Cytokeratin- 18 as a Marker of Non-alcoholic Fatty Liver Disease in Obese Children and Adolescents

Amel El-Faramawy[1], Rasha T. Hamza[1], Nermine H. Mahmoud[2] and Rania H. Elkabarity[2]

Departments of Pediatrics[1] and Clinical Pathology, [2]Faculty of Medicine - Ain Shams University Cairo Egypt
nermine_helmy70@hotmail.com

Abstract: Objective: To assess the clinical utility of serum cytokeratin 18 (CK-18) fragment levels in diagnosis of non-alcoholic steatohepatitis (NASH) and its severity in obese children with suspected non-alcoholic fatty liver disease (NAFLD). **Subjects and Methods:** Fifty obese children and adolescents were compared to 25 non-obese, age-, sex- and pubertal stage- matched healthy controls. Among the obese children, forty had NAFLD (80 %), of which seven (17.5%) had NASH. Following the anthropometric measurements, the ultrasonography and the routine laboratory tests of liver functions, fasting blood glucose, fasting insulin, Homeostasis model assessment (HOMA) index and fasting glucose/insulin ratio (G/I), CK-18 was assessed by an enzyme linked immunosorbent technique. **Results:** CK-18 levels were significantly higher in obese children than controls; and in obese children with fatty liver than those without fatty liver; and in obese children with fatty liver and elevated liver enzymes. Moreover, CK-18 correlated positively with liver enzymes, total cholesterol, triglycerides and low density lipoproteins; and negatively with high density lipoproteins. **Conclusion:** NAFLD is a common complication of childhood obesity. Noninvasive monitoring of CK-18 fragment levels in sera of obese patients may be used as a reliable tool to identify those with NAFLD and to differentiate NASH from simple liver steatosis.

[Amel El-Faramawy, Rasha T. Hamza, Nermine H. Mahmoud and Rania H. Elkabarity. **Cytokeratin- 18 as a Marker of Non-alcoholic Fatty Liver Disease in Obese Children and Adolescents.** *Life Sci J* 2013;10(4): 3262-3272]. (ISSN:1097-8135). http://www.lifesciencesite.com. 434

Keywords: Cytokeratin-18, NAFLD, NASH.

1. Introduction:

Non-alcoholic fatty liver disease (NAFLD) is a common clinicopathological condition characterized by significant lipid deposition in the hepatocytes of the liver parenchyma, its pathological picture bears a striking resemblance to that of alcohol-induced liver injury, but it occurs in individuals who deny a significant history of alcohol ingestion. NAFLD comprises a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. Steatosis represents fat accumulation in liver tissue without inflammation and is the earliest recognizable stage[(1)]. Nonalcoholic steatohepatitis (NASH) is characterized by balloon degeneration of hepatocytes, inflammation and fibrosis. It is considered to be a form of chronic hepatitis with the potential for progressing to cirrhosis and end-stage liver disease. Therefore, it's important to determine among the spectrum of the patients with NAFLD those with simple fatty liver and those with NASH[(2)].

NAFLD is identified as an important liver disease in children, occurring even in the very young ages. The incidence in the general population is 2.6% but increases to 53% in obese children, thus NAFLD is expected to become the most common cause of pediatric chronic liver disease in the near future[(3)]. NAFLD may also be a result of secondary causes such as medications as corticosteroids, methotrexate, amiodarone and tamoxifen. Nutritional causes such as rapid weight loss or total parenteral nutrition or metabolic diseases such as lipodystrophy[(1)]. In some cases, altered growth hormone secretion resulting from cranial irradiation may play an etiologic role. Children who become hyperphagic and experience rapid and extensive weight gain resulting from hypothalamic or pituitary dysfunction, such as that occurring after resection of a cranio-pharyngioma, are prone to developing NAFLD and seem to be at risk for rapid development of cirrhosis[(4)]. Patients with resistance to insulin and obesity are at a higher risk of developing the NASH subtype[(5)].

Liver biopsy remains the only reliable way of diagnosing NASH, however, it's an invasive procedure, therefore, there's an urgent need to develop and validate a simple, non invasive test that distinguish NASH from NAFLD and determines the stage and grade of the disease[(6)].

Cytokeratin 18 (CK-18) is the major intermediate filament protein in the liver and one of the most prominent substrates of caspases during hepatocyte apoptosis. Apoptotic cell death of hepatocytes is associated with release of caspase-cleaved CK-18 fragments into the blood stream[(7)]. The increased apoptotic rate as a consequence of the hepatic inflammatory response is reflected by elevation of serum CK-18 fragments that may therefore distinguish NASH from simple steatosis[(8)].

Thus, our study aimed at assessing the clinical utility of serum CK-18 fragment levels in diagnosis of

NASH and assessment of its severity in obese children with suspected non-alcoholic fatty liver disease.

2.Subjects and Methods

Study population:

This cross sectional case-control study was conducted on 50 obese children and adolescents. Obesity [BMI SDS (standard deviation score) > +2] was defined according to the reference ranges of **(Cole, 2002)**[9]. Patients were randomly collected from the Pediatrics Obesity Clinic, Children's Hospital, Ain Shams University, Cairo, Egypt during the period from June 2012 to January 2013. They were 26 males (52%) and 24 females (48%) whose ages ranged between 1.8 and 16 years with mean age of 8.9±3.5years.

Obese patients were compared to 25 healthy age-sex-and pubertal stage- matched non obese children and adolescents [15 males (60%) and 10 females (40%)] whose ages ranged between 2 to15 years with mean age of 8±3.8 years. They were recruited from the Pediatrics Outpatient Clinic of the same hospital.

Obese patients with genetic syndromes, endocrine diseases, psychiatric disorders or cardiac problems were excluded from the study. Children with history of steatohepatitis or any of its alternative causes as Wilson`s disease, alpha-one-antitrypsin deficiency, and iron deficiency anemia or those with infectious causes of hepatitis as hepatitis B and C.; as well as children who received medications known to precipitate steatohepatitis as valproate, amiodarone or prednisone were also excluded from the study.

An informed written consent was signed by the parents or legal guardians of the studied subjects. This study was approved by the Ethics Committee, Faculty of Medicine, Ain Shams University, Cairo, Egypt which complies with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals.

Methods:

All studied children were subjected to:

- Medical history including therapeutic history and symptoms related to liver disease e.g. right hypochondrial pain or swelling, jaundice, abdominal distension, general fatigue, intestinal bleeding or itching.
- Clinical assessment including systemic examination and dysmorphic features e.g. almond eyes. Blood pressure (BP) was measured together with comparison of its values to normal reference percentiles for age / height according to National High Blood Pressure Education Program**(10).** Normal BP was defined when systolic blood pressure (SBP) and diastolic blood pressure (DBP) were<90th percentile. Pre-hypertension was defined when SBP and/or DBP were 90th to95th percentile; and hypertension was defined when SBP and / or DBP were ≥ 95th percentile**(10).**
- Anthropometric measurements:

- Height was measured to the nearest 1.0 mm with a Harpenden wall mounted stadiometer and weight to the nearest 0.1 kg on electronic scales together with calculation of height for age SDS**(11).** BMI was calculated using the formula weight (in kg)/height2 (in meters) together with calculation of BMISDS from the age- and sex-specific reference values[9].
- Waist circumference (WC): was measured midway between the lowest rib and the top of the iliac crest according to**(Eisenmann,)(12).** Waist circumference SDS was calculated and compared to normal references for age and sex according to **Cole and Green, (13).** Central obesity was defined if WC SDS was > +2[14].
- Hip circumference (HC): was measured in a horizontal plane at the maximum extension of the buttocks according to National Health and Nutrition Examination Survey[15].
- The waist / hip ratio (W/H): was calculated and compared to normal age- and sex- reference ranges according to **Troiano, (16).**
- Abdominal fat thickness: was measured by taking the skin fold thickness according to International Society for the Advancement of Kinanthropometry[17].

- Tanner pubertal staging: for assessment of pubertal status according to the standards of **Tanner and Whitehouse (18)**.
- Laboratory assays:

- Liver function tests (ALT, AST, bilirubin) (assayed on the Synchron CX7 Beckman Coulter Incorporation (Brea, CA, USA).
- Fasting lipid profile: including total cholesterol (TC), triglycerides (TG), high density lipoproteins (HDL) assayed on the Synchron CX7 Beckman Coulter Incorporation (Brea, CA, USA). Low density lipoproteins (LDL) concentration was calculated using the Friedwald-Fredrickson equation[19]. Dyslipidemia was defined if one or more of these lipids or lipoprotein levels were abnormal. Values were compared with the reference ranges of the National Cholesterol Education Program, 1992, which set values for acceptable, borderline, and high and low lipid concentrations in children and adolescents[20].
- Fasting blood glucose (FBG):was measured by glucose oxidase method on the Synchron CX7Beckman Coulter Incorporation. A value of >100 mg/dl was the cutoff used to define impaired fasting blood glucose and a value of > 126 mg/dl defined frank diabetes mellitus[21].

- Fasting serum insulin (FI): was measured by the Immulite 2000 Analyzer by chemiluminescent immunometric assay using insulin (INS) chemiluminescence immunoassay test kit (Tokyo, Japan). A cutoff value <10µIU/ml was considered normal[22].
- Fasting glucose/insulin (G/I) ratio: was calculated**.** Normally, fasting G/I ratio should exceed 4.5, and insulin resistance is diagnosed when the G/I ratio is <4.5[23].
- Homeostasis model assessment (HOMA) index was calculated by the equation: fasting insulin concentration (µU/ml) × fasting glucose concentration (mg/dl) /405. A value of >2.5 was the cutoff used as an index of insulin resistance[24].
- Fragmented Cytokeratin 18 level: was assayed using commercially available Tissue Polypeptide Specific antigen (TPS®) ELISA kit using one step enzyme linked sandwich immunoassay*(IDL BiotecAB, Karlsbodavagen 39 P.O.Box 11151,SE -161 11 Bromma, Sweden.)*. TPS(R) ELISA is a one step enzyme liked sandwich immunoassay. Standards, controls and samples react during incubation simultaneously with a solid phase monoclonal catcher antibody and the HRP-conjugated detector antibody (M3). After washing, the TMB substrate is added and after an incubation time the reaction is stopped and the absorbance at 450nm is measured. The developed color is directly proportional to the concentration of the analyte.

- Abdominal ultrasonography: Multiple transverse and longitudinal gray scale images of the abdomen were acquired by ultrasonographers using commercially available equipment (Acuson, Sequoia, Mountain View, CA, USA) with a 4-MHz vector transducer. Patients were scanned in the supine and left lateral decubitus position, utilizing subcostal and intercostals approaches. Sonograms were performed under fasting conditions. The time-gain compensation was set to adjust the tissue echogenicity as constant as possible regardless of depth and the following data were collected: liver size (in centimeters) and echogenecity, echo penetration and visibility of diaphragm, clarity of liver blood vessel structure, echogenicity of both kidneys, fibrosis which can be recognized in the presence of steatosis by coarse echoes (‘‘pin-head echoes’’) within the fine-echo pattern of steatosis. Based on previous data, the grade of fatty liver was categorized as follows: Mild steatosis: increased echogenicity of liver compared with renal cortex or spleen, moderate steatosis: obscured hepatic and portal vein walls and severe steatosis: impaired visibility of the diaphragm[25].

Statistical Analysis

The results were analyzed using the Statistical Package for the Social Science (SPSS) version number 10, Echosoftcorp; USA, 2005. Description of quantitative variables was in the form of mean ± standard deviation and qualitative variables as frequencies and percentages. Student’s t-test of 2 independent samples was used to compare 2 quantitative parametric variables while Chi-square test was used to compare two qualitative groups of data., p<0.05 was considered significant and p <0.01 was considered highly significant and p >0.05 was considered non-significant. Pearson`s correlation coefficient test (r-test) was used to rank different parametric variables against each other either directly or indirectly. Furthermore, the diagnostic performance of the studied paramaters was evaluated using receiver operating characteristic curve analysis, in which sensitivity % was plotted on the Y axis and 100-specificity on the x-axis. The best cutoff value (the point nearest to the left upper corner of the curve) was determined

3.Results

Descriptive Clinical and Laboratory Data of Studied Subjects:

Of the 50 studied obese subjects, 28(56%) had breathing problems, 27(54%) had sleep disorders, 12(24%) had right hypochondrial pain, 21(42%) had abdominal distension and 14(28%) had generalized fatigue; none of the patients had intestinal bleeding or itching. In addition, positive family history of obesity [34(68%) vs 12(48%) respectively] and type 2 diabetes mellitus [28(56%) vs 6(24%) respectively] was higher in obese cases than controls, while the frequency of fatty liver did not differ between both groups[6(12%) vs 3(12%) respectively].

On comparing obese subjects to non obese ones, all clinical and anthropometric parameters were significantly higher in obese patients (Table 1).In addition, 15 obese children (30%) were found to be hypertensive when compared to age- matched blood pressure percentiles and started antihypertensive therapy.

Regarding laboratory parameters, liver function tests and fasting lipid profile were significantly higher in obese children vs non-obese ones, except HDL which was lower. On comparing glucose homeostasis parameters between obese children and controls, FBG, fasting insulin and HOMA index were significantly higher while G/I ratio was significantly lower in obese cases than controls (Table 2). Of the 50 obese cases, 10(20%) had a FBG >126 mg/dl and 15(30%) had a FI > 10 µIU/ml; and started oral hypoglycemic therapy.

Table 1. Clinical and anthropometric data among cases and controls.

	Cases (n=50)	Controls (n=25)	t	*p*
Age (years)	8.9±3.5 (1.8-16)	8.0±3.8 (2.0-15)	1.10	0.32
Height SDS	+1.10±0.1 (-0.66-2.56)	-0.67±1.12 (-0.86-1.64)	-2.97	0.035*
BMI (kg/m2)	28.55±4.90 (26.49-39.71)	18.3±1.8 (16.02-21.9)	9.79	<0.01**
BMI SDS	+3.17±0.73 (2.62-4.71)	0.09±+0.88 (0.99-1.21)	13.38	<0.001***
Liver span (cm)	12.23±1.88 (8.0-16.80)	7.97±1.51 (4.0-9.21)	9.88	<0.001***
SBP Percentile	66.02±34.21 (54.12-99.55)	17.12±9.1 (15.88-62.11)	12.06	<0.001***
DBP Percentile	78.48±18.98 (56.12-98.80)	43.16±16.51 (35.70-59.99)	10.55	0.002**
WC (cm)	100.6±16.8 (52-136)	61.4±8.9 (47.2-76.2)	8.80	<0.001***
WC SDS	+3.28±1.21 (2.88-4.56)	+0.89±0.55 (0.69-1.30)	10.87	<0.001***
HC (cm)	72.7±6.9 (55.70-85.56)	53.4±10.8 (45.60-72.04)	16.61	<0.001***
W/H ratio	1.20±0.1 (0.81-1.97)	0.6±0.15 (0.37-0.91)	11.40	0.002**
AFT (mm)	32.7±6.9 (18-50)	18.3±4.3 (12-29)	12.24	<0.01**

Results are expressed as mean±SD and range, *p<0.05, ** p <0.01, *** p <0.001, SDS: standard deviation Score, BMI: body mass index, SBP: systoloic blood pressure, DBP: diastolic blood pressure, WC: waist circumference, HC: hip circumference, W/H ratio: waist hip ratio, AFT: abdominal fat thickness.

Table 2. Laboratory data among cases and controls.

	Cases (n=50)	Controls (n=25)	t	*p*
ALT (U/L)	34.7±19.9 (10-100)	18±5.9 (10-25)	7.55	0.035*
AST (U/L)	25.9±20 (6-89)	14.4±5.5 (6-25)	6.98	0.028*
Serum Bilirubin (mg/dl)	0.6±0.3 (0.1-1.2)	0.6±0.2 (0.2-1.1)	0.1	0.88
TC (mg/dl)	222.9±35.8 (160-325)	152.8±10.9 (127-170)	11.30	0.002**
TG (mg/dl)	120.9±26.8 (75-200)	102.8±11.8 (85-118)	6.56	<0.01**
HDL (mg/dl)	40.9±8.6 (29-65)	55.3±5.7 (48-69)	5.89	0.030*
LDL (mg/dl)	155.7±22.1 (118-206)	98.9±14.6 (75-105)	13.20	<0.001***
FBG (mg/dl)	109.2±18 (75-152)	82.4±7.2 (77-96)	11.56	0.01*
FI (µIU/ml)	10.8±4.8 (5-25)	6.1±1.3 (5-10)	6.40	0.03*
G/I ratio	7.8±5.8 (1.2-30)	13.18±5.3 (8.5-31.3)	0.51	0.04*
HOMA Index	2.88±1.5 (1-8.7)	1.8±0.5 (0.6-3)	6.10	0.022*
CK18(U/l)	710±288.02 (200-1200)	364±134.2 (100-600)	14.33	<0.001***

Results are expressed as mean±SD and range, ***p <0.05, **p <0.01, ***p <0.001, ALT: alanine aminotransferase, AST: aspartate aminotransferase, TC: total cholesterol, TG: triglycerides, HDL: high density liopoproteins, LDL: low density lipoproteins, FBG: fasting blood glucose, FI: fasting insulin, G/I ratio: glucose/ insulin ratio, HOMA: homeostasis model assessment, CK18: cytokeratin 18.

Cytokeratin- 18 in Studied Subjects:

ROC curve was done to set a cutoff point for serum CK-18 to discriminate cases from controls. The area under the ROC curve (AUROC) for CK-18 in predicting NAFLD was 0.82. Accordingly, the cutoff point for CK-18 between cases and controls was 575 U/L with a sensitivity 71%, specificity 77% (Fig. 1).

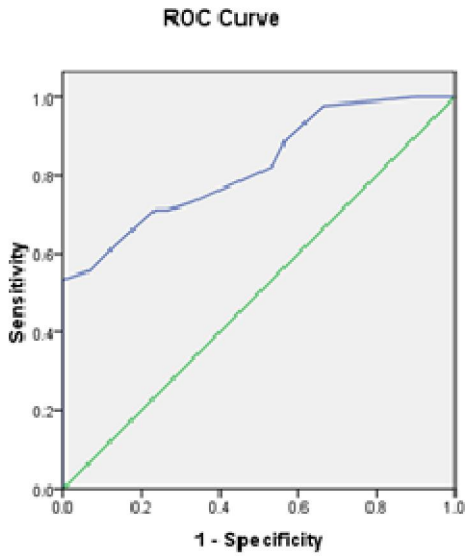

Figure (1): ROC Curve analysis showing the diagnostic performance of CK-18 in discriminating obese children from healthy controls.

Ultrasonographic Findings of Studied Subjects:

The frequency of fatty liver was significantly higher in obese cases than controls. Moreover, all controls having fatty liver were of the mild degree. In addition, the frequency of kidneys echogenicity indicating excess visceral fat and also the presence of liver fibrosis were significantly higher in obese cases than in controls (Table 3). Also, there was a progressive increase in serum CK-18 levels as severity of fatty liver proven by ultrasound increased [mild: 480±201.5 (U/L), moderate: 837.5±143.2 (U/L), severe: 1083.33±79.05 (U/L), (P<0.001 when comparing each group to the other)].

Correlations between Cytokeratin- 18 and; Clinical, Laboratory and Ultrasonographic Findings in Obese Cases:

On correlating CK-18 to clinical parameters, CK-18 correlated positively with BMISDS (r=0.88, P <0.001), SBP percentile (r=0.69, P < 0.05), DBP percentile (r=0.64, P < 0.05), liver span (r=0.65, P < 0.05), WC (r=0.69, P < 0.05) and its SDS (r=0.77, P < 0.05), HC (r=0.71, P < 0.05), W/H ratio (0.66, P < 0.05), AFT (r=0.80, P < 0.05) while height SDS was not significantly correlated with CK-18.

On correlating CK-18 to laboratory parameters, CK-18 correlated positively with ALT (r=0.59, P < 0.05) and AST (r=0.55, P < 0.05) but did not correlate with serum bilirubin (r=0.07, P > 0.05). Regarding fasting lipid profile, CK-18 correlated positively with TC (r=0.80, P <0.001), TG (r=0.70, P < 0.05), LDL (r=0.69, P < 0.05) and negatively with HDL (-0.81, P <0.01) but did not significantly correlate with glucose homeostasis parameters (P >0.05). In addition, CK-18 correlated positively with liver size measured by ultrasound (r=0.73, P <0.01).

Obese Cases with and without Fatty Liver:

Of the 50 obese cases, 40(80%) were found to have fatty liver proven by ultrasound. All clinical and laboratory parameters were significantly higher, while HDL was significantly lower in obese cases with fatty liver compared to those without fatty liver. On the other hand, height SDS, serum bilirubin and all glucose homeostasis parameters did not differ between obese cases with and without fatty liver (Tables 4 and 5).

When a ROC curve was done to compare CK-18 levels in cases with (n=40) and cases without (n=10) fatty liver as proved by ultrasound, the AUROC was 0.79, with a cut off value of 625 U/L giving a sensitivity of 70% and a specificity of 60% (Fig. 2).

Obese Cases with Fatty Liver and Elevated Liver Enzymes:

Of the 40 obese cases with fatty liver, 7(17.5%) had elevated liver enzymes(mean ALT: 74.5±20 IU/L and AST:64.8±22.9 IU/L) after exclusion of other causes. The seven cases showed hepatomegaly by ultrasound, one of them (14.3%) had mild degree, 2(28.5%) had moderate degree and 4(57.2%) had severe degree of fatty liver. Liver fibrosis detected by ultrasound was more common in cases with elevated liver enzymes [4/7 (57.2%)] than those with normal liver enzymes [8/33(24.2%), p=0.02]. Moreover, CK-18 was significantly higher in cases with elevated liver enzymes (1042.8±83.8 U/L) than those with normal liver enzymes (709+271.9 U/L, p <0.0001). Other clinical and laboratory parameters did not differ between cases with and without elevated liver enzymes (p >0.05).

When a ROC curve was done to compare CK-18 levels in cases of fatty liver with (n=7) and cases of fatty liver without (n=33) elevated liver enzymes, the AUROC was 0.87, with a cut off value of 925 U/L as a possible cut off value to differentiate fatty liver from NASH giving a sensitivity of 100% and a specificity of 73% (Fig. 3).

Table 3. Ultrasonographic findings in cases and controls.

	Cases (n=50)	Controls (n=25)	*X2*	*p*
No FL	10(20)	20(80)	28.1	<0.001***
FL	40(80)	5(20)		
Grades of FL Mild Moderate Severe	 15(37.5) 16(40) 9(22.5)	 5(100) 0(0) 0(0)	16.55	<0.001***
Kidneys echogenicity Normal Increased	 40(80) 10(20)	 25(100) 0(0)	9.54	0.01*
Liver fibrosis No Yes	 37(74) 13(26)	 25(100) 0(0)	7.98	0.01*

Results are expressed as frequency and percentage, * p <0.05, ** p <0.01, *** p <0.001, FL: fatty liver.

Table 4. Clinical data among cases with and without fatty liver.

	Fatty liver(n=40)	No fatty liver(n=10)	t	*p*
Height SDS	+1.1±0.2 (-0.9 - 2.56)	+0.9±0.11 (-0.6 – 1.78)	1.5	0.12
BMI (kg/m2)	33±3.5 (28.50 - 39.71)	27±2.7 (26.49-33.11)	8.17	0.01*
BMI SDS	+3.52±0.55 (2.91-4.71)	+2.89±0.67 (2.62-4.10)	6.55	0.021*
Liver span (cm)	14.01±1.52 (9.5-16.80)	9.21±0.21 (8.0-10.30)	7.11	0.01*
SBP Percentile	89±10 (60.5 - 99.55)	73±20 (54.12 - 95.5)	8.18	<0.01**
DBP Percentile	82±15 (60.5 - 98.80)	63±20 (56.12 - 97.55)	6.98	0.020*
WC (cm)	90.2±13.9 (64 - 136)	75.8±12.7 (52 - 125)	9.11	<0.001***
WC SDS	+3.95±0.1 (2.90 - 4.56)	+2.43±0.2 (2.88 – 3.81)	6.99	0.01*
HC	75±5.3 (56.70-85.56)	60±5 (55.70-83.56)	9.99	<0.001***
W/H ratio	1.1±0.06 (0.81-1.97)	0.86±0.08 (0.83-1.17)	5.88	0.03*
AFT (mm)	42±5.4 (20-50)	20±8.7 (18-45)	13.22	<0.001***

Results are expressed as mean±SD and range, * p <0.05, ** p <0.01, *** p <0.001, SDS: standard deviation Score, BMI: body mass index, SBP: systoloic blood pressure, DBP: diastolic blood pressure, WC: waist circumference, HC: hip circumference, W/H ratio: waist hip ratio, AFT: abdominal fat thickness.

Table 5. Laboratory data among cases with and without fatty liver.

	Fatty liver (n=40)	No fatty liver (n=10)	t	*p*
ALT (U/L)	37.06±21.2 (15-100)	25.2±8.8 (10-45)	6.7	0.01*
AST (U/L)	28.2±21.4 (11-89)	16.6±8.9 (6-39)	7.66	0.01*
Serum Bilirubin (mg/dl)	0.57±0.28 (0.4-1.0)	0.63±0.19 (0.1-1.2)	0.7	0.53
TC (mg/dl)	246.8±36.9 (174-325)	207.8±28.02 (160-260)	8.55	<0.001***
TG (mg/dl)	142.4±28.9 (75-200)	115.2±15.35 (96-142)	7.89	<0.001***
HDL (mg/dl)	34.3±5.8 (29-56)	42.5±5.4 (35-65)	5.88	0.031*
LDL (mg/dl)	166.4±22.5 (120-206)	142.8±21.06 (118-179)	6.04	0.01*
FBG (mg/dl)	112.4±18.5 (96-152)	108.6±16.5 (75-150)	0.77	0.51
FI (µIU/ml)	11.6±4.2 (7-25)	10.8±6.63 (5-22)	0.81	0.46
G/I ratio	11.6±5.5 (1.2-26)	12.5±7.6 (5.2-30)	0.44	0.65
HOMA Index	2.7±1.4 (1-8.7)	3.2±2 (1.4-8)	0.83	0.40
CK18(U/l)	767±279.8 (300-1200)	480±197.4 (200-700)	19.33	<0.001***

Results are expressed as mean±SD and range, * p <0.05, ** p <0.01, *** p <0.001, ALT: alanine aminotransferase, AST: aspartate aminotransferase, TC: total cholesterol, TG: triglycerides, HDL: high density liopoproteins, LDL: low density lipoproteins, FBG: fasting blood glucose, FI: fasting insulin, G/I ratio: glucose/ insulin ratio, HOMA: homeostasis model assessment, CK18: cytokeratin 18.

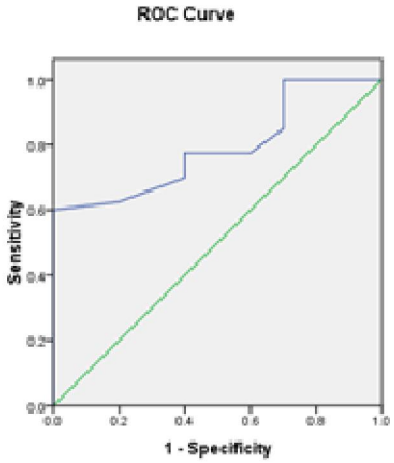

Figure (2): ROC Curve analysis showing the diagnostic performance of CK-18 in discriminating obese children with fatty liver from obese children without fatty liver.

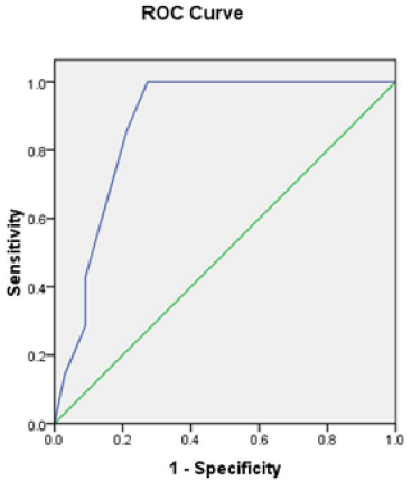

Figure (3): The ROC curve showing the diagnostic performance of CK-18 in discriminating cases with fatty liver proved by U/S with elevated liver enzymes and those without elevated liver enzymes

4. Discussion

Liver biopsy remains the gold standard for obtaining an accurate diagnosis of NASH, as well as for differentiating this condition from simple steatosis. Unfortunately, biopsy is a costly and invasive diagnostic procedure, and possibly subject to inter- and intra-observer variability[26].

Therefore, there is an urgent need to develop and validate a simple, reproducible, non-invasive test that accurately distinguishes NASH from simple steatosis and determines the stage of the disease. Such a test would not only aid clinicians in the identification of patients with NASH, but also allow for non-invasive frequent monitoring of disease status, response to therapy, and prediction of disease progression risk[27].

In our study, the mean FBG and FI levels of cases were significantly higher than controls. Similar results were reported by ***Manco et al.,*** [28] who conducted a study on 80 obese children and adolescents (5-17 years) and found that the mean FBG and FI in obese children were higher than in normal weight controls. This confirms the relationship between fasting hyperinsulinemia, insulin resistance, impaired glucose tolerance and occurrence of NAFLD in children. ***Manco et al., 2009***[28] reported that studies in adults with NAFLD have shown that components of metabolic syndrome may contribute to severe liver steatosis, NASH activity and fibrosis.

Our study revealed that 66% of the obese children had increased FI levels, which was higher than that found by ***Kawasaki et al.,*** [29] who conducted a study on 228 obese Japanese children suspected to have NAFLD, the study was undertaken in an attempt to clarify the relationship between fatty liver and hyperinsulinemia in obese children and they found that it was present in 32% of patients. This confirms that hyperinsulinemia appears to be one of the most important contributing factors to the development of fatty liver even in childhood. Finally, they concluded that; among variables such as anthropometric data and the levels of serum lipids and blood glucose, pediatricians should pay more attention to hyperinsulinemia in addition to the degree of obesity when checking and treating obese children.

In the present work, the HOMA-IR was significantly higher while G/I ratio was lower in cases than in controls. This is similar to the study by ***Keskin et al., 2005***[30] who conducted a study on 57 obese children who were categorized into 2 separate groups according to presence or absence of insulin resistance. There was a significant difference in the mean HOMA and G/I ratio between the two groups. In their study, they compared the diagnostic performance of both tests and they found that HOMA had high sensitivity and specificity for measuring insulin resistance. Insulin resistance promotes disturbances in lipid metabolism, which increases delivery of free fatty acids (FFAs) to the liver. Impaired mitochondrial β-oxidation of FFAs, 'de novo' lipogenesis and decreased β export from the liver, all of which result in fatty liver development[31]

In our work, fatty liver was diagnosed by ultrasound in 40 cases (80%). Most children were affected by mild to moderate fatty infiltration of the liver; it was mild in 15 (37.5%), moderate in 16 (40%), and severe in 9 (22.5%). This is in concordance to ***Chan et al.,*** [32] who conducted a study on 84 obese Chinese children; according to the ultrasonographic findings they found that fatty liver was present in 65 (77%), mild in 32 (38%), moderate in 20 (24%), and severe in 13 (16%). In their study, the final scoring used for the detection and grading of fatty infiltration of the liver was similar to that described by ***Tominaga et al.,***[33] on the basis of liver echotexture, liver-diaphragm differentiation in echo amplitude, and clarity of hepatic blood vessels, which was also similar to our work scoring that was described by ***Xiaozhou et al.,*** [25]

In the current study, the mean ALT and AST levels in obese children with sonographic evidence of fatty liver was significantly higher than in cases without fatty liver, and the difference was significant, however both were within normal for age. This is in concordance to the study by ***Brunt et al.*** [34]. ***Jou et al.*** [35] reported a series of 14 children with idiopathic hepatic steatosis, identified by retrospective review of results of all liver biopsies performed in a tertiary care pediatric hospital, all of them were obese and had elevated AST and ALT. In the study by ***Rafeey et al.,*** [36] they found that a strong positive correlation was demonstrated between ultrasonic severity of fatty liver and elevation of ALT and AST.

There was a high prevalence of fatty liver in our obese pediatric population (80%). Seven patients (17.5%) had a combination of fatty liver by U/S and elevated liver enzymes (presumptive NASH). This is similar to the study by ***Sagi et al.,***[37] who conducted a study on 58 obese children aged 8-18 years, ALT and AST were elevated in 9 of the 35 patients (25.7%) who were diagnosed with fatty liver by U/S. Also in an Italian screening study by ***Bergomi et al.,*** [38] which was conducted on 195 obese children, they found fatty liver changes by ultrasound in 55%, elevated AST and ALT in 20% and both features in 15%.

Franzese et al., [39] demonstrated a lack of agreement between ultrasonography and serum aminotransferase levels in cases of fatty liver. Of the 53% of their obese children with fatty liver identified by U/S, only 32% had abnormalities in serum

aminotransferases. They found that in subjects with more severe steatosis, a higher proportion of abnormalities in aminotransferase levels existed. These findings suggest that heavy infiltration is required for abnormalities in ALT and AST levels to occur.

Our study revealed that the mean CK18 levels were significantly higher in obese children with fatty liver than in obese children without. We also proved that CK-18 levels were significantly higher in obese children with fatty liver and elevated liver enzymes than in those with fatty liver without elevated liver enzymes. This was confirmed by ***Fitzpatrick et al.,*** [40] who carried out a study on 45 children with biopsy- proven NAFLD and 13 age-matched controls. Children with NAFLD showed considerably elevated levels of CK-18 fragments as compared with those of healthy controls. In addition, those with established NASH showed significantly higher levels versus those with hepatic steatosis. The cut off value for NASH prediction was 207 U/L giving a sensitivity of 84% and a specificity of 88%. In addition, ***Vos et al.,*** [41] assessed the usefulness of plasma caspase-generated Cytokeratin 18 (CK 18) fragment as a novel biochemical marker for NAFLD in a pediatric cohort. Results showed that CK 18 levels were significantly higher in children with suspected NAFLD detected by ultrasound compared with those of obese children without suspected NAFLD and healthy lean children. The authors concluded that their results supported the potential utility of this biochemical test for the diagnosis of NAFLD in a pediatric setting. This is also in concordance to the study of ***Feldstein et al.,*** [42] in which they measured the level of CK 18 fragments in serum samples obtained from 21 children (aged 4.5-16.8 years) with biopsy proven NAFLD and 13 age matched normal control children using the M30-Apoptosense enzyme-linked immunosorbent assay (ELISA) kit. They found that mean serum level of CK-18 fragments was significantly higher in children with NAFLD compared to controls.

In our study**,** CK-18 levels were lower in cases with fatty liver than controls. This is in concordance to the study of ***Vos et al.,*** [41] who conducted a study on 62 children who were categorized into; group (1): 28 normal-weight subjects, group (2): 14 obese children with no fatty liver, and group (3): 20 obese children with fatty liver, they found that serum CK18 values were significantly higher in group (3) than in group (1) and group (2).

Our study also revealed that the CK18 fragment level of cases of fatty liver with elevated liver enzymes was significantly higher than cases without elevated liver enzymes. Supporting our findings, a report by ***Wieckowska et al., 2006***[7] revealed that measurment of serum CK18 fragment levels may allow discrimination of definitive NASH patients from simple fatty liver with high sensitivity and specificity.

Our study revealed that there was a significant positive correlation between CK18 and components of the lipid profile, FG and FI in obese children. This is in concordance to the study by ***Tsutsui et al.,*** [43] in which serum levels of fragmented CK-18 were measured in 118 NAFLD patients at time of liver biopsy. They found that CK-18 significantly associated with fasting cholesterol, triglycerides and LDL as well as fasting blood glucose and insulin.

In our study, the mean CK-18 fragment level was significantly higher in cases with severe degree of hepatic steatosis than cases with mild degree and moderate degree. This was documented by ***Tsutsui et al.,*** [43] who found that serum levels of CK 18 was positively correlated with the degree of hepatic steatosis.

In the present study the incidence of fibrosis in cases of fatty liver with elevated liver enzymes (57.2%) was higher than in cases of fatty liver with normal liver enzymes (24.2%). Our results showed that there was significant positive correlation between CK-18 and fibrosis. Similar results were reported by ***Feldstein et al.,*** [44] who found that CK-18 fragment levels were significantly higher in patients with fibrosis as compared to those without fibrosis. ***Tarantino et al.,*** [45] demonstrated that levels of CK-18 increase in a stepwise fashion according to fibrosis stages in a cohort of patients with NASH. This was confirmed by ***Diab et al.,*** [46] who found that CK-18 fragment levels are increased with the severity of fibrosis in liver biopsy.

Using the area under the receiver operating characteristic (AUROC) curve approach, we next calculated potential cut off values to detect patients with NASH from those with simple steatosis. In the present study the AUROC for predicting NAFLD was 0.79. A cut off value of 625 U/L gave a sensitivity and specificity of 70% and 60%, respectively. While the AUROC for CK-18 to discriminate NASH from simple steatosis was 0.87, a cut off value of 925U/L giving a sensitivity of 100% and a specificity of 73%. ***Wieckowska et al.,*** [7] reported an AUROC of 0.93 to distinguish NASH from simple fatty liver. We found a lower AUROC curve for diagnosis, however, still with acceptable sensitivity and specificity to distinguish simple steatosis from NASH. ***Fitzpatrick et al.,*** [40] conducted a study on 45 children with biopsy-proven NAFLD. They found that the AUROC for CK18 as a predictor of NASH was 0.85, with a cut off value of 207 U/L giving a sensitivity of 84% and a specificity of 88%. The difference in the diagnostic levels in different studies could be attributed to the difference

in the used kits and the difference in the studied population. **Conclusion:** NAFLD is a common complication of childhood obesity. Noninvasive monitoring of CK-18 fragment levels in sera of obese patients may be used as a reliable tool to identify those with NAFLD and to differentiate NASH from simple liver steatosis.

References

1. *Neuschwander-Tetri BA and Caldwell SH:* Nonalcoholic steatohepatitis: Summary of an AASLD single topic conference. Hepatology, 2003; 37: 1202-1219.
2. *Saadeh S, Younossi Z and Remer E:* The utility of radiological imaging in nonalcoholic fatty liver disease. Gastroenterology, 2002; 123: 745-750.
3. *Patton HM, Sirlin CB, Schwimmer JB and Lavine JE:* Pediatric nonalcoholic fatty liver disease. J Pediatr Gastroenterol Nutr, 2006; 43: 413-427.
4. *Molleston JP, White F and Teckman J:* Obese children with steatohepatitis can develop cirrhosis in childhood. Am J Gastroenterol, 2002; 97(9): 2460-2462.
5. *Chitturi S and Farrel GC:* Etiopathogenesis of nonalcoholic steatohepatitis. Semin liver Disease, 2001; 21:27-41.
6. *Campos GM, Bambha K, Vittinghoff E, Rabl C, Posselt AM, Ciovica R, et al.:* A clinical scoring system for predicting nonalcoholic steatohepatitis in morbidly obese patients, Hepatology 2008; 47:1916-1923.
7. *Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ and Feldstein AE: In vivo* assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology, 2006; 44: 27-33.
8. *Yilmaz Y, Dolar E, Ulukaya E, Akgoz S, Keskin M, Kiyici M, Aker S, et al.:* Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steato-hepatitis. World J Gasteroenterol, 2007; 13: 837-844.
9. *Cole TJ:* A chart to link child centiles of body mass index, weight and height. Eur J Clin Nutr 2002, 56:1194–1199.
10. *NHBPEP:* Working Group on High BP in Children and Adolescents. The 4th report on the diagnosis, evaluation, and treatment of high blood pressure in children and adolescents. Pediatrics 2004; 114:555-576.
11. *Tanner JM, Whitehouse RH, Takaishi M:* Standards from birth to maturity for height, weight, height velocity, and weight velocity: British children, 1965. Arch Dis Child 1966, 41:454–613.
12. *Eisenmann JC:* Waist circumference percentiles for 7 to 15 years old Australian children. Acta Paediatr, 2005; 94:1182–1185.
13. *Cole TJ and Green PJ:* Smoothing reference centile curves: the LMS method and penalized likelihood. Stat Med, 1992; 11:1305–1319.
14. *Kissebah AH, Vydelingum N, Murray R, Evans DJ, Hartz AJ, Kalkhoff RK:* Relation of body fat distribution to metabolic complications of obesity. J Clin Endocrinol Metab, 1982;54:254–260.
15. *National Center for Health Statistics*. Plan and operation of the third National Health and Nutrition Examination Survey, 1988-94.Vital Health Stat 1(32). 1994.
16. *Troiano RP, Flegai KM, Kuczmarski RJ, Campbell SM, Johnson CL:* Overweight prevalence and trends for children and adolescents. The National Health and Nutrition Examination Surveys, 1963 to 1991. Arch Pediatr Adolesc Med, 1995; 149: 1085 – 1091.
17. *International Society for the Advancement of Kinanthropometry (ISAK):* International Standards for Anthropometric Assessment, Underdale SA, Australia, 2001.
18. *Tanner JM, Whitehouse RH:* Clinical longitudinal standards for height, weight, height velocity, weight velocity and stages of puberty. Arch Dis Child 1976, 51:170–179.
19. *Friedewald WT, Levy RI, Fredrickson DS:* Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18:499-502.
20. *National cholesterol education program expert panel on blood cholesterol levels in children and adolescents:* Highlights of the report of the expert panel on blood cholesterol levels in children and adolescents. Am Fam Physician 1992; 45: 2127-2136.
21. *Young-Hyman D, Schlundt D, Herman L, De Luca F, Counts D:* Evaluation of the insulin resistance syndrome in 5 to 10 year old overweight/OB African American children. Diabetes Care, 2001;24:1359-2364.
22. *Sacks DB:* Carbohydrates. In: Burtis CA, Ashwood ER, editors: Tietz textbook of Clinical Chemistry, 2nd edition. Philadelphia: Saunders, 1994.
23. *Legro RS, Finegood D, Dunaif A:* A fasting glucose to insulin ratio is a useful measure of insulin sensitivity in women with polycystic ovary syndrome. J Clin Endocrinol Metab 1998; 83:2964-2968.
24. *Madeira TR, Carvalho CN, Gazolla FM, De Mallos HJ, Borges MA and Bordallo MA:* Cutoff point for homeostatic model assessment for insulin resistance (HOMA-IR) index established from receiver operating characteristic (ROC)curve in the detection of metabolic syndrome in overweight pre-pubertal children. Arq Bras Endocrinol Metabol, 2008;52(9): 1466-1473.
25. *Xiaozhou M, Holalkere NS, Kambadakone R, Kenudson, MM and Sahani DV:* Imaging-based

Quantification of Hepatic Fat: Methods and Clinical Applications. Radio Graphics, RSNA,2009; 29:1253–1280.
26. *Wieckowska A and Feldstein AE:* Diagnosis of nonalcoholic fatty liver disease: invasive versus noninvasive. Semin Liver Dis, 2008; 28(4): 386-395.
27. *Feldstein A E and Gores GJ:* Apoptosis in alcoholic and nonalcoholic steatohepatitis. Front Biosci, 2005; 10: 3093-3099.
28. *Manco M., Marcellini M.; Nobili V.; Devito R.. and Sartorelli M..:* Metabolic syndrome and liver histology in pediatric non alcoholic steatohepatitis.Int. J. Obes. 2008, 32: 381- 387.
29. *Kawasaki T.; Hashimoto H.; Kikuchi T.; Takahahi H. and Uchiyema M.:* The relationship between fatty liver and hyperinsulinemia in obese Japanese children. Nephrol Dial Transplant 1997, 11 (8): 24 - 31.
30. *Keskin M.; Kurtoglu M.; Kendirci M.; Atabck M. E. and Yazici C.:* Homeostasis model assessment is more reliable than the fasting glucose/insulin ratio and quantitative insulin sensitivity check index for assessing insulin resistance among obese children and adolescents. Pediatrics 2005, 115: 500- 503.
31. *Edmison J and McCullough AJ:* Pathogenesis of nonalcoholic steatohepatitis. Clin liver Dis, 2007; 11: 85-93.
32. *Chan D. F.; Li A. M.; and Chin W. C.:* Hepatic steatosis in obese chinese children. Int.J.Obes.Relat.Metab.Disord.2004, 28: 1257 - 1263.
33. *Tominaga K.; Kunata J. H.; Chen Y. K.; Fujimoto E. and Miyajawa S.:* Prevalence of fatty liver in Japanese children and relationship to obesity – an epidemiologic ultrasonographic survey.Dig Dis Sci.1995: 40:2002 – 2009.
34. *Brunt EM, Kleiner DE, Wilson LA, Unalp A, Behling CE, Lavine JE and Neuschwander-Tetri BA:* Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): a histologic marker of advanced NAFLD Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. Hepatology, 2009; 49:809–820.
35. *Jou J, Choi SS and Diehl AM:* Mechanisms of disease progression in nonalcoholic fatty liver disease. Semin Liver Dis, 2008; 28: 370-379.
36. *Rafeey M.; Mortazavi F.; Mogaddasi N. and Robobeh G.:* Fatty liver in children. Therapeutics and Clinical Risk Management. 2009, 5: 371-374.
37. *Sagi R.; Reif S. and Neuman C.:* Non-alcoholic fatty liver disease in overweight children and adolescents. Acta. Paediatr. 2007, 96:1209 – 1213.
38. *Bergomi A.; Lughetti L. and Corcuilo N.:* Italian multi center study on liver damage in pediatric obesity. Obes Relat Metab Disord 1998, 22-S22.
39. *Franzese A.; Argenziano A.,Puzzeillo A,Iannucci M.P..and Savianco M.C:* Liver involvement in obese children. Ultrasonography and liver enzymes levels at diagnosis and during follow up of an Italian population. Dig. Dis. Sci.1997,41;1428 – 1432..
40. *Fitzpatrick E.; Mitry R. R.; Quoglia A.; Hussain M. J. and Dhawan A:* Serum levels of CK-18 M30 and leptin are useful predictors of steatohepatitis and fibrosis in pediatric NAFLD J. Pediatr Gasteroentrol Nutr 2010, 51: 500-506.
41. *Vos MB, Barve S, Joshi-Barve S, Carew JD, Whitington PF and McClain CJ:* Cytokeratin 18, a marker of cell death, is increased in children with suspected nonalcoholic fatty liver disease. J Pediatr Gastroenterol Nutr, 2008; 47: 481-485.
42. *Feldstein A. B.; Alkhoui N.; De Vito R.; Alisi A.; Lopez R. and Nobili V.:* Serum cytokeratin - 18 fragment levels are useful biomarkers for non alcoholic steatohepatitis in children. Am J. Gasteroentrol 2013, 108 (9), 1526-1531.
43. *Tsutsui, M.; Tanaka, N.; Kawakubo, M.; Sheema, Y.; Horiuhi, A. and Sanok.:* Serum Fragmented cytokeratin 18 levels reflect the histologic activity score of non-alcoholic fatty liver disease more accurately than serum alanine aminotransferase levels. (2010), Journal of Clinical Gastroenterology 44(6): 440-447
44. *Feldstein A E, Wieckowska A.,Lopez AR and Zein NN:* Cytokeratin 18 fragment level as a noninvasive biomarker for nonalcoholic steatohepatitis. A multicenter validation study. Hepatology 2009, 50 ; 1072 – 1078.
45. *Tarantino G, Conca P, Pasanisi F, Ariello M and Mastrolia M:* Could inflammatory markers help diagnose nonalcoholic steatohepatitis? Eur J Gastroenterol Hepatol, 2009; 21: 504-511.
46. *Diab DL, Yerian L, Schauer P, Kashyap SR, Lopez R, et al:* Cytokeratin 18 fragment level as a noninvasive biomarker for nonalcoholic steatohepatitis in bariatric surgery patients. Clin Gastroenterol Hepatol, 2008; 6(11): 1249-1254.

12/5/2013