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BASIC RESEARCH ARTICLE

Methylene Tetrahydrofolate Reductase Gene Polymorphism is Associated with Severity of Liver Steatosis in Chronically Infected Patients with HCV Genotype 4

Reham M. Dawood [1], Eman M. Mahmoud [1], Marwa K. Ibrahim [1], Noha G. Bader El Din [1], Ahmed Aboul-Enein [2], Naglaa Zayed [3], Yasmine S. El Abd [1], Hadeel G. Eldeen [3], Rasha Eletreby [3], Marwa Elsharkawy [4], Moataza Omran [1], Reem El-Shenawy [1], Sayed A. Fayed [2], Mostafa K. El Awady [1]

[1] *Microbial Biotechnology Department, Genetic Engineering Division, National Research Center, El-Tahrir Street, Dokki, 12622 Giza, Egypt*
[2] *Department of Biochemistry, Faculty of Agriculture, Cairo University, Cairo, Egypt*
[3] *Department of Endemic Medicine, Faculty of Medicine, Cairo University, Cairo, Egypt*
[4] *Department of Chemical and Clinical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt*

SUMMARY

Background: **Methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism was reported as a genetic variant in liver steatosis and fibrosis. This is a study of the association between MTHFR C677T polymorphism and HCV core with severity of steatosis in HCV GT4 patients.**
Methods: **111 HCV patients and 112 control subjects were recruited. Polymorphism was detected by RFLP analysis, core Ag was detected by ELISA.**
Results: **Combined HCV infection and MTHFR C677T polymorphism increases the risk to develop steatosis by 3.63- and 5.21-fold in subjects with single (CT) and double (TT) substitutions, respectively. Patients with chronic HCV infection had a 2.88- and 8.57-fold higher risk to develop steatosis in CT and TT genotypes, respectively, than patients with the (CC) genotype. No significant difference in core Ag titers were observed.**
Conclusions: **MTHFR C677T polymorphism is a valuable genetic marker for steatosis, while HCV core Ag titer had no association with grades of steatosis in GT4 infections.**
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Correspondence:
Dr. Yasmine El Abd
Assistant Professor Microbial Biotechnology Department
Genetic Engineering and Biotechnology Division
El Bohous Street, Dokki
National Research Centre
12622 Giza
Egypt
Phone: 00201223405272
Fax: 0020233387758
Email: yasminco@yahoo.com

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KEY WORDS

HCV, MTHFR C677T polymorphism, liver steatosis

INTRODUCTION

Liver steatosis is the accumulation of triglycerides in hepatocytes and is a frequent histological finding of chronic hepatitis C (CHC) infection, where it was detected in 30% - 70% of HCV patients [1]. Liver steatosis is formed due to the imbalance between the fatty acid formation and the impairment of fatty acid removal. The presence of steatosis in chronic HCV infected patients leads to the development of liver fibrosis and ultimately ends up with hepatocellular carcinoma [2]. Epidemiological data revealed that the prevalence of

HCV-related steatosis is 2.5 times higher than non-alcoholic fatty liver disease (NAFLD) and CHC [3]. Steatosis developed in HCV infected patients can be caused due to host metabolic disorders or appeared as a result of the cytopathic effect of HCV genotype 3 [4] which eventually ameliorated after viral clearance. The host metabolic disorders that are commonly implicated with liver steatosis are lipid metabolism disorders and type 2 diabetes mellitus. Several studies provide evidence of the involvement of both viral and host genetic factors in the development of liver steatosis. The methylenetetrahydrofolate reductase (MTHFR) gene polymorphism has been repeatedly associated with liver steatosis besides other host factors as BMI, age, hepatic enzyme levels, lipid profile, blood sugar levels [5]. It is worth mentioning that there has been a remarkable association between the polymorphism C677T and the high levels of plasma homocysteine. MTHFR gene codes for methylenetetrahydrofolate reductase enzyme that reduces 5,10 methylenetetrahydrofolate into 5 methyltetrahydrofolate. The latter is an essential cofactor for the homocysteine methylation. A point mutation in exon 4 (C677T) results in an alanine to valine substitution in the expressed enzyme. This mutation was shown to produce an MTHFR enzyme with reduced activity. The MTHFR dysfunction is accompanied by an increased level of homocystein in plasma [6]. Therefore, patients with C677T polymorphism develop hyperhomocysteinemia that plays a key role in development of steatosis, which in turn accelerates the progression of liver fibrosis in chronic hepatitis C (CHC) [7].
The role of the HCV viral proteins such as core and/or NS5A in inducing steatosis has been investigated. It has been proven that the severity of steatosis is genotype dependant and the high accumulation of fats in the liver has been observed with HCV genotype 3. An *in vitro* study has shown that expressed HCV core protein has the ability to induce liver steatosis in the transgenic mice [8] and it has been reported that HCV core genotype 3 is associated with steatosis severity.
The expression of HCV core protein from different genotypes in Huh7 cell lines were examined and the highest triglycerides accumulation level was noticed with HCV core protein derived from genotype 3. The ultimate mechanism describing the development of steatosis in HCV infected patients is not yet clear. However, there are some reports describing the coexistence of steatosis and HCV infection [9]. It was noted that HCV core protein impairs the microsomal triglyceride transfer protein (MTP) activity which has a direct role in the assembly of very low density lipoprotein and consequently leads to triglycerides accumulation and alteration of lipid metabolism [10,11]. An alternative mechanism is that HCV core protein induces a redox alteration status (oxidative stress) through the upregulation of the reactive oxygen species (ROS) production with a subsequent dysfunction in the antioxidant system. The detection of the viral core protein around the mitochondria reinforces the pro-oxidant role of HCV [12-14]. Accordingly, the aims of the present study were: (1) To study the association of MTHFR 677 C/T polymorphism with different grades of steatosis in chronic HCV genotype-4 patients; (2) To examine the hypothesis that HCV core protein but not viral RNA levels are correlated with the degree of steatosis in the studied cohort.

MATERIALS AND METHODS

This study was conducted on 111 adult HCV (G4) chronically infected naïve patients with various stages of steatosis. Patients were recruited from Kasr El Eini hospital, Faculty of Medicine, Cairo University between December 2013 and December 2014. The enrolled patients were classified into two groups based on the degree of steatosis: 87 patients with varying degrees of steatosis (mild, moderate or severe) and 24 patients without steatosis. Different grades of necro-inflammation, stages of fibrosis, and grades of steatosis were assessed according to Metavir score by an expert pathologist. All enrolled samples were tested for anti-HCV antibody using third generation ELISA Kit (Dia Sorin, Torino, Italy) and for HCV-RNA by real time PCR Artus HCV QS RGQ Kit (Qiagen, Hilden, Germany). Liver enzymes, blood sugar, and lipid profile were measured for all enrolled patients. The BMI was calculated as weight in kg/(height in meters)^2.
The score for homeostasis model assessment of insulin resistance (HOMA IR) was determined. The HCV genotype was determined using the INNO-LiPA HCV assay (Innogenetics, Belguim). Patients with co-morbid conditions (DM, hypertension, dyslipidemia) were excluded. 112 individuals were enrolled as a control group.
For participation in the study, informed written consent was obtained from all enrolled subjects. The study protocol was approved by Ethical Committees of Faculty of Medicine, Cairo University. The study followed the principles of the Helsinki Declaration.

DNA Extraction

Total genomic DNA was extracted from whole blood using the QIA Amp DNA blood mini kit according to the manufacturer's protocol. Purity and quality of DNA were determined by spectrophotometric analysis.

Genotyping of MTHFR C677T polymorphism in exon 4

The polymorphism of MTHFR C677T was detected by Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) assay.
For amplification: forward primer: 5' TCC CTG TGG TCT CTT CAT CC 3', reverse primer: 5' ACT CAG CAC TCC ACC CAG AG 3'. Amplification was performed in a 50 µL reaction mixture containing 20 ng genomic DNA, 200 µM of each dNTPs, 50 pmole from each primer, and 2 units of Taq DNA polymerase (Promega, Madison, WI, USA) in a 1 x buffer supplied with

the enzyme containing 1.5 mM $MgCl_2$. Amplification was performed on a Biometra T1 Thermal Cycler (Biometra, Germany) using the following program: A 95°C incubation for 5 minutes for initial denaturation was followed by 35 cycles at 95°C for 60 seconds, 60°C for 60 seconds, and 72°C for 60 seconds, and a final 10-minute incubation at 72°C. After amplification, PCR products were resolved and visualized in a 2% agarose gel stained with ethidium bromide.

Digestion of the PCR product was carried out using 10 U Hinfl (Promega, Madison, WI, USA) for MTHFR C677T polymorphism. The Hinfl restricted products of MTHFR C677T for genotypes CC, CT and TT had band sizes of (360 bp), (360bp/210bp/150bp) and (210bp/150bp), respectively. Ethidium bromide stained bands were photographed on a photo documentation system (Biometra, Germany).

Detection of HCV core antigen titer by using QuickTiter™ HCV Core Antigen ELISA Kit

The core antigen titer was measured by Cell Biolabs' QuickTiter™ HCV Core Antigen ELISA Kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions.

Core monoclonal Ab was pre-coated onto a microtiter plate. Sera of the recruited patients were added and incubated at 37ºC for 2 hours. The plates were washed and incubated with a FITC-conjugated mouse anti-HCV core antibody at room temperature for 1 hour on an orbital shaker. Following incubation and wash steps, a HRP-conjugated mouse anti-FITC antibody was added and bound to the FITC conjugated anti-HCVcAg provided with the kit. Unbound HRP-conjugated mouse anti-FITC antibody was removed during a wash step, and substrate solution reactive with HRP was added to the wells.

The reaction was stopped by the addition of 2 N H_2SO_4 and absorbance was measured at 450 nm. A standard curve was prepared from recombinant HCV core antigen and sample concentration was then determined.

Statistical analysis

Data management and analysis were performed using Statistical Package for Sciences (SPSS) vs. 21. Numerical data were summarized using means and standard deviation or medians and ranges. Categorical data were summarized as percentages. Comparisons between 2 groups with respect to normally distributed numeric variables were done using the *t*-test. Non-normally distributed numeric variables were compared by Mann-Whitney test. All p-values are two-sided. P-values < 0.05 were considerd significant.

RESULTS

Demographic Data

The study included 56 (51%) males and 55 (49%) females. The median age of the recruited patients was 39.4 ± 8.6 years. The mean of the body mass index (BMI) was 26.6 ± 2.38 kg/m^2. The degree of steatosis was correlated with age (p-value < 0.018), BMI (p-value < 0.003), platelets (p-value < 0.001), albumin (p-value < 0.001), and Hb (p-value < 0.023) but there were no statistically significant differences between different grades of steatosis and serum ALT, AST, WBCs, bilirubin, HCV RNA concentration, and gender. Further statistical analysis showed a strong correlation between steatosis and the progression rate of fibrosis Table 1.

Distribution of genotype frequency of the MTHFR C677T polymorphism among controls and patients

The frequencies of MTHFR 677C→T genotypes (CC, CT, and TT) among chronic HCV patients who have steatosis were 32%, 50%, and 18%, respectively. The corresponding frequencies among controls were 65%, 28%, and 7%, respectively, as shown in Table 2, whereas the corresponding frequencies among patients who have no steatosis were 63%, 33%, and 4%, respectively, as shown in Table 2.

Genotyping of MTHFR and the risk of developing liver steatosis

In comparing the chronic HCV patients with steatosis to control group, the data in Table 3 revealed that the risk of having liver steatosis was 3.63-fold higher in patients who have steatosis with the 677C→T substitution in one allele than in patients without the T substitution (95% CI 1.92 - 6.82; (p-value < 0.001) and 5.21-fold in patients with two alleles (95% CI 2.01 - 13.54; (p-value < 0.001).

On the other hand, when comparing the chronic HCV patients with steatosis to those without steatosis, the data in Table 4 showed that patients with single allele substitution i.e., CT had 2.88-fold more risk to develop steatosis than those having the CC genotype. (95% CI 1.08 - 7.68; p-value is less than or equal to 0.031) while those with 2 allele substitutions i.e., TT have an 8.57-fold higher risk to develop steatosis (95% CI 1.03 - 71.08; p-value is less than or equal to 0.022). Those data reveal the importance of the host genetics in determining the risk to develop steatosis. The risk of having liver steatosis increased by 2.88-fold when the patient had one of mutant (T) allele at nucleotide 677.

Distribution of allele frequency of the MTHFR gene among HCV patients with steatosis and controls: Table 5

a) The 677 (C) allele frequency:

The normal (C) allele frequency of MTHFR at position 677 in 87 chronic HCV patients who have steatosis was 57% (99/174 allele) of the overall (C) allele number of chronic HCV patients who have steatosis, while in con-

Table 1. The clinical characteristics and laboratory findings (n = 111).

Variables		No steatosis	Mild steatosis	Moderate steatosis	Severe steatosis	p-value
Age	mean ± SD	39.417 ± 10.517	46.327 ± 8.867	45.684 ± 9.135	47.167 ± 8.622	0.018 significant
	min max	19 57	21 60	25 59	32 60	
BMI	mean ± SD	26.67 ± 3.965	31.61 ± 7.714	30.231 ± 3.526	26.436 ± 2.380	0.003 significant
	min max	19.71 35.20	22.2 77.02	24 36.78	22.40 29.70	
Gender	male	17	22	11	6	0.067 not significant
	female	7	33	8	7	
Platelets cmm3	mean ± SD	263,100 ± 82,948	204,740 ± 80,342	220,940 ± 92,197	145,170 ± 63,361	0.001 significant
Albumin	mean ± SD	4.31±0.48	4.13±0.49	4.09 ± 0.62	3.56 ± 0.56	0.001 significant
WBCs cmm3	mean ± SD	6,652 ± 2,478	5,748 ± 2,105	7,106 ± 2,146	5,927 ± 1,913	0.128 not significant
ALT	mean ± SD	50.04 ± 35.60	55.12 ± 47.31	54.00 ± 26.48	68.08 ± 58.71	0.709 not significant
AST	mean ± SD	41.70 ± 24.44	49.34 ± 32.74	55.00 ± 41.29	63.50 ± 47.66	0.326 not significant
Hb g/dL	mean ± SD	13.65 ± 1.73	12.81 ± 1.84	13.17 ± 1.54	11.73 ± 1.44	0.023 significant
HCV RNA (IU/mL)	mean ± SD	1257200.00 ± 2243500	1364200.00 ± 2658870.0	8145300.00 ± 25698800	3989600.00 ± 10107600	0.167 not significant
Total bilirubin	mean ± SD	0.69 ± 0.39	0.65 ± 0.39	0.63 ± 0.35	0.65 ± 0.40	0.964 not significant

Data are presented as mean ± SD, $p < 0.05$ is considered significant. Differences between groups were analyzed by analysis of variance (ANOVA).

trols it was 79% (117/224 allele) of the overall (C) allele number of controls.

b) The 677 (T) allele frequency:
The mutant (T) allele frequency of MTHFR at position 677 in 87 chronic HCV patients who have steatosis was 43% (75/174 allele) of the overall (T) allele number of chronic HCV patients who have steatosis, while in controls it was 21% (47/224 allele) of the overall (T) allele number of controls.

Distribution of allele frequency of the MTHFR gene among HCV patients with and without steatosis: Table 6

a) The 677 (C) allele frequency:
The normal (C) allele frequency of MTHFR at position 677 in 87 chronic HCV patients who have steatosis was 57% (99/174 allele) of the overall (C) allele number while in patients who do not have steatosis it was 79% (38/48 allele) of the overall (C) allele number.

b) The 677 (T) allele frequency:
The mutant (T) allele frequency of MTHFR at position 677 in 87 chronic HCV patients who have steatosis was 43% (75/174 allele) of the overall (T) allele number, while in chronic HCV patients that do not have steatosis it was 21% (10/24 allele) of the overall (T) allele number

Relation between HCV core antigen titer and degree of steatosis using HCV core antigen ELISA
We investigated the level of the circulating HCV core protein in the recruited patients.
The data illustrates that there is no significant difference between the core antigen titer and degree of steatosis as shown in Table 7.

Table 2. MTHFR genotype distribution in chronic HCV patients with or without steatosis compared with controls.

Genotype	Chronic HCV infected patients with steatosis (n = 87)	Chronic HCV patients without steatosis (n = 24)	Control Group (n = 112)
CC	28 (32%)	15 (63%)	73 (65%)
CT	43 (50%)	8 (33%)	31 (28%)
TT	16 (18%)	1 (4%)	8 (7%)

Genomic DNA from each subject was analysed for genotyping at nucleotide 677 in exon 4 of the MTHFR gene using PCR-RFLP as in Materials and Methods. The percentage of each genotype was calculated in each patient group and in controls.

Table 3. Shows the relationship between MTHFR 677C→T genotypes among chronic HCV patients who have steatosis and controls and risk of steatosis.

	Chronic HCV patients with steatosis (n = 87)	controls (n = 112)	0dds ratio	95% CI	p-value
CC	(28) 32%	(73) 65%			
CT	(43) 50%	(31) 28%	3.62	1.92 - 6.82	≤ 0.000 significant
TT	(16) 18%	(8) 7%	5.21	2.01 - 13.54	≤ 0.000 significant
CT + TT	(59) 68%	(39) 35%	3.94	2.18 -7.15	≤ 0.000 significant

The risk of having liver steatosis increased by 3.62-fold when the patient had one mutant (T) allele at nucleotide 677.

Table 4. Shows the relationship between MTHFR 677C→T genotypes among chronic HCV patients who have steatosis and chronic HCV patients who do not have steatosis and risk of steatosis.

	Chronic HCV patients with steatosis (n = 87)	Chronic HCV patients without steatosis (n = 24)	0dds ratio	95% CI	p-value
CC	(28) 32%	(15) 63%			
CT	(43) 50%	(8) 33%	2.88	1.08 - 7.68	≤ 0.032 significant
TT	(16) 18%	(1) 4%	8.57	1.03 - 71.08	≤ 0.023 significant
CT + TT	(59) 68%	(9) 37%	3.51	1.37 - 9.00	≤ 0.007 significant

DISCUSSION

Liver steatosis is characterized by the excessive accumulation of fat droplets in hepatocytes. Steatosis has been associated with accelerating fibrosis, low response to the interferon treatment, and the risk of developing HCC [15]. Steatosis in HCV patients is associated with more severe histological injury and higher fibrosis scores, suggesting that fat in the liver is a biologically active tissue.

Core antigen level in HCV genotype 3 infected patients was shown to be associated with a higher degree of steatosis [16]. Whether the core protein of genotype 4 has a similar function is currently unknown. In the current study, the concomitant effects of host (MTHFR polymorphism) and viral (core antigen level) factors on development of steatosis were investigated.

Among the routine investigations of the current patient

Table 5. The number of C and T alleles in exon 4 of the MTHFR gene in 87 chronic HCV infected patients with steatosis and in 112 controls were calculated by the Mann-Whitney *U* test.

Genotype	Allele	HCV patients with steatosis (n = 87)	Controls (n = 112)	z	p-value
		Alleles (%) (n = 174)	Alleles (%) (n = 224)		
at nucleotide 677	C	99 (56.8%)	177 (79%)	-4.742	0.000
	T	75 (43.1%)	47 (20.9%)		

The allele distribution among the total number of studied chromosomes reveals the frequencies of C and T alleles in chronic HCV patients with steatosis compared to controls.

Table 6. The table illustrates the numbers of C and T alleles in exon 4 of the MTHFR gene in 87 chronic HCV infected patients with steatosis and in 24 HCV patients without steatosis calculated by the Mann-Whitney *U* test.

Genotype	Allele	HCV patients with steatosis (n = 87)	HCV patients without steatosis (n = 24)	z	p-value
		Alleles (%) (n = 174)	Alleles (%) (n = 48)		
at nucleotide 677	C	99 (56.8%)	38 (79.1%)	-2.804.	0.005
	T	75 (43.1%)	10 (20 %)		

The allele distribution among the total number of studied chromosomes shows the frequencies of C and T alleles in chronic HCV patients with and without steatosis.

Table 7. Detection of the circulating HCV core protein in chronic HCV infected patients with different grades of steatosis.

Variables	No steatosis	Mild steatosis	Moderate steatosis	Severe steatosis	p-value
Core antigen level	209.21 ± 100.75	155.10 ± 100.48	152.42 ± 72.42	168.46 ± 88.43	0.225 not significant

Differences between groups were analyzed by analysis of variance (ANOVA).

cohort, several host factors were found strongly correlated with the severity of steatosis including age, platelet counts, serum albumin, and hemoglobin concentrations. Of particular interest is the mean BMI value which increased significantly, as expected, with severity of steatosis. The present results confirm the data reported by Hourigan et al. [17] and pave the road towards a therapeutic intervention via reducing the BMI value. The present data confirmed that MTHFR C677T polymorphism strongly contributed to liver steatosis among the studied patient group. The difference between the frequency of mutant (T) allele and the normal (C) allele carriers at nucleotide 677 when comparing controls with patients having HCV/steatosis was significant (p-value equal to 0.000) indicating the strong association between the mutant (T) allele and the risk of developing liver steatosis. Moreover, a significant difference was also observed between allele frequency of T versus C in HCV patients with steatosis when compared with those who have no steatosis (p-value is less than or equal to 0.005) thus confirming the above conclusion that the T allele at position 677 of the human MTHFR gene represents a high-risk host factor for development of steatosis in chronically infected patients with HCV genotype 4. The genotype TT increased the risk of developing steatosis by 5.21-fold whereas the genotype CT increased the risk of developing steatosis by 3.62-fold when compared to controls. These findings are consis-

tent with those reported [18-20]. Adinolfi and his colleagues [21] estimated that heterozygous patients with CT genotype had a 6-fold higher risk, while homozygous TT patients had a 20-fold higher risk to develop steatosis than wild type CC patients.
Since MTHFR C677T polymorphism increases homocystein levels i.e., hyperhomocysteinemia as well as liver steatosis and fibrosis in chronic HCV patients. Toniutto et al. provided an explanation that the activation of the tissue inhibitor metalloproteinase 1 (TIMP1) is a key player in the observed steatosis and fibrosis in chronic HCV infected patients [22].
Toniutto et al. also showed the role of the MTHFR C677T polymorphism in promoting liver fibrosis in recurrent HCV patients after liver transplantation [23]. Studies in a transgenic mouse model [8,24,25], showed that expression of the HCV core and NS5A were associated with severe steatosis. The core protein role in triglycerides (TG) accumulation in mouse hepatocytes is still unclear. Nevertheless, several studies have demonstrated the binding of the core protein with apolipoproteins which are involved in lipid metabolism and consequently leads to fatty liver. Moreover, there was a direct correlation between core protein levels in the liver and grade of steatosis in those transgenic mice. In chronic HCV patients with genotypes 1b and 3, the core protein was detected in the different liver cell types such as stellate, Kupffer, and lymphocytes. The core protein was detected in both cytoplasm as well as the nucleus of the hepatocytes. Besides, it was colocalized with fat droplets describing its role in modulating liver steatosis [26].
The present data failed to show any increase in serum core levels in patients with severe steatosis, thus suggesting a different role of core protein derived from G4 HCV than those originally described in HCV G3 infected patients. Hourioux et al. and others [27,28] reported similar findings.
The lack of association between the level of core protein of genotype 4 HCV and grade of steatosis is further supported in the present cohort by absence of significant association between viral RNA load and the grade of steatosis. This creates a contrast to data obtained from HCV G3 infected patients who showed significant association between viral load and steatosis grade [29,30]. Multiple sequence alignment for the core protein among different genotypes revealed the presence of phenylalanine residue at position 164 specific to genotype 3. The wild type of the HCV G1a core protein as well as genotype 4 carried the tyrosine residue at position 164 [27]. *In vitro* studies revealed that both constructs have shown their capability to associate with lipid droplets. However, the lipid droplet area was found to be larger in the phenylalanine construct transfected cells. Phenylalanine has a higher affinity for lipid droplets than tyrosine.

CONCLUSION

The majority of Egyptian patients chronically infected with HCV genotype 4 frequently develop liver steatosis and the severity of the progression is significantly associated with the presence of the T allele at nucleotide 677 in exon 4 of the MTHFR gene.

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Authors' Contributions:
Study concept and design: Mostafa El Awady, Naglaa Zayed, Reham Dawood. Analysis and interpretation of data: Reham Dawood, Eman Mahmoud, Yasmine El Abd, Marwa Khalil Ibrahim, Noha Bader El Din. Drafting of the manuscript: Reham Dawood. Critical revision of the manuscript for important intellectual content: Mostafa El Awady, Ahmed Aboul-Enein. Sample collection and clinical evaluation: Naglaa Zayed, Rasha Eletreby, Hadeel Gamal Eldeen, Marwa Elsharkawy. Statistical analysis: Yasmine El Abd, Moataza Omran, Reem El-Shenawy.

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Declaration of Interest:
The authors disclose no potential conflicts of interest.

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