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Original Article

Serum microRNA-486-5p expression in obese Egyptian children and its possible association with fatty liver

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ABSTRACT

Aims: Several microRNAs (miRNAs) are involved in regulating the process of adipogenesis. White adipose tissue is a major source for these miRNAs. We aimed to evaluate the expression of miR-486-5p in children with obesity and its possible association with nonalcoholic fatty liver disease (NAFLD).
Method: This case-control study included 100 obese and overweight children and 100 normal-weight children of matched age and sex. All children were subjected to anthropometric measurements and evaluation of miR-486-5p expression levels using the SYBR green-based real-time RT-PCR technique.
Results: Obese children showed significantly up-regulated miR-486-5p gene expression (p value < 0.001) when compared to control group. MiR-486-5p gene expression showed significant positive correlation with weight ($r = 0.924$), BMI ($r = 0.497$), waist circumference ($r = 0.387$), fat mass ($r = 0.361$), LDL($r = 0.351$), TG ($r = 0.867$), TC ($r = 0.875$) and presence of fatty liver ($r = 0.760$). The best cutoff value of miR-486-5p gene expression in the prediction of obesity was 0.44 with AUC 0.736 that has a sensitivity 60% and specificity 90%,
Conclusion: The serum level of the miR-486-5p gene is up-regulated in obese and overweight children and might be an independent predictor for obesity and fatty liver susceptibility.

1. Introduction

The obesity epidemic has developed rapidly over the last few decades as a public health problem. Particularly worrisome is the rapid rise in obesity and its related comorbidities (e.g. hypertension, lack of mobility, type 2 diabetes, dyslipidemia, and obstructive sleep apnea) among children [1]. The combined prevalence of obesity and overweight among Egyptian school adolescents aged 11 to 17 was 40.7%, with a lower prevalence of obesity among females (7.6%) than males (8.6%) [2], with diagnosis at progressively younger ages pointing to early childhood environmental origins. Hence, it is a priority to identify reliable biomarkers that can predict childhood obesity and its complications [1].

MicroRNAs are small non-coding RNAs with a length of about 19–24 nucleotides. They function to inhibit protein synthesis by binding to complementary 3′-untranslated regions (3′ UTR) of messenger RNA (mRNA), and either accelerating mRNA degradation or inhibiting translation [3].MiRNAs play an important role in the development of adipogenesis and the maintenance of specific metabolic functions. Interestingly, during adipogenesis, up-regulated miRNAs were downregulated in obesity, suggesting a reciprocal expression of miRNAs in obesity and adipogenesis [4].MiRNAs regulate white adipose tissue function; their levels are changed in obesity. Some miRNAs' levels are down-regulated or up-regulated in obesity which ultimately contributes to the dysfunction of the white adipose tissue [5].

MiR-486 is transcribed from an Ank1 gene intron that encodes an ankyrin repeat protein which binds the cytoskeleton to the plasma membrane. In erythroid cells under the control of an erythroid-specific promoter, Ank1 is expressed specifically [6]. MiR-486 inhibits the transcription factor forkhead box O1, which is

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one of the important mediators of insulin action and triglyceride metabolism. MiR486 activates the nuclear factor-beta signaling pathway by inhibiting multiple nuclear factor-beta negative regulators [7]. We aimed to evaluate the expression of miR-486-5p in children with obesity and its possible association with nonalcoholic fatty liver disease (NAFLD).

1.1. ***Patients and methods***

Study participants: A total number of 200 children who were recruited from the Pediatrics Department of Benha University Hospitals were included in this study during the period from June 2018 to April 2019 and they were divided into two groups, 100 obese and overweight children, some of them have nonalcoholic fatty liver, and 100 normal-weight children of matched age and sex. All Obese children group having body mass index (BMI) above 95th percentile, while the overweight group was between the 85th and 95th percentile. The control group consists of children having (BMI) between the 5th and 85th percentile [8].

Children with abnormal liver function, major congenital abnormalities, preexisting liver condition, and abnormal thyroid function, or kidney function, chronic use of medication, or evidence of chronic disease were excluded from the study.

Informed written consent was obtained from parents of all children before inclusion in the study and the study was approved by the ethical committee of Benha Faculty of Medicine.

All participants were subjected to full history taking including age, sex, dietary habits, physical exercise, medication, chronic disease and anthropometric measurement including height was measured by a tape measure, weight and fat mass were measured on a digital electronic scale using In Body (USA) then we plotted them on percentile for age and calculating (BMI), Waist circumference was measured by a tape measure, and blood pressure (BP) was measured using the standardized mercury sphygmomanometer. Tanner staging was assessed to all participants [9]. Laboratory and radiological investigations were done for all participants in the study, abdominal ultrasonography was performed on all participants for detection of the non-alcoholic fatty liver using GE logic p6 (TIWAN).

Sample collection Under complete aseptic conditions, 6 ml of venous blood were withdrawn from each participant after overnight fasting. Then 1 ml was put into tubes containing K3EDTA anticoagulant which was used for measuring complete blood count (CBC) includes: Hb(g/dl), WBC($X10^9$/L), platelet count($X10^9$/L) were performed by Sysmex XS-500i (JAPAN) and 5 ml were put in serum separating tubes then left for 30 min till clotting then centrifuged at 1500 rpm for 5 min. The separated serum was divided into 2 parts, one was used immediately for measuring fasting blood sugar, lipid profile (TG, HDL, LDL, total cholesterol), ALT, and AST was performed using Biosystem A15 autoanalyzer using kit supplied by biosystem (Barcheloiena, Spain) according to the manufacturer's instructions, while the other part was aliquoted and stored at −80 °C for evaluation of serum microRNA-486-5p expression using reverse transcriptase real-time polymerase chain reaction technique (RT-PCR).

RNA Extraction Using miRNeasy [Mini Kit supplied by (Qiagen, Hilden, Germany) cat. No. 217004], extraction of total RNA which includes microRNA was done from each sample according to the manufacturer's instructions. Thermo scientific nanodrop spectrophotometer (USA) was used for measuring RNA purity and concentration of each sample.

1.2. *Quantification of micro RNA using SYBR green-real time RT-PCR (qRT-PCR)*

First, using miRCURY LNA RT Kit supplied by (QIAGEN, Germany) (Cat.No 339340) a reverse transcription (RT) step was performed for conversion of RNA to its complementary deoxynucleic acid (cDNA) according to manufacturer's instructions.

Second, using miRCURY LNA SYBR Green PCR Kit supplied by (QIAGEN, Germany) (cat. No 339345) and miRCURY LNA miRNA PCR Assay (Cat. No. 339306) using SYBR green qRT-PCR assay real-time PCR was performed for quantification of miRNA by real-time PCR cycler Applied Biosystems (Singapore). The normalization control used in our research was SNORD68. The relative quantities of miRNA were normalized against the relative quantities of SNORD68. Relative expression of miRNA and the fold change of expression was calculated using the equation of the threshold cycle (Ct) value, relative quantity (RQ) = $2-\Delta\Delta Ct$, $\Delta\Delta Ct$ = obesity (Ct miRNA −Ct SNORD68) - mean normal (Ct miRNA - Ct SNORD68) [10].

2. Statistical analysis

Using the statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).The obtained data were fed to the computer and tabulated. The following tests were used for data analysis: Student T-test, Mann−Whitney *U* test, Fisher's exact, Kruskal−Wallis and Chi-Square test. Mean values ± standard deviations (SD) represent the parametric numerical data while median and range represent the non-parametric numerical data. For detection of the specificity and sensitivity for prediction of the optimal cut-off values of case-control status for serum miR-486-5p expression level receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used. p values are significant if < 0.05 at confidence interval 95%.

3. Results

The mean age of one hundred obese and overweight cases was 8.6 ± 2.3 years ranged from 6.3 to 10.9 years; twenty-seven of them had non-alcoholic fatty liver, while the age of matched control group was 7.9 ± 2.4 years ranged from 6.1 to 9.9 years. The obese group had a positive family history of obesity in 74% of them, in comparison with 10% in the control group, with a significant differences between the studied groups ($p < 0.001$).

The obese children had significantly heavier body weight/centile ($p < 0.001$), higher BMI ($p < 0.001$), higher BMI\centile ($p < 0.001$), higher waist circumference ($p < 0.001$), higher fat mass ($p < 0.001$), higher frequency of fatty liver by ultrasound ($p = 0.012$) when compared to control group. Concerning laboratory data, the obese children had significantly higher low-density lipoprotein (LDL), triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT), significantly lower high-density lipoprotein (HDL) when compared to the control group. Complete blood count (CBC) and aspartate aminotransferase (AST) did not show a statically significant differences between the studied groups. Table 1.

4. Serum miRNA-486-5pexpression profile in studied groups

The obese and overweight group showed significantly up-regulated miR-486-5p gene expression when compared to control group (median = 0.79 versus 0.24 respectively, $p = 0.001$)Table 1, Fig. 1.

Receiver operating characteristic curve(ROC) of miR-486-5p gene expression was conducted for discrimination between obese cases and control groups as miR-486-5p gene expression showed a fair area under the curve (AUC = 0.736), at cut off value of 0.44 had sensitivity 60%, specificity 90%, PPV was 88.9%, NPV was 62.8%, and accuracy was 72.9%. Fig. 2.

Table 1
Clinical and laboratory data of the studied groups.

variables			Control group N = 50		Obese group N = 50		*p*
Weight/centile (kg)		Mean ± SD	28.3	±9.1	52.1	±17.1	<0.001
Height/centile (cm)		Mean ± SD	126.4	±20	133.9	±25.6	0.192
BMI (kg/m^2)		Mean ± SD	16.9	±1.4	27.8	±5.6	<0.001
BMI/centile	>97	N, %	0	0%	38	38%	<0.001
	>95	N, %	0	0%	58	58%	
	90–95	N, %	0	0%	4	4%	
	5–85	N, %	100	100%	0	0%	
Waist circumference (cm)		Mean ± SD	64.6	±6.4	89.5	±12.1	<0.001
Fat mass (%)		Mean ± SD	27.9	±2.4	35.6	±2.8	<0.001
SBP (mmHg)		Mean ± SD	104.5	±3.8	100.2	±14.8	0.134
DBP (mmHg)		Mean ± SD	64.8	±4.3	63.3	±4.4	0.155
Tanner staging	I	N, %	18	18%	20	20%	0.116
	II	N, %	42	42%	50	50%	
	III	N, %	32	32%	24	24%	
	IV	N, %	8	8%	6	6%	
Fatty liver by ultrasound		N, %	0	0%	27	27%	0.012
Hemoglobin (g/dL)		Mean ± SD	12	±0.5	11.5	±0.5	0.196
WBCs (X10^9/L)		Mean ± SD	8.1	±1.6	7.7	±1.5	0.374
Platelet (X10^9/L)		Mean ± SD	263.1	±83.8	282.5	±89.2	0.360
HDL cholesterol (mg/dL)		Mean ± SD	45.4	±3.5	41.2	±3.2	0.007
LDL cholesterol (mg/dL)		Mean ± SD	101.7	±8.5	116.6	±9.7	0.027
TG (mg/dL)		Mean ± SD	135.8	±27.5	180.8	±23.3	<0.001
TC (mg/dL)		Mean ± SD	177	±13.1	186.9	±16.5	0.008
ALT (U/L)		Mean ± SD	28.1	±1.7	35.9	±7	<0.001
AST (U/L)		Mean ± SD	31	±2.5	33.6	±3.6	0.230
MiR-486-5p gene expression		Mean ± SD	0.26	±0.03	1.55	±0.27	<0.001

BMI: body mass index, SBD: systolic blood pressure, DBP: diastolic blood pressure, WBCS: white blood cells, HDL: high density lipo protein, LD: low density lipo protein, TG: triglyceride, TC: total cholesterol, ALT: alanine aminotransferase, AST: aspartate aminotransferase.

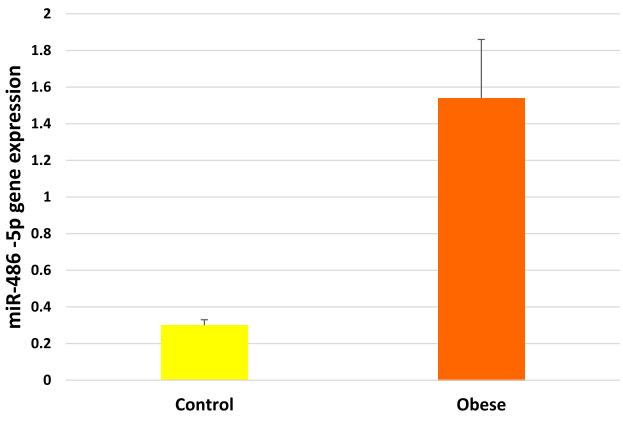

Fig. 1. Bar chart for miR-486 gene expression level in the studied groups.

5. Association and correlation of miR-486-5p gene expression level with other parameters in the obese and overweight group

Higher miR-486-5p gene expression level was significantly associated with higher BMI/centile ($p < 0.001$), as well as with fatty liver ($p = 0.001$). Table 2.

MiR-486-5p gene expression showed a significant positive correlation with (weight, BMI BMI/centile, waist circumference, fat mass, LDL, TG, TC, presence of fatty liver, and significant negative correlation with HDL. Table 3.

For prediction of obesity susceptibility, logistic regression analysis was conducted and revealed that positive family history of obesity and higher miR-486-5p were positively associated with risk of obesity occurrence in univariate analysis. However, in multivariate analysis taking significant risk factors revealed that only miR-486-5p gene expression was considered as an independent predictor for obesity susceptibility. Table 4.

Ordinal regression analysis was also conducted for prediction of obesity severity using age, gender, consanguinity, family history of obesity (FH), HDL, LDL, TG, TC and miR-486-5p gene expression as covariates and has revealed that lower HDL, higher LDL, TG, TC and miR-486-5p gene expression levels were associated with higher grades of obese in univariable analysis. While in multivariable analysis, higher TG concentration and miR-486-5p gene expression up-regulation were considered as one of the independent risk predictors for more severe obesity. Table 4.

For fatty liver prediction, regression analysis was conducted and revealed that positive family history of obesity, lower HDL, higher TG, TC, miR-486-5p were significantly associated with the risk of fatty liver occurrence in univariate analysis. However, taking significant risk factors into multivariate analysis, revealed that only miR-486-5p gene expression was considered as one of the independent predictors for fatty liver susceptibility. Table 4.

6. Discussion

In the present study, there was a statistically significant increase of miR-486-5p in obese and overweight children when compared to the normal-weight group. These findings were consistent with other studies [11,12] as they concluded that miR-486 exhibited an increased expression level in obese subjects versus healthy controls. Moreover, our results were consistent with Marzano et al., [13] who reported that miR-486-3p was up-regulated in both obese appropriate for gestational age (OB-AGA) and obese small for gestational age (OB-SGA) compared to normal-weight children. Pathway analysis suggested that this miRNA was particularly involved in insulin signaling, glucose transport, insulin resistance, cholesterol, and lipid metabolism.

Also, Mohany et al., [14] stated that in obese children with or without type2 diabetes mellitus, the circulating levels of

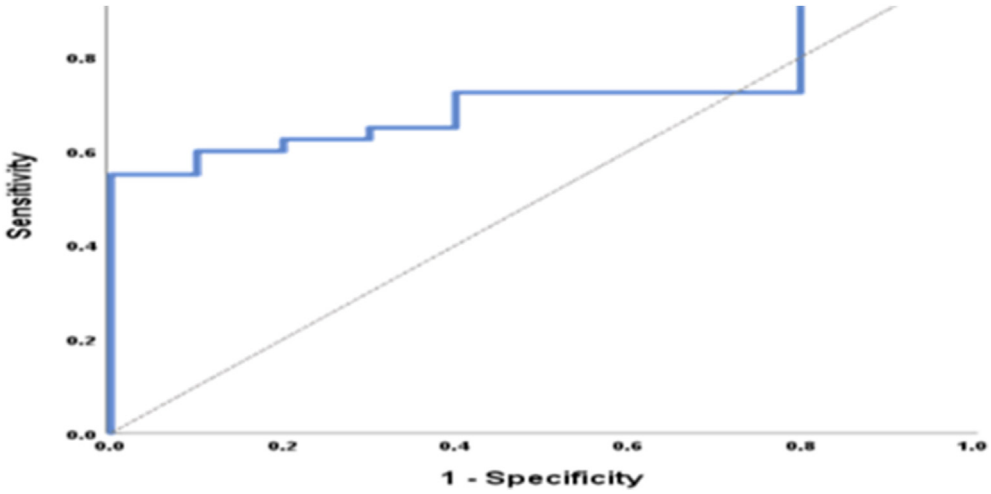

Fig. 2. ROC curve of miR-486-5p gene expression for differentiation between the studied groups.

Table 2
Association between miR-486-5p gene expression level and other parameters in obese children group.

\		miR-486 gene expression level			*p*
		Median	Range		
Sex	Male	0.6	0.2	8.5	0.605
	Female	0.8	2.9×10^{-4}	5.8	
Family history of obesity	Negative	0.6	0.1	4.2	0.351
	Positive	0.9	2.9×10^{-4}	8.5	
BMI/centile	>97	1.9	0.7	8.5	<0.001
	>95	0.3	0.02	5.1	
	90–95	0.05	2.9×10^{-4}	0.1	
Tanner staging	I	1.5	0.2	1.9	0.380
	II	0.3	0.02	5.8	
	III	1.2	2.9×10^{-4}	8.5	
	IV	0.8	0.2	5.1	
Presence of fatty liver	No	0.5	2.9×10^{-4}	5.8	0.001
	Yes	3.1	0.8	8.5	

Table 3
Correlations of miR-486 gene expression with other parameters in obese group.

	MiR-486 gene expression level	
	R	P
Age	0.058	0.724
Weight (kg)	0.924	<0.001
Height (cm)	0.048	0.771
BMI	0.497	0.001
Percentile	0.688	<0.001
Waist circumference (cm)	0.387	0.013
Fat mass (%)	0.361	0.022
SBP(mm/hg)	0.154	0.344
DBP(mm/hg)	0.243	0.131
Tanner staging	0.064	0.702
Hemoglobin (g/dl)	−0.013	0.931
WBCs ($X10^9$/L)	0.24	0.136
Platelet ($X10^9$/L)	0.007	0.971
HDL (mg/dl)	−0.894	<0.001
LDL (mg/dl)	0.351	0.026
TG (mg/dl)	0.867	<0.001
TC (mg/dl)	0.875	<0.001
ALT (u/l)	0.237	0.142
AST (u/l)	0.184	0.257
Fatty liver	0.760	<0.001

r, correlation coefficient,BMI:body mass index,SBD:systolic blood pressure,DBP:diastolic blood pressure,WBCS:white blood cells,HDL:high density lipo protein,LDL:low density lipo protein,TG: triglycride TC:total cholesterol,ALT: alanine aminotransferase,AST: aspartate aminotransferase.

microRNA-486 were higher than its levels in healthy controls. Furthermore, its levels significantly correlated with the serum fasting glucose, BMI and HbA1c%. This highlights their importance as one of the metabolic regulators in the pathogenesis of type2 diabetes mellitus and obesity. White adipose tissue is one of the most important sources for several microRNAs in obese children including microRNA-486. MicroRNA-486 and MicroRNA-146b are famous contributors in the development of obesity and enhancement of preadipocyte growth [12].

The current study showed that serum miRNA-486-5p gene expression showed a significant positive correlation with weight/centile, BMI/centile, waist circumference, fat mass, LDL, TG, TC, and significant negative correlation with HDL. These results are in accordance with other studies [5,7] as they stated that (miR-486-5p, -486-3p) were found to be positively correlated with body mass index among other anthropometric measurements, such as waist/hip circumference, fat mass percentage, other clinical variables related to obesity and laboratory variables including circulating lipids. Also *Liu et* al., [15] who stated that miR-486 mimic and miR-486 inhibitor were injected into (THP-1 macrophage-derived foam cells). They found that miR-486 bound directly to histone acetyltransferase-1 (HAT1) 3′UTR, and down-regulated its mRNA and protein expression also it promotes cholesterol accumulation in THP-1 macrophages.

In the present study, the frequency of fatty liver detected by ultrasound was higher in obese and overweight children compared

Table 4
Univariate and multivariate analysis of miRNA-486-5p expression as a predictor of risk of obesity.

Variables		Univariate analysis of miRNA-486-5p expression				Multivariate analysis of miRNA-486-5p expression			
		P	OR	95% CI		*P*	OR	95% CI	
Prediction of obesity susceptibility	Age	0.173	1.065	0.972	1.164				
	Gender	0.831	0.934	0.510	1.718				
	Consanguinity	0.874	0.946	0.481	1.861				
	Positive family history	<0.001	5.909	2.931	11.913	0.377	1.714	0.508	2.983
	miR-486-5p	0.005	4.845	1.645	14.287	0.014	2.943	1.419	4.913
Prediction of obesity severity	Age	0.225	0.941	0.848	1.041				
	Gender	0.766	0.892	0.418	1.894				
	Consanguinity	0.188	0.563	0.235	1.331				
	Positive family history	0.502	0.745	0.316	1.753				
	HDL(mg/dl)	<0.001	0.695	0.567	0.849	0.223	0.830	0.612	1.127
	LDL(mg/dl)	0.036	1.038	1.011	1.081	0.469	1.019	0.966	1.078
	TG(mg/dl)	0.002	1.040	1.015	1.067	0.009	1.026	1.002	1.063
	TC(mg/dl)	0.001	1.076	1.028	1.130	0.326	1.028	0.967	1.095
	miR-486-5p gene expression	0.002	1.694	1.216	2.361	0.028	1.106	1.06	1.608
Prediction of fatty liver susceptibility	Age	0.393	1.038	0.953	1.131				
	Gender	0.720	0.895	0.489	1.639				
	Consanguinity	0.415	0.754	0.382	1.487				
	Positive family history	0.001	2.850	1.536	5.288	0.122	2.302	1.307	3.392
	HDL(mg/dl)	<0.001	0.771	0.685	0.868	0.575	0.937	0.748	1.175
	LDL(mg/dl)	0.888	1.002	0.972	1.033				
	TG(mg/dl)	<0.001	1.036	1.021	1.051	0.162	1.037	0.998	1.078
	TC(mg/dl)	<0.001	1.059	1.032	1.088	0.719	1.690	0.939	1.944
	miR-486-5p gene expression	<0.001	1.476	1.119	2.287	0.006	1.576	1.102	1.928

to the control group. This goes in line with a study by *Kelsey et al.,* [16] who stated that obesity significantly increases the risk for non-alcoholic fatty liver disease, which is defined as the presence of hepatic steatosis in the absence of alcohol use which leads to hepatic inflammation, fibrosis, cirrhosis, and even hepatocellular carcinoma. *Lee* [17] reported that the pathogenic mechanism of non-alcoholic steatohepatitis (NASH) may result from a combination of hyperlipidemia, increased oxidative stress, and insulin resistance. Insulin resistance increases the insulin level which stimulates lipolysis and also fatty acid synthesis in hepatocytes, which increases fatty acid uptake by liver cells and hypertriglyceridemia. This leads to the triglycerides accumulation in the hepatocytes, which stimulates cytochrome P450 2E1 activity and free oxygen radicals generation, causing lipid peroxidation, cytokines, and Fas ligand induction, so the net result is cell death and fibrosis.

In the present study, there was a significant increase in ALT level in the patient group compared to the control group, while there was no significant difference between both groups regarding AST level. These results were supported by a study conducted by *Dokumacioglu et* al., [18] who concluded that obese children displayed significantly higher levels of ALT, compared to the control group. Moreover *Deeb* et al., [19]stated that in obese children, elevated liver enzyme (alanine aminotransferase) has been considered as one of the indicators for non-alcoholic fatty liver disease and its elevation represents an indicator for biopsy. However, significant histological abnormalities of non-alcoholic fatty liver disease, which includes advanced fibrosis can be seen in children with normal or slightly elevated (alanine aminotransferase) levels. So the use of (ALT) alone may underestimate the degree of liver injury.

The current study showed that serum miRNA-486-5P expression showed a significant positive correlation with the presence of fatty liver detected by ultrasound. these results run in accordance with Niculescu et al., [20]as they reported that miR-486 directly regulates Sterol O-Acyltransferase2 and sterol regulatory element-binding protein (SOAT2 and SREBF1), which are two important enzymes involved in the lipid metabolism. Acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2 or SOAT2) is responsible for the cholesteryl esters formation in the liver, which protects against the cytotoxic effect of accumulation of free cholesterol. Therefore, inhibition of SOAT2 may result in cytotoxic accumulation of free cholesterol in the hepatic cells, but can also stimulate excretion of free cholesterol to the bile. The liver and plasma miR-486 levels significantly were increased by induction of a high-fat diet. They have demonstrated that restoring (SOAT2) expression by in vivo inhibition of miR-486 reduces plasma cholesterol levels and hepatic cholesterol content. To the best of our knowledge, no previous studies investigated the correlation of serum microRNA 486-5p expression with fatty liver in obese children.

For prediction of obesity susceptibility logistic regression analysis was conducted and revealed that positive family history of obesity and higher miR-486-5p expression was significantly correlated with the risk of obesity occurrence in univariate analysis and multivariate analysis revealed that only miR-486-5p gene expression was considered as one of the independent predictors for obesity susceptibility. Moreover, for prediction of obesity severity, ordinal regression analysis revealed that higher TG concentration and miR-486-5p gene expression up-regulation were considered as one of the independent risk predictors for more severe obesity in multivariate analysis. Fatty liver susceptibility prediction was detected by logistic regression analysis, which revealed that only miR-486-5p gene expression was considered as one of the independent predictors for fatty liver susceptibility in multivariate analysis. These results come with *Prats-Puig et al.,* [7] who stated that in multiple linear regression models, the combined increase in the circulating levels of (miR-486-5p or miR-486-3p) and miR-142-3p and HOMA-IR, together with the decreased level of miR-28-3p, explains 60% of BMI variability.

7. Conclusion

The serum level of the miR-486-5p gene is up-regulated in obese and overweight children. Up-regulation of miR-486-5p was considered as an independent predictor for obesity and fatty liver susceptibility.

Conflicts of interest

The authors declare that they have no conflict of interest.

Funding

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Statement of contribution

1 M.A: contributed to the design and implementation of the research, aided in choosing the patients and helped shape the research, supervised the findings of this work, discussed the results and contributed to the final manuscript.
2 E. B: contributed to the design and implementation of the research, aided in choosing the patients and helped shape the research, supervised the findings of this work, discussed the results and contributed to the final manuscript.
3 O. B: contributed to the design and implementation of the research, aided in choosing the patients and helped shape the research, supervised the findings of this work, discussed the results and contributed to the final manuscript.
4 H. A: contributed to the design and implementation of the research, aided in choosing the patients and helped shape the research, supervised the findings of this work, discussed the results and contributed to the final manuscript.
5 A. E: contributed to the design and implementation of the research, aided in choosing the patients and helped shape the research, supervised the findings of this work, discussed the results and contributed to the final manuscript.
"All authors have read and approved the manuscript"

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