simple steatosis or normal liver.

The hepatic IL-6 expression in their cohort study has showed to be correlated with the severity of inflammation and fibrosis[(36)]. At the same time, a positive correlation was observed between the plasma IL-6 levels measured with ELISA and hepatic IL-6 expression[(36)]. However, discrepant results have been reported regarding IL-6 in NAFLD studies. Haukeland *et al*[(37)] determined IL-6 in 47 biopsy-proven NAFLD and 30 controls with enzyme immunoassays and revealed no significant changes in NASH group compared to simple steatosis. Again, in our study, although IL-6 was higher in diabetic patients with NAFLD than in healthy subjects (control group), yet this difference was insignificant. So we agreed with Fitzpatrick E et al[(30)] as previously mentioned and with Giannitrapani L,[(38)] who declared that, insulin resistance and augmentation of hepatic inflammation could be modulated by other factors than TNF-α and IL-6.

MCP-1 is a potent chemoattractant for monocytes**.** In our study, we found a significant increase in MCP -1 in diabetic patients with NAFLD in comparison with healthy subjects (control group). MCP -1 showed a sensitivity of 88% and a specificity of 91% for detection of NAFLD in diabetic patients. This is in consistence with other studies, as interestingly, Haukeland *et al*[(37)] further evaluated serum level of CC-chemokine ligand 2 /monocyte chemoattractant protein-1 (CCL2/MCP1) and demonstrated increasing levels from healthy controls to simple steatosis and reached the highest levels in NASH.

It has been proposed that an increased circulating CCL2/MCP-1 levels may be related to the development of NASH [(35,37)]. Furthermore, in another study conducted by Huda Jaber Waheed et al[(39)] showed that; there is a significant increment in MCP-1 in diabetic subjects when compared to a healthy group. These findings are corresponded to the well-known metabolic changes that occur in normal and diabetic subjects [(39)].

CONCLUSION

In view of the high prevalence of NAFLD in the population, in both adults and children, and the fact that up to a one-third will develop end-stage liver disease and/or hepatocellular carcinoma, it is important that we develop noninvasive methods (noninvasive biomarkers either in blood or imaging techniques) other than liver biopsy (though the disease is still defined histologically), to diagnose and monitor this liver condition. A differentiation needs to be made between those with advanced disease/or are at risk of developing advanced disease from those who have simple steatosis and are unlikely to progress.

Advances in proteomic technologies have contributed to the discovery of clinically important protein biomarkers, which are the key molecules that reflect biological reactions. From our study, insulin resistance and augmentation of hepatic inflammation could be modulated by other factors than TNF-α and IL- 6. The results of TNF-α and IL-6 as predictors of disease may not be sensitive or specific enough to act as serum protein markers in isolation that could differentiate NAFLD and NASH. On the other hand, MCP-1 show promising results. Further validations of these proteins markers in larger cohorts are required to reflect the degree of hepatic fibrosis. In general, further breakthrough and investigation of changes in protein expression levels are still warranted to understand the pathophysiology of NAFLD.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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