

## Osteocalcin: A New Biomarker for Non Alcoholic Fatty Liver Disease (NAFLD) in Children and Adolescents

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### Abstract

**Background:** Nonalcoholic fatty liver disease (NAFLD) is a common liver disease in adults but uncommon in Pediatrics. Patients with chronic liver disease are at increasing risk of developing metabolic bone disease which may be attributed to decreased Osteocalcin which is involved in bone extracellular matrix mineralization.

**Aim of the work:** was to measure serum osteocalcin levels in children and adolescents with NAFLD and to investigate the relationship with variables degrees of NAFLD.

**Materials and methods:** This study was carried out upon 60 children with NAFL detected by abdominal ultrasonography. Forty apparently healthy children, matched for age, sex and body mass index (BMI) were chosen as controls. They were subjected to history taking, clinical examination, investigations included liver function tests, fasting serum glucose and insulin levels, Homeostasis Model Assessment method of Insulin Resistance (HOMA-IR), serum osteocalcin level and abdominal Ultrasonography to assess semiquantitatively the degree of steatosis.

**Results:** Mean serum osteocalcin level was significantly lower in patients than in controls. Mean serum level of ALT, total cholesterol and triglycerides, fasting insulin and HOMA-IR were markedly increases as the hepatic steatosis advancing in severity. There was a parallel decrease of mean serum osteocalcin level with advancing the degree of hepatic steatosis by ultrasound. Serum osteocalcin levels were inversely correlated with the values of W/H ratio, fasting insulin and HOMA-IR.

**Conclusions:** NAFLD had lower serum levels of osteocalcin compared to controls. It were inversely associated with the degrees of steatosis so can considered as a biomarker for severity NAFLD in pediatric age.

**Keywords:** Osteocalcin; Non-alcoholic fatty liver disease; Children

### Background

Nonalcoholic Fatty Liver Disease (NAFLD) is a common liver disease in adults but uncommon in Pediatrics. Obesity is increasing worldwide at a dramatic speed among children and adolescents. NAFLD was estimated to affect up to one third of obese children [1]. NAFLD ranges from accumulation of fat in the liver (Hepatoesteatosis) that may be accompanied by inflammation (Steatohepatitis) to necrosis, fibrosis and even cirrhosis resembling alcoholic hepatitis in the absence of alcoholic abuse [1]. Paralleling the increasing prevalence of obesity in the pediatric population, NAFLD is expected to become one of the most common causes of liver diseases in obese children and young adults [1]. NAFLD is mainly associated with obesity, diabetes mellitus and hypertension which are the main features of the metabolic syndrome. The pathogenesis of NAFLD is poorly understood. The “multi-hit” theory suggests that in the first “hit”, insulin resistance leads to the accumulation of fat within hepatocytes by lipolysis and hyperinsulinemia. The second “hit” mitochondrial release of reactive oxygen species (ROS), lipid peroxidation and cytokine induction [2]. In addition, recent data suggest a potential role for leptin hormone in the pathogenesis of non-alcoholic Steatohepatitis (NASH) by inducing dephosphorylating of insulin-receptor substrate [2]. Patients with chronic liver disease are at increasing risk of developing metabolic bone disease [2]. Which may be attributed to decreased osteocalcin (bone gla protein) which is involved in bone extracellular matrix mineralization [3]. Recent studies suggest that serum osteocalcin plays a key role in the pathogenesis of insulin resistance (IR) as experimentally, osteocalcin-gene deficiency exhibited metabolic syndrome in form of increased fat mass, IR and glucose intolerance [3]. There are few studies, mostly reported in adults that have explored the association between osteocalcin and NAFLD. This prompted us to investigate this relationship in children and adolescents with NAFLD.

The aim of the work was to measure serum osteocalcin levels in children and adolescents with NAFLD and to investigate the relationship between serum osteocalcin levels and both the degree and the clinico-laboratory variables of NAFLD.

### Materials and Methods

#### Design of the study and setting

This study was carried out upon 60 children and adolescents diagnosed as NAFL which based on the findings of hepatic steatosis detected by abdominal ultrasonography. Their ages ranged from 5-17 years with a mean value of  $10.6 \pm 2.7$  years who were attending the outpatient clinics and inpatient wards of Pediatric Department of Benha Teaching Hospital in the period from July 2015 to July 2017. Forty apparently healthy children, with normal liver function tests and normal liver examination by ultrasonography, matched for age, sex and body mass index (BMI) were chosen as controls. The study was conducted after approval from the ethical committee of the Faculty of Medicine, Benha University and informed oral or written parental consents.

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## Inclusion criteria

Children and adolescents with overweight or simple obesity who were diagnosed as NAFL by abdominal ultrasonography.

## Exclusion criteria

-Patients with known causes of fatty liver, e.g., diabetes mellitus, glycogen storage disease and or Wilson's disease.

-Patient with syndromic obesity.

-Patients with viral hepatitis B or C by performing serological markers (HBs Ag, Anti HBC Ab and HCV Ab).

-Patients receiving long term use of drugs which may cause steatosis or affect bone metabolism, e.g., glucocorticoids.

All participants included in this study were subjected to the following:

- Full history taking
- Through clinical examination which included

Anthropometric measurements including weight, height, BMI, waist circumference (WC), hip circumference (HC) and waist-to-hip ratio (WHR). They were obtained by using standardized equipments following the recommendations of the International Biological program [4]. Each measurement was recorded as the mean of three consecutive readings. The instruments were periodically checked for accuracy and standardization.

**Body weight** was measured by using Seca scale which was set on a hard, flat and uncarpeted surface. The child should be in light clothes and no shoes were worn, stood straight, calm on the scale, hands close to the trunk. The reading of weight was obtained in kilogram unit to the nearest 0.1 kilogram [5].

**Body height** was measured by using Harpenden stadiometer which with the same precautions as discussed before. The back of child should be against the scaled board of the stadiometer with the buttocks, back and the occiput touching the stadiometer. The child's eyes should face forward, knees kept unbent, arms close to the trunk and heels together. A special piece of the stadiometer was descended on the highest point of the head without pressure. The reading of height was obtained in meter unit to the nearest 0.1 centimeter [5].

**Body mass index (BMI)** was calculated according to the known formula:

$$BMI = \frac{Weight(kg)}{Height(m^2)}$$

We interpret BMI according to the Egyptian Growth Charts (2002). All the participants had a body mass index (BMI) that was above the 85<sup>th</sup> percentile for their age and sex, based on the national reference data. Children were defined as overweight if their BMI was equal to or above 85<sup>th</sup> percentile and as obese if BMI was equal to or above 95<sup>th</sup> percentile [6].

**Waist circumference** was measured at the level midway between the lowest rib margin and the iliac crest [7].

**Hip circumference** was measured at the widest level over the greater trochanters in a standing position, by the same examiner.

**Waist-to hip (W/H) ratio** was calculated. WHR was considered abnormal if its value exceeds 0.86. Values of WC and HC were plotted on American growth curves [2,4,7]. Patients with WHR values

exceeded 0.86 were considered abnormal.

- Laboratory investigations

Liver function tests included total and direct serum bilirubin levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total serum protein, serum albumin, prothrombin time (PT) and serological markers (HBs Ag, Anti HBC Ab and HCV Ab) [8].

Lipid profile included total serum cholesterol, high density lipoproteins cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and triglycerides (TG) [9]. Fasting serum glucose level [10]; Fasting serum insulin level [11]. Homeostasis Model Assessment method of Insulin Resistance (HOMA-IR) for evaluation of insulin resistance was calculated as follow:

$$HOMA = \frac{Fasting\ serum\ glucose [mmol / L] \times fasting\ serum\ insulin [\mu U / ml]}{22.5}$$

(Values  $\geq 3$  indicating insulin resistance);

Measurement of serum osteocalcin level [12].

**Sampling:** Two samples of blood were taken between the hours of 8 and 10 in the morning after 8 hours fasting. The first was 5 ml left to clot at 2-8°C and then the serum was separated using centrifugation then stored frozen at -20°C till time of analysis. This sample was used to analyze liver function tests and fasting blood glucose level, lipid profile and osteocalcin. The second sample for performing fasting serum insulin level, 1 ml of blood left to clot at 2-8°C and then the serum was separated using centrifugation.

Determination of serum insulin by enzyme linked immunosorbent assay (ELISA) method, using insulin ELISA kit which is a solid phase enzyme-linked immunosorbent assay based on the sandwich principle, Sigma Company [11].

Determination of serum osteocalcin in human serum: [12] by an immunoassay in which a sample of the serum or plasma containing the h-osteocalcin to be determined is incubated, Reference values for Pediatric age:

5-9 years: 47-142 ng/ml.

10-13 years: 49-167 ng/ml.

**Abdominal ultrasonography:** US to confirm diagnosis of liver fatty infiltration and to assess semiquantitatively the degree of steatosis. Liver ultrasound was carried out by using a convex 3.5-5.0 MHz probe. Sagittal hepatic sections that encompassed longitudinal images of the right liver lobe and the ipsi-lateral kidney were obtained. Moreover, hepatic US can provide a good estimate of the degree or extent of hepatic steatosis present based on a series of US characteristics including hepatorenal echo contrast, liver echogenicity, visualization of intrahepatic vessels and visualization of liver parenchyma and the diaphragm [13]. The diagnosis of hepatic steatosis was made on the basis of characteristic sonographic features: increased echogenicity of liver; increased liver contrast compared to kidney, vascular blurring-mainly of portal veins, attenuation of echogenic level in deep seated area [13].

## Grading of nonalcoholic fatty liver on ultrasonography

**Grade I:** Minimal diffuse increase in the fine echoes. Liver appears bright compared to the cortex of the kidney.

**Grade II:** Moderate diffuse increase in the fine echoes. Slightly

impaired visualization of the intrahepatic vessels and diaphragm.

*Grade III:* Marked increase in the fine echoes. Poor or no visualization of intrahepatic vessels and diaphragm and poor penetration of the posterior segment of the right lobe of the liver [14].

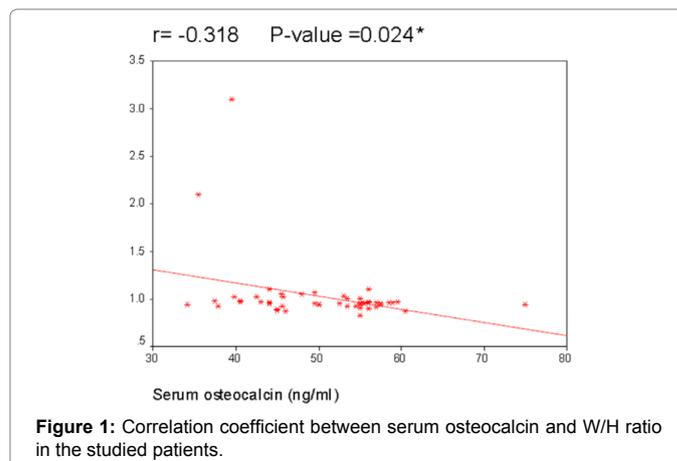
### Statistical analysis

Statistical presentation and analysis of the present study was conducted, using the mean, standard error, student t- test, Chi-square, Linear correlation coefficient (r), ROC curve Test and Analysis of variance (ANOVA) tests by SPSS V17. Unpaired Student T-test was used to compare between two groups in quantitative data. Chi-square hypothesis that the row and column variables are independent, without indicating strength or direction of the relationship. Pearson chi-square and likelihood-ratio chi-square. Fisher's exact test and Yates' corrected chi-square are computed for 2 × 2 tables. Linear Correlation Coefficient (r) was used for detection of correlation between two quantitative variables in one group. Analysis of variance (ANOVA) test was used for comparison among different times in the same group in quantitative data. Significance was adopted at p<0.05 for interpretation of results of tests of significance [15].

### Results

Patients enrolled in this study were classified according to BMI into 6 (10%) were overweight and 54 (90%) were obese. They were sonographically sub grouped into 3 grades: 40 (66.7%) patients had grade I, 16 (26.7%) had grade II and 4 (6.7%) had grade III steatosis.

Table 1 summarized demographic data of studied patients and controls, there was no significant difference between patients and controls as regard age, sex, Out of the 60 studied patients, 40 female (66.7%) and 20 male (33.3%). Mean value of waist and hip circumferences was significantly higher in patients than controls. Mean value of weight, height, BMI and WHR was statistically insignificant between patients and controls. Hepatomegaly, hypertension and acanthosis nigricans were found in 14 (23.33%), 16(26.6%) and 10 (16.67%) of the studied patients respectively. Splenomegaly, ascites or



edema were not detected in any studied patients (Figure 1).

Tables 2a and 2b summarized laboratory data of studied patients and controls; there was no statistically significant difference in CBC, serum creatinine, serum bilirubin, albumin, ALT, AST, HDL or LDL. Mean serum level of total cholesterol, and triglycerides (TG) was significantly higher in patients than control. Mean fasting serum glucose level was significantly higher in patients (85.7 ± 9.3 mg/dl) than controls (79.8 ± 4 mg/dl), (p=0.009) (Figure 2). Mean fasting serum insulin level was significantly higher in patients (15.5 ± 2.6 µU/ml) than in controls (14.1 ± 1.5 µU/ml), (p=0.025). Mean HOMA-IR value was significantly higher in patients (3.4 ± 0.8) than in controls (2.8 ± 0.3), (p=0.004). Mean serum osteocalcin level in was significantly lower in patients (48.3 ± 7.8 ng/ml) than in controls (52.8 ± 7.6 ng/ml), (p=0.049) (Figure 3).

Table 3 compared between demographic and laboratory data of studied patients according to grading of hepatic steatosis. Mean serum level of ALT, total cholesterol and triglycerides was increasing paralleling the severity of steatosis. Mean value of insulin resistance (HOMA-IR) markedly increases as the hepatic steatosis advancing in severity. Mean serum level of total cholesterol and triglycerides was increasing paralleling the severity of steatosis. Mean values of serum level of fasting insulin and insulin resistance (HOMA-IR) increase paralleling the severity of steatosis. Mean level of osteocalcin was (51 ± 7.32 ng/ml) in grade I, (44 ± 5.8 ng/ml) in grade II and (39.1 ± 7 ng/ml) in grade III. There was a parallel decrease of mean serum osteocalcin level with advancing the degree of hepatic steatosis by ultrasound. Table 4 compared between Patients with metabolic syndrome (MS) and patients without MS according to laboratory data. Values of HOMA-IR and alkaline phosphatase were higher in patients with metabolic syndrome than patients without metabolic syndrome Mean level of serum osteocalcin was lower in patients with metabolic syndrome (43.850 ± 9.963 ng/ml) than in patients without metabolic syndrome (48.965 ± 7.410 ng/ml) with p ≤ 0.05 (Figure 4).

Table 5 summarized correlations between serum osteocalcin and different studied variables in patients. Serum osteocalcin levels were inversely correlated with the values of W/H ratio, ALT, triglycerides, fasting insulin and HOMA-IR.

### Discussion

Pediatric NAFLD is considered as a global problem which has been increased in prevalence with the dramatic rise in obesity in children during the past three decades [16]. NAFLD is an important cause of

		Group I (n=60)	Group II (n=60)	Statistical test	P value
Age (years)	Range	5-17	5-15	1.7	0.16
	Mean ± SD	10.6 ± 2.7	9.1 ± 3.5		
Gender	Male: No (%)	20(33.3%)	24(60%)	3.5	0.06
	Female: No (%)	40(66.7%)	16(40%)		
Weight (kg)	Range	33.5-100	27-29	1.57	0.12
	Mean ± SD	72±19.4	46 ± 13.9		
Height (cm)	Range	105.5-159.5	107-154	1.3	0.22
	Mean ± SD	141.6 ± 15.2	136.6 ± 11.3		
BMI (Kg/m <sup>2</sup> )	Range	29.8-48.2	13.4-50.8	0.29	0.78
	Mean ± SD	35.1 ± 4.6	34.6 ± 7.9		
Waist circumference (cm)	Range	76-117	70-98	5.7	<0.001*
	Mean ± SD	99.4 ± 11	82.4 ± 9.5		
Hip circumference (cm)	Range	79-137	69-100	5.6	<0.001*
	Mean ± SD	105 ± 14.3	84.5 ± 9.8		
Waist/Hip circumference (cm)	Range	0.1-1.1	0.9-1.1	1.41	0.16
	Mean ± SD	0.9 ± 0.2	0.97 ± 0.04		

BMI: body mass index; p: p values for comparing between the studied groups; \*Statistically significant at p ≤ 0.05

**Table 1:** Demographic data of the studied groups.

		Group I (n=60)	Group II (n=60)	Statistical test	P value
Hemoglobin (gm/dl)	Range	9.5-14	12-15	1.595	0.116
	Mean ± SD	11.79 ± 1.38	12.23 ± 0.61		
TLC (× 10 <sup>3</sup> /mm <sup>3</sup> )	Range	4.7-14.9	4.1-13.9	0.013	0.99
	Mean ± SD	8.24 ± 3.08	8.25 ± 2.89		
Platelets count (× 10 <sup>3</sup> /mm <sup>3</sup> )	Range	153-409	159-386	0.652	0.519
	Mean ± SD	252.56 ± 75.68	266.28 ± 73.72		
Total serum Bilirubin (mg/dl)	Range	0.5-1.4	0.5-1.4	1.263	0.217
	Mean ± SD	0.91 ± 0.27	0.82 ± 0.26		
Serum albumin (gm/dl)	Range	3.5-4.5	3.4-4.5	0	1
	Mean ± SD	3.92 ± 0.29	3.92 ± 0.32		
ALT (U/L)	Range	21-40	20-39	0.398	0.694
	Mean ± SD	28.93 ± 5.77	28.23 ± 5.65		
AST (U/L)	Range	24-46	22-41	1.187	0.245
	Mean ± SD	32.63 ± 5.91	30.57 ± 5.71		
Serum creatinine (mg/dl)	Range	0.4-1.2	0.4-1.1	0.521	0.606
	Mean ± SD	0.77 ± 0.71	0.67 ± 0.7		

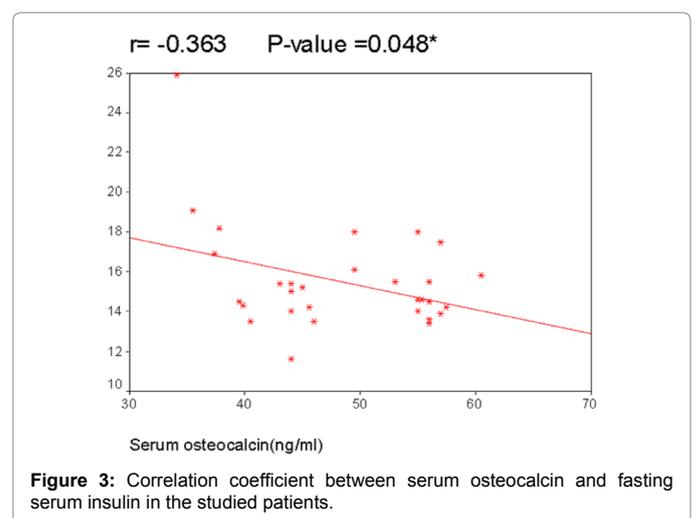
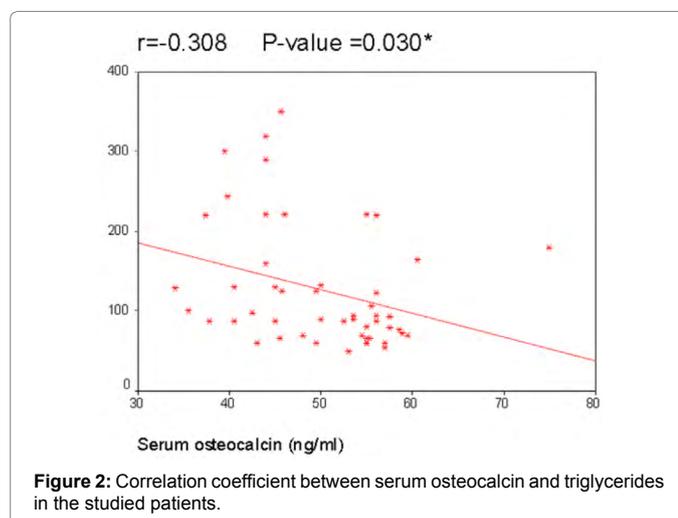
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; p: p values for comparing between the studied groups; \*Statistically significant at p ≤ 0.05

Table 2a: Laboratory characteristics of patients and controls.

		Group I (n=60)	Group II (n=40)	Statistical test	P value
Total cholesterol (mg/dl)	Range	87-278	99-240	2.95	0.005*
	Mean ± SD	193.4 ± 41	157.3 ± 44.5		
Triglycerides (mg/dl)	Range	60-194	60-180	2.4	0.02*
	Mean ± SD	117.2 ± 365	93.4 ± 29.3		
HDL (mg/dl)	Range	20-57	15-59	0.2	0.8
	Mean ± SD	40.8 ± 11.1	41.4 ± 11.5		
LDL (mg/dl)	Range	63-195	49-160	1.67	0.1
	Mean ± SD	110.6 ± 32.4	95.4 ± 30.2		
Fasting serum glucose (uU/ml)	Range	69-103	70-85	2.71	0.009*
	Mean ± SD	85.7 ± 9.3	79.8 ± 4		
Fasting insulin (µu/ml)	Range	11.6-25.9	12.3-17.5	2.31	0.03*
	Mean ± SD	15.5 ± 2.6	14.1 ± 1.5		
HOMA-IR	Range	2.4-6.7	2.5-3.3	3.05	0.004*
	Mean ± SD	3.7 ± 0.8	2.8 ± 0.3		
Serum osteocalcin (ng/ml)	Range	34.1-60.5	40.5-74.9	2.02-	0.05*
	Mean ± SD	48.3 ± 7.8	52.8 ± 7.6		

P: p values for comparing between the studied groups; p. value: patients vs control; LDL-C (low density lipoprotein cholesterol); HDL-C (high density lipoprotein cholesterol).; \*: Statistically significant at p ≤ 0.05

Table 2b: Laboratory characteristics of patients and controls (Cont.).



		Grade I NAFLD	Grade II NAFLD	Grade III NAFLD	Statistical test	
					F=ANOVA	P value
Serum osteocalcin (ng/ml)	Range	37.4-60.9	35.5-56	34.1-44	4.74	0.02
	Mean ± SD	50.9 ± 7.3	44 ± 5.8	39.1 ± 7		
Age (years)	Mean ± SD	10.3 ± 2.9	11.9 ± 1.7	7.8 ± 1.8	2.3	0.12
BMI (Kg/m <sup>2</sup> )	Mean ± SD	34.4 ± 3.9	36.1 ± 5.3	38.5 ± 8.8	1.03	0.37
Waist /Hip circumference (cm)	Mean ± SD	0.94 ± 0.1	0.85 ± 0.3	0.95 ± 0.01	0.94	0.4
ALT (U/L)	Mean ± SD	28.5 ± 9.8	32.8 ± 11.9	39.5 ± 17.7	0.23	0.02*
ALP (IU/L)	Mean ± SD	244.9 ± 131.5	244.4 ± 106.3	400 ± 70.7	1.83	0.18
Total cholesterol (mg/dl)	Mean ± SD	186 ± 41.4	194.5 ± 25.5	284 ± 19.8	3.99	0.03*
Triglycerides (mg/dl)	Mean ± SD	11131 ± 32.9	122.8 ± 47.3	154.5 ± 21.9	0.32	0.01*
HDL (mg%)	Mean ± SD	43 ± 9.8	37.5 ± 12.7	31 ± 15.6	1.6	0.22
LDL (mg%)	Mean ± SD	108 ± 31.2	108 ± 34.4	142.5 ± 38.9	1.04	0.37
Fasting insulin (µu/ml)	Mean ± SD	15.3 ± 1.4	16.4 ± 2.6	189 ± 10.1	2.3	0.03*
HOMA-IR	Mean ± SD	3.2± 0.5	3.5± 0.03	4.8 ± 2.8	5.1	0.01*

HOMA-IR: Homeostasis module assessment of insulin resistance; \*Significant; p value: patients vs control

Table 3: Comparison between demographic and laboratory data of studied patients according to grading of hepatic steatosis.

Laboratory findings	Patients without metabolic syndrome (No=52)		Patients with metabolic syndrome (No=8)		Statistical test	
	Mean	SD	Mean	SD	t value	P value
ALT (U/L)	29.2	10.6	29	10.6	0.04	0.97
AST (U/L)	30.7	9	36.5	8.6	-1.2	0.24
Alkaline Phosphatase (IU/L)	220.6	122	379.3	56.8	-2.5	0.02*
HOMA-IR	3.3	0.5	4.1	1.8	-2.2	0.04*
Serum osteocalcin (ng/ml)	49	7.4	83.9	8	2.2	0.005*

**HOMA-IR**

Table 4: Comparison between patients with metabolic syndrome and patients without metabolic syndrome according to laboratory data.

Variable	r	p
Age (years)	0.155	0.415
BMI (kg/m <sup>2</sup> )	0.104	0.584
W/H ratio	-0.318	0.024 *
ALT (U/L)	-0.342	0.015*
ALP (IU/L)	-0.286	0.126
Total cholesterol (mg/dl)	-0.108	0.571
Triglycerides (mg/dl) -0.308	-0.308	0.03*
HDL (mg/dl)	0.078	0.683
LDL (mg/dl)	0.078	0.68
Fasting insulin (µu/ml)	-0.363	0.048*
HOMA-IR	-0.381	0.038*

\*Significant; BMI: body mass index; W/H ratio: Waist to hip ratio; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; HOMA-IR: Homeostasis Model Assessment method of Insulin Resistance

Table 5: Correlations serum osteocalcin and different studied variables in patients.

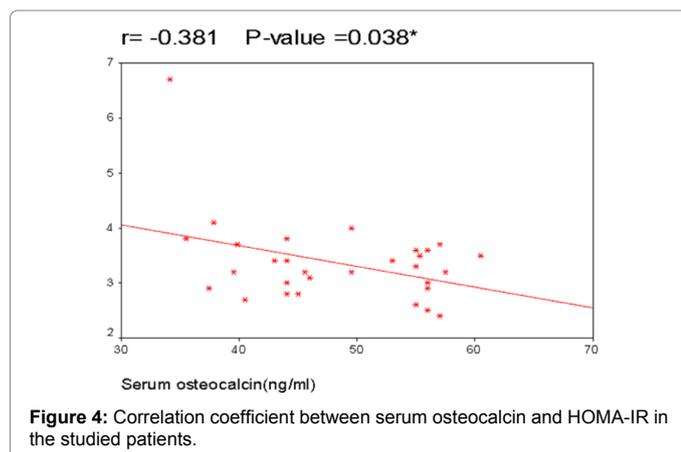


Figure 4: Correlation coefficient between serum osteocalcin and HOMA-IR in the studied patients.

chronic liver disease in children.

An Egyptian study gave the prevalence of NAFLD in 38.5% in obese children and adolescents [17]. Mechanisms behind the reduced BMD in NAFLD are still not completely understood [2]. Currently the information on the relationships between serum osteocalcin and NAFLD is lacking. The present study was designed to analyse the relationship between serum osteocalcin levels and NAFLD in children and adolescents. In our study, we diagnosed NAFLD patient by US which is reliable modality for the screening for fatty liver in clinical

settings. Dasarathy et al. [18] reported that US had a sensitivity of 81% and a specificity of 98% in identifying hepatic steatosis. The present study showed that patients and controls had high waist circumference exceeding the 95<sup>th</sup> percentile. The mean value of waist circumference in NAFLD patients was significantly higher than in those without NAFLD. Waist circumference is considered a surrogate measure of visceral fat and may predict the development of NAFLD in children [19] and considered as integral part of the definition of the metabolic syndrome both in children and adults [20].

This was in agreement with Duarte and Silva [21] study. By ultrasound we observed that 40 (66.7%) of our patients had grade I fatty infiltration, 8 patients (26.7%) had grade II and 4 patients (6.7%) had grade III fatty liver which was in agreement of study of Duarte and Silva [21] and Papandreou et al. [22]. Hepatic synthetic functions were assessed by measuring serum albumin and prothrombin time values showing no significant difference between patients and controls. These results coincided with that reported by other authors studied obese patients with NAFLD [23,24].

In this study, there was elevation in ALT levels paralleled the advancing grades of grading of NAFL. Previous reports have shown controversial results regarding the correlation between ALT elevation and the presence of hepatic fat on imaging. In the study conducted by Franzese et al. [25], about two thirds of their NAFLD had normal amino transaminases. In the study by Tazawa et al. [26], 18% of Japanese

school children with normal ALT levels had ultrasound findings of NASH.

The present work showed a clear evidence of abnormal lipid profile in the studied patients. The mean serum level of total cholesterol and triglycerides was significantly higher in patients than in controls. Also, there was an increase in the mean serum level of total cholesterol and triglycerides with increasing the degree of hepatic steatosis. This results was in agreement with Sartorio et al. [24], El-Karakasy et al. [17] and Duarte and Silva study [26], On the other hand, Papandreo et al. [22] reported that none of their studied obese children with NAFLD had abnormal lipid profile or liver indices.

In the present work, we found that 8 (13.3%) fulfilled the criteria of the metabolic syndrome. This estimated value was lower than that reported in other pediatric studies. Jun-Fen et al. [27] gave a prevalence of metabolic syndrome (MS) of 26% among their studied obese children and of 40% in those with NAFLD. The lower incidence of metabolic syndrome in our study may be attributed to the presence of mild and moderate fatty infiltration of the liver in the majority of the currently studied patients (93.3% in grade I and II versus 6.7% in grade III steatosis). It is known that the presence of severe steatosis carries a high risk of development of MS [28]. This highlights that NAFLD is closely associated with features of the metabolic syndrome.

In this study, the mean level of fasting serum glucose and insulin was significantly higher in patients with NAFLD than that of controls.

There was an increase in mean level of fasting serum insulin with increasing the degree of hepatic steatosis. Sartorio et al. showed similar results [24] Mean value of insulin resistance, as measured by HOMA-IR was markedly higher in the studied patients when compared to controls.

Among the 60 patients with NAFLD, 38 cases (63.3%) exhibited abnormal HOMA-IR values. We also observed that there was an increase in mean HOMA-IR values with increasing the degree of hepatic steatosis. All patients with grade III and 87.5% with grade II and 50% of those with grade I had IR. These results may indicate that insulin resistance, in addition to hyperinsulinemia, occurs early and worsen in children with grade II and III (presumed to have NASH). IR seems to be a common finding in NASH and it was described in 85% of the patients tested by Willner et al. [29]. IR plays a key role in the pathogenesis and development of NASH [30] Studies performed by Xanthakos et al. [27] and Finucane et al. [31] showed that HOMA-IR values were significantly different between obese patients with NAFLD and obese subjects without NAFLD. Chan et al. [28] had presented parameters of insulin sensitivity, including the HOMA index in 84 obese children, 65 of whom showed hepatic steatosis proven by ultrasonography. It was found that the severity of fatty liver was positively related to the HOMA index. Schwimmer et al. [32] screened 43 children with biopsy-proven NAFLD for insulin resistance and concluded that IR was correlated with the severity of liver histology. However, Eminoglu et al. [33] did not find any correlation between the presence and the severity of steatosis and the increased levels of HOMA-IR in their study. Radetti et al. [34] showed decreased insulin sensitivity in all of the obese children but no difference was found in insulin sensitivity between children with or without NAFLD.

The most important observation in the current study was the finding that serum osteocalcin level was significantly lower in the studied NAFLD patients, compared to those matched controls. Out of 60 patients, 42 (70%) had low serum osteocalcin concentrations. Also, there was a parallel decrease in serum osteocalcin levels

with increasing the grades of fatty liver infiltration as detected by ultrasound. Correlation analysis in the present study demonstrated that serum osteocalcin concentrations were inversely associated with HOMA-IR, fasting insulin, ALT and W/H ratio in children with NAFLD. Previous data concerning estimation of serum osteocalcin levels in children still limited and controversial [35]. Yalmiz et al. [36] showed that serum osteocalcin levels were significantly lower in their biopsy proven NAFLD patients than in controls. Serum osteocalcin levels were inversely associated also with ALT, AST and HOMA-IR. The authors also demonstrated that serum osteocalcin concentrations are weakly, but significantly associated with the degree of hepatocyte ballooning, independent of other risk factors (including IR). As serum transaminases levels are conventionally believed to be surrogate biomarkers of hepatocyte injury, the association observed in our work and in other studies [36], seems to suggest that serum osteocalcin level may possibly reflect liver injury. However, we did not examine the severity of liver injury histopathologically among the ultrasonographic - proven NAFLD children.

In a study of 28 obese patients, Fernández-Real et al. [37] have shown that circulating osteocalcin concentrations are negatively associated with blood markers of liver injury and liver disease, including ALT and AST. In addition, the changes in ALT levels following weight loss in obese individuals were linearly associated with changes in osteocalcin concentrations. A Chinese adults study was consistent with our findings [38] reporting that serum osteocalcin levels were significantly associated with the scale of NAFLD. Osteocalcin showed a decreased trend with the scale of NAFLD. These results implied that osteocalcin could be a potential novel marker to assess the progression of NAFLD in children. In another study, Dou et al. [39] found that serum osteocalcin levels were significantly lower in subjects with NAFLD than those without NAFL. This study also showed that there was a statistical negative. Aller et al. [40] and Sinn et al. [41] studied the relation of osteocalcin with NAFL and found similar results. The observation that NAFLD patients with metabolic syndrome (MS) in this study had significantly lower levels of osteocalcin compared to those without MS is similar to that shown in adults e.g., Pittas et al. [42], Saleem et al. [43]. It is uncertain which mechanisms lead to lower osteocalcin levels in patients with NAFLD. Relying upon findings in patients with primary biliary cirrhosis and in patients with chronic alcoholic liver disease showing low serum osteocalcin levels [44], it is supposed that the chronic liver disease by itself can influence the osteoblast activity. One of the potential key contributor is the pro-inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) that inhibits osteoblast differentiation and promotes osteoblast apoptosis [45]. An increase in circulatory level of TNF- $\alpha$  has been reported in NAFLD patients [46]. Additionally, the oncofetal fibronectin produced by activated star cells in chronic liver disease suppresses osteoblast function [47]. Consequently, osteocalcin synthesis is suppressed resulting in low serum osteocalcin concentrations in these patients. Previous studies have revealed that serum osteocalcin concentrations were positively associated with insulin secretion and inversely correlated with adiposity [35]. It has been suggested that decreased osteocalcin could lead to a worsening of liver fat infiltration in a vicious cycle. Insulin resistance (IR) might be the underlying mechanism linking the endocrine organ bone (osteocalcin) and liver lipogenesis.

Pomiedzy et al. [48] found that serum osteocalcin level decreases with increasing percentage of body fat and waist circumference as well as insulin concentration and HOMA-IR in obese children and adolescents. This observation indicates that osteocalcin acts as a negative regulator of fat mass and has favorable effects on fat and glucose metabolism in obese children. The present work and other

related studies could suggest a novel cross – talk between bone and adipose tissue and liver. This association raises new prospects for future research in this area of work.

## Conclusion

Our NAFL patients had significantly lower serum levels of osteocalcin compared to controls. Patients who had metabolic syndrome (MS) showed significantly lower serum levels of osteocalcin compared to those without MS. Serum osteocalcin levels were inversely correlated with the values of W/H ratio, ALT, triglycerides, fasting insulin and insulin resistance (HOMA-IR) so serum osteocalcin levels were inversely associated with the degree of steatosis. So, osteocalcin is considered as a biomarker for severity NAFLD in children and adolescents.

## Recommendations

Measurements of serum osteocalcin concentrations, as a marker of bone metabolism should be included among the biochemical tests performed in obese children and adolescents with NAFLD as the change in serum osteocalcin level was significantly associated with advancing liver fat infiltration.

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