

Genetic Polymorphisms in Non-alcoholic Fatty Liver Disease in Obese Egyptian Children

Nehal M. El-Koofy, Hanaa M. El-Karaksy, Iman M. Mandour¹, Ghada M. Anwar,
Mona S. El-Raziky, Ahmad M. El-Hennawy²

Departments of Pediatrics,
¹Chemical Pathology,
²Pathology, Cairo University,
Egypt

Address for correspondence:

Dr. Nehal Mohammed El-Koofy,
Department of Pediatrics, Cairo
University, Egypt.
E-mail: elkoufynehal@yahoo.
com

ABSTRACT

Background/Aim: Polymorphisms in the promoter of microsomal triglyceride transfer protein (MTP) lead to decreased MTP transcription, less export of triglyceride from hepatocytes, and greater intracellular triglyceride accumulation. Therefore, functional polymorphisms in MTP may be involved in determining susceptibility to nonalcoholic steatohepatitis (NASH). The aim of this study is to examine the effect of some genetic influences among a group of obese Egyptian children. **Patients and Methods:** A cross-sectional study was conducted on 76 overweight and obese children presenting to the Pediatric Endocrinology Unit, Cairo University Children's Hospital, Egypt, as well as on 20 healthy controls. Anthropometric measurements were taken for all the patients and they underwent clinical examination, ultrasonographic examination of the liver, and liver biopsy when appropriate. Liver functions, blood glucose, serum insulin, C-peptide, and lipid profile were assessed and HOMA-IR calculated. Blood samples from biopsy-proven NASH patients and controls were analyzed by polymerase chain reaction (PCR) and restriction fragment length polymorphism for the -493 G/T polymorphism in the promoter of MTP and the 1183 T/C polymorphism in the mitochondrial targeting sequence of manganese superoxide dismutase (MnSOD). **Results:** Eight had biopsy-proven simple steatosis and 7 had NASH. NASH patients had a much higher incidence of the MTP G/G genotype ($P = 0.002$, CI: 2.9-392) compared with the controls. NASH patients also had a 100% prevalence of the MnSOD T/T genotype. **Conclusion:** Certain genotypes in MTP and MnSOD are significantly more prevalent among obese children with NASH and may be responsible for such a phenotype.

Key Words: Egyptian children, microsomal triglyceride transfer protein, manganese superoxide dismutase, nonalcoholic steatohepatitis, obesity

Received 28.05.2010, Accepted 17.09.2010

How to cite this article: El-Koofy NM, El-Karaksy HM, Mandour IM, Anwar GM, El-Raziky MS, El-Hennawy AM. Genetic polymorphisms in non-alcoholic fatty liver disease in obese Egyptian children. Saudi J Gastroenterol 2011;17:265-70.

The "two-hit" hypothesis has been elaborated to explain the pathogenesis of nonalcoholic fatty liver disease (NAFLD).^[1] Genetic variation in lipid metabolism is the first hit. One enzyme is the microsomal triglyceride transfer protein (MTP). It regulates synthesis, storage, and export of hepatic triglyceride content. Low levels of MTP result in failure to excrete triacylglycerol from the liver and hepatic steatosis. A genetic variation of the MTP gene affects

the susceptibility for the development of NAFLD.^[2] A common genetic variation of the MTP gene is -493 G/T polymorphism.^[3]

An excessive free fatty acid oxidation leads to oxidative stress causing hepatocyte apoptosis and liver injury is the second hit. Manganese superoxide dismutase (MnSOD) is the main reactive oxygen species (ROS) scavenger in mitochondria. A 1183 T/C polymorphism in the mitochondrial targeting sequence of MnSOD gene leads to a less efficient transport of MnSOD to the mitochondria and increases the susceptibility for the development of NAFLD.^[3,4]

The aim of the current study was to examine the frequency of the MTP gene -493 G/T polymorphism and the 1183 T/C polymorphism in the mitochondrial targeting sequence of MnSOD among biopsy-proven NASH patients.

Access this article online	
Quick Response Code: 	Website: www.saudijgastro.com
	DOI: 10.4103/1319-3767.82582

PATIENTS AND METHODS

This cross-sectional study included 76 children that were referred to the Pediatric Endocrinology Unit at Cairo University Children's Hospital for medical assessment of obesity. The study was approved by the university ethical committee and the patients were enrolled after obtaining an informed consent from their parents.

Patients

Inclusion criteria

Simple obesity, both sexes, age 2–15 years

Exclusion criteria

Patients with known disorders to cause fatty liver (eg, HCV, diabetes, glycogen storage disease, and Wilson's disease), long-term use of drugs known to cause steatosis (eg, glucocorticoids, aspirin) and any case with syndromic obesity.

Controls: Twenty healthy age- and sex-matched control subjects were included for insulin and C-peptide estimation as well as molecular studies.

Methods

All patients were subjected to the following

- Full medical history with special emphasis on nutritional history, drug intake, symptoms of liver disease, diabetes, and family history of liver disease.
- Thorough clinical examination including
 - Abdominal examination with focus on liver examination
 - Anthropometric assessment including the following: Height (Ht), weight (Wt), body mass index (BMI) (Wt in kg and Ht in m²) were plotted on Standard Egyptian growth curves.^[5] In children and adolescents, BMI > 85th percentile is defined as overweight and BMI > 95th percentile is defined as obesity.^[6] Subscapular skin fold thickness was measured by skin fold caliper (Holtain LTD, UK) and was plotted on Egyptian growth curves. Waist circumference (WC) and hip circumference (HC) were measured and waist/hip (W/H) ratio was calculated.^[7] The W/H ratio was considered abnormal if >0.86.^[8]

Ultrasonographic abdominal examination was performed for all the enrolled patients by a single sonographer following not less than 8 h fasting using FFsonic (UF-4100, Fukuda Denshi Co, Tokyo, Japan) UF-4100. Liver echopattern was graded according to Mottin *et al.*^[9]

Percutaneous liver biopsy

Forty-one patients were indicated for liver biopsy: clinical hepatomegaly and/or elevated liver enzymes and/or increased liver echogenicity by ultrasonography. However, it was obtained in 33 patients only due to parental refusal. All patients were examined by a single pathologist.

The main histologic features commonly described in NALFD/NASH, including steatosis, inflammation (portal and lobular), hepatocyte ballooning, and fibrosis, were scored according to the scoring system for NAFLD.^[10]

Laboratory investigations

Sampling

Laboratory investigations were performed in the morning, following a not less than 12-h fasting period. Blood samples were collected as follows: fasting clotted samples for lipid profile and liver functions and EDTA blood for DNA analysis.

Routine laboratory investigations

Serum triglycerides, total cholesterol (total-C), and high-density lipoprotein cholesterol, fasting blood sugar, total bilirubin, direct bilirubin, alanine transaminase, aspartate aminotransferase, alkaline phosphatase (AP), and gamma-glutamyltransferase, were estimated using commercial kits on the synchron CX5 analyzer from Beckman Instruments Inc., California, USA. Low-density cholesterol was calculated using Friedewald's formula.^[11]

Special laboratory investigations

Fasting serum insulin and C-peptide measurement using a commercial solid phase chemiluminescent enzyme immunometric assay (IMMULITE 2000, Diagnostic Products Corp. IL, USA).^[12]

Insulin resistance was calculated for both patients and controls using the following equation:

The homeostasis model assessment method (*HOMA-IR*) = [fasting insulin (μ U/mL) \times fasting glucose (mg/dL)/405].^[13]

PCR-restriction fragment length polymorphism

Determination of MTP and MnSOD genotypes was performed for 8 and 7 patients with histologically proven steatosis and NASH, respectively, as well as for the controls.

Genomic DNA was isolated using the standard salting out technique.^[14] The following primers were used, 5'-GGATTTAAATTTAAACTGTTAATTCATATCAC (forward) 5'-AGTTTCACACATCAAGGACAATCATCTA (reverse) for the MTP polymorphism and 5'-ACCAGCAGGCAGCTGGCGCCCGG-3' (forward) and 5'-GCGTTGATGTGAGGTTCCAG-3' (reverse) for the MnSOD polymorphism. This was followed by HphI and NgoMIV restriction enzymes digestion for MTP

and MnSOD polymorphisms, respectively. Restriction fragments were then separated on 3% agarose gel and sized by comparison to a DNA step ladder Puc 18 (Promega, WI, USA). This restriction digestion, in case of -493 G/T MTP polymorphism gives rise to one full-length fragment of 109 base pairs (bp) for homozygotes for the T variant, two fragments of 89 and 20 bp for homozygotes for the G variant, and three fragments of 109, 89, and 20 bp for G/T heterozygotes. As for the 1183 T/C MnSOD polymorphism 107 bp with C allele (Ala) was digested into two fragments (89 and 18 bp), whereas the PCR product with T allele (Val) cannot be cut by NgoMIV.^[3,15]

Statistical methods

The statistical package for social sciences (SPSS, version 10.0; Chicago, IL, USA) was used for data management and analysis and Microsoft power point for charts. Parametric quantitative data were presented as mean \pm SD and compared by Student's *t* test. Nonparametric quantitative data were expressed as median (range) and compared by Mann-Whitney *U* test. Qualitative data were expressed as frequency and percentage. Risk estimate was done by odds ratio. *P* value was considered significant at 0.05.

RESULTS

Our study included 76 patients: 37 were overweight (17 males and 20 females) and 39 were obese (21 males and 18 females). Their age ranged between 2 and 15 years with a mean of 7.7 ± 3.5 years.

Table 1 shows the comparison of insulin, C-peptide, and HOMA-IR between patients and controls. The patients had higher values of fasting serum insulin, insulin C peptide, and insulin resistance using HOMA ratio.

Five patients (6.5%) had clinical hepatomegaly and 22 patients (29%) had elevated aminotransferases levels. Otherwise, gamma glutamyltranspeptidase (GGT), alkaline phosphatase, serum total protein, albumin, and total bilirubin levels of all the patients were within normal limits. Forty-one patients (54%) had echogenic liver parenchyma by ultrasound.

A percutaneous liver biopsy was obtained in 33 patients: 18 cases (54.6%) had normal liver histology, 8 cases (24.2%) were diagnosed as fatty liver (simple steatosis), and 7 cases (21.2%) as NASH. A significant association between the grade of hepatic echogenicity by ultrasonography and the degree of hepatic steatosis as determined by biopsy ($P < 0.0001$) was found [Table 2]. Similarly, a significant association between insulin resistance and the degree of hepatic steatosis was found ($P < 0.0001$). Six out of 7 children (86%) with NASH had insulin resistance.

Table 1: Comparison between patients and controls with regard to insulin, C-peptide, and HOMA-IR

Variables	Abnormal values in patients N = 76	Patients n = 76	Controls n = 20	P value
Insulin (μ U/mL)	23 (30.3)	14.3 ± 6.0	9.3 ± 4.4	0.0001
C-peptide (ng/mL)	21 (27.6)	1.6 ± 0.8	0.4 ± 0.02	0.0001
HOMA-IR	21 (27.6)	3.2 ± 1.4	1.9 ± 0.9	0.0001

HOMA-IR: Homeostasis model assessment method. Statistically significant difference at $P = 0.05$. Data expressed as mean \pm standard deviation or n (%) as appropriate.

Table 2: Relation between grade of liver echogenicity and result of liver biopsy

Variables	Normal liver echogenicity (n = 35)	Grade I echogenicity (n = 17)	Grade II echogenicity (n = 14)	Grade III echogenicity (n = 10)
Normal liver histology (n = 18)	0	11	6	0
Fatty liver (n = 8)	0	0	5	3
NASH (n = 7)	0	0	0	7

NASH: Nonalcoholic steatohepatitis

The frequency of the polymorphism resulting in a G/T substitution in the sequence of MTP as well as that resulting in a T/C substitution in the MnSOD mitochondrial targeting sequence was studied among the NAFLD patients as well as in the healthy controls [Tables 3 and 4]. As regards MTP (-493 G/T), no T/T genotype was detected in either the NASH or the simple steatosis groups. Combining the MTP G/G and the MnSOD T/T genotypes, a significant risk for developing NASH was obtained when compared with the control group (odds ratio [OR]: 54; 95% confidence interval [CI]: 4.1-707; $P = 0.001$).

Sixty percent of NAFLD patients having a G/G genotype for MTP (-493 G/T) had a high triglyceride level (> 150 mg/dL). A significant association between the G/G genotype and high triglycerides level was found ($P = 0.01$).

DISCUSSION

Childhood obesity is the new pandemic of the new millennium, with significant adverse effects on childhood health. The prevalence of NAFLD is increasing in parallel with the growing proportions of childhood obesity. The prevalence of NAFLD among children is unknown, but some data indicate that 2.6%-9.6% of children have NAFLD, increasing up to 38%-53%

Table 3: Odds ratio of MTP and MnSOD genotype in simple steatosis and healthy controls

Gene	Polymorphisms	Simple steatosis n = 8		Controls n = 20		OR	95% CI	P value
		Frequency	%	Frequency	%			
MTP (-493 G/T)	G/G	5	62.5	3	15	9.4	1.4–62	0.022
	G/T and T/T	3	37.5	17	85			
MnSOD (1183 T/C)	T/T	8	100	9	45	NA	NA	0.9
	T/C and C/C	—	—	11	55			
Combined MTP and MnSOD	G/G and T/T	5	62.5	2	10	15	1.9–115.9	0.009

OR: Odds ratio; CI: Confidence interval; MTP: Microsomal triglyceride transfer protein; MnSOD: Manganese superoxide dismutase; NA: Not applicable

Table 4: Odds ratio of MTP and MnSOD genotype in NASH and healthy controls

Gene	Polymorphisms	NASH (n=7)		Controls 9 (n=200)		OR	95% CI	P value
		Frequency	%	Frequency	%			
MTP (-493 G/T)	G/G	6	85.7	3	15	34	2.9–392	0.002
	G/T and T/T	1	14.3	17	85			
MnSOD (1183 T/C)	T/T	7	100	9	45	NA	NA	0.02
	T/C and C/C	-	-	11	55			
Combined MTP and MnSOD	G/G and T/T	6	85.7	2	10	54	4.1–707	0.001

OR: Odds ratio; CI: confidence interval; MTP: Microsomal triglyceride transfer protein; MnSOD: Manganese superoxide dismutase; NASH: Nonalcoholic steatohepatitis; NA: Not applicable

among obese children.^[16] NAFLD prevalence in this study was 20% (10.5% simple steatosis and 9.2% NASH). This prevalence could have increased if biopsy was performed in all biopsy-indicated patients. Similarly, de Silva *et al.*^[17] reported NASH prevalence of 18% among obese patients. Higher frequencies were reported by other authors.^[18–20]

Insulin resistance was assessed by measuring fasting serum insulin level, glucose, and calculating HOMA-IR. Eighty-six percent of our NASH patients were found to have insulin resistance, which plays a key role in the development of NASH.^[21,22]

The pathogenesis and progress of NASH remain unclear and the most advocated theory is the “two-hit hypothesis.”^[1] Briefly, the first hit is the deposition of fatty acid in hepatocytes triggered by different factors, whereas the second hit is the concomitant liver damage induced by oxidative stress and lipid peroxidation. The fact that NASH is observed only in a fraction of patients with NAFLD suggests genetic predisposition to this disease. Remarkably, the NASH patients included in this study had both an increased frequency of the G allele (92.8%) and of the G/G genotype (85.7%) of MTP, indicating a genetic background favorable for the development of steatosis. This is in accordance with the study by Namikawa *et al.*^[4] who showed an increased frequency of the G allele (91%) and of the G/G genotype (83%). No T/T genotype was detected among our NASH patients. This was also observed in the work of Namikawa *et al.*^[4] who did not find any T/T genotypes among their NASH

patients, but in partial agreement to the work of Gambino *et al.*^[23] who found a 66% G/G frequency among NASH patients and a T/T genotype of 7%.

The frequency of the G/G genotype among the simple steatosis group was significantly higher than that in healthy controls, which makes them more prone to develop NAFLD (OR: 9.4; 95% CI: 1.4–62; $P = 0.022$). The G/G genotype in the MTP promoter would render a patient more susceptible to steatosis. Similar results have been described by Bernard *et al.*^[2] who demonstrated that the MTP G allele confers genetic susceptibility to liver steatosis in patients with type 2 diabetes.

The impact of the G/G genotype as a susceptibility factor to steatosis is proved by the fact that 100% of NASH patients carrying the G/G genotype had elevated triglycerides levels (>150 mg/dL) ($P = 0.01$). These findings are consistent with the current understanding of NASH pathogenesis. The G allele has been previously shown to produce less MTP gene transcription than the T allele.^[2,4] Less MTP activity, in turn, would lead to less triglyceride excretion, and greater accumulation of lipid inside the hepatocytes.

Having dealt with the first hit, analyzing another gene that could possibly confer predisposition to develop a second insult, such as oxidative stress, has been performed. One enzyme that is important in detoxifying mitochondrial ROS is MnSOD. A limited number of polymorphisms have been described for MnSOD, including a T/C polymorphism

in the mitochondrial targeting sequence leading to a valine-to-alanine amino acid change. In turn, this amino acid substitution may alter the helical structure of the mitochondrial targeting sequence, enhancing transport and increased localization of MnSOD into the mitochondrial matrix.^[4,24] This localization pattern may increase the ability of MnSOD to process superoxide anion produced in the mitochondria, and on the contrary, the presence of valine would be associated with increased generation of ROS. In agreement with this hypothesis, a higher frequency of the T/T genotype was observed in our NASH patients in comparison to the control group (100% vs 45%). A higher frequency of the T/T genotype among NASH patients was also observed by Namikawa *et al.*^[4] However, this high frequency observed in our study was not statistically significant. Thus, this group of patients has an increased frequency of genotypes predisposing not only to fatty liver, but also to oxidative stress, one of the factors believed to be involved in severe injury and the development of NASH.

Neither the T/T genotype nor the T alleles of MnSOD (OR: 0.5; 95% CI: 0.3–0.8; $P = 0.9$ and OR: 0.64; 95% CI: 0.5–0.8; $P = 0.2$, respectively) were increased among our simple steatosis patients. Although our results were in accordance with the work of Namikawa and coworkers,^[4] concerning the T allele, they were contradictory as regards the T/T genotype. The T allele was, therefore, not found to impose an increased risk for NASH in the simple steatosis group in the present study.

The exact significance of the polymorphism in the MnSOD gene is still unclear. It is possible that the presence of more MnSOD in the mitochondrial matrix affords a better protection against pro-oxidant stimuli because of a more efficient conversion of superoxide anion.^[3] However, others speculate that under the same conditions, more hydrogen peroxide would be generated, resulting in more severe cell damage. Thus, in many studies investigating genetic polymorphisms it would be important to provide evidence for a genotype–phenotype correlation.^[25]

The combined presence of G/G and T/T genotypes of MTP and MnSOD, respectively, in simple steatosis determines their greater susceptibility to developing NAFLD (OR: 15; 95% CI: 1.9–115.9; $P = 0.009$).

In conclusion, the G/G genotype of MTP may impact NASH by modulating postprandial lipemia and lipoprotein metabolism. Homozygous G/G carriers have a more atherogenic lipid profile than other genotypes. This may mandate establishment of treatment modalities for carriers of this haplotype to prevent progression to NASH.

REFERENCES

- Day CP, James OF. Steatohepatitis: A tale of two 'hits'? *Gastroenterology* 1998;114:842-4.
- Bernard S, Touzet S, Personne I, Lapras V, Bondon PJ, Berthezene F. Association between microsomal triglyceride transfer protein gene polymorphism and the biological features of liver steatosis in patients with type II diabetes. *Diabetologia* 2000;43:995-9.
- Karpe F, Lundahl B, Ehrenborg E, Eriksson P, Hamsten A. A common functional polymorphism in the promoter region of the microsomal triglyceride transfer protein gene influences plasma LDL levels. *Atheroscler Thromb Vasc Biol* 1998;18:756-61.
- Namikawa C, Shu-Ping Z, Vyselaar JR, Nozaki Y, Nemoto Y, Ono M, *et al.* Polymorphisms of microsomal triglyceride transfer protein gene and manganese superoxide dismutase gene in non-alcoholic steatohepatitis. *J Hepatol* 2004;40:781-6.
- Standard Egyptian Growth. Diabetes Endocrine Metabolism Pediatric Unit Cairo University Children's Hospital. Available from: <http://www.dempuegypt.blogspot.com/>. [Last accessed on 2009 Aug 13] [Last revised 2008 Nov 29].
- Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: International survey. *BMJ* 2000;320:1240-3.
- Fernández JR, Redden DT, Pietrobelli A, Allison DB. Waist circumference percentiles in nationally representative samples of African-American, European-American, and Mexican-American children and adolescents. *J Pediatr* 2004;145:439-44.
- Giugliano R, Melo A. Diagnosis of overweight and obesity in school children: Utilization of BMI international standards. *J Pediatr* 2004;80:129-34.
- Mottin CC, Moretto M, Padoin AV. The role of ultrasound in the diagnosis of hepatic steatosis in morbidly obese patients. *Obes Surg* 2004;14:635-7.
- Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313-21.
- Freidewald WT, Levy RJ, Fredrickson DS. Estimation of concentration of LDL-C in plasma without use of the preparative ultracentrifuge. *Clin Chem* 1972;8:499.
- Wiedmeyer HM, Polonsky KS, Myers GL, Little RR, Greenbaum CJ, Goldstein DE, *et al.* International Comparison of C-Peptide Measurements. *Clin Chem* 2007;53:784-7.
- Yokoyama H, Emoto M, Fujiwara S, Motoyama K, Morioka T, Komatsu M, *et al.* Quantitative insulin sensitivity check index and the reciprocal index of homeostasis model assessment in normal range weight and moderately obese type 2 diabetic patients. *Diabetes Care* 2003;26:2426-32.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: A laboratory manual*. Vol. 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989. p. 1-62.
- Cai Q, Shu XO, Wen W, Cheng JR, Dai Q, Gao YT, *et al.* Genetic polymorphism in the manganese superoxide dismutase gene, antioxidant intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study. *Breast Cancer Res* 2004;6:R647-55.
- Angulo P. GI Epidemiology: Nonalcoholic fatty liver disease. *Aliment Pharmacol Ther* 2007;25:883-9.
- De Silva KS, Wickramasinghe VP, Gooneratne IN. Metabolic consequences of childhood obesity—a preliminary report. *Ceylon Med J* 2006;51:105-9.
- Rashid M, Roberts EA. Non alcoholic steatohepatitis in children. *J Pediatr Gastro Nutr* 2000;30:48-53.

19. Xanthokos S, Miles L, Bucuvalas J, Daniels S, Garcia V, Inge T. Histologic spectrum of nonalcoholic fatty liver disease in morbidly obese adolescents. *Clin Gastroenterol Hepatol* 2006;4:226-32.
20. Sagi R, Reif S, Neuman G, Webb M, Phillip M, Shalitin S. Nonalcoholic fatty liver disease in overweight children and adolescents. *Acta Paediatr* 2007;96:1209-1.
21. Dixon JB, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: Predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology* 2001;121:91-100.
22. Finucane FM, Teong L, Pittock S, Fallon M, Hatunic M, Costigan C, *et al.* Adverse metabolic profiles in a cohort of obese Irish children. *Ann Clin Biochem* 2008;45:206-9.
23. Gambino R, Cassader M, Pagano G, Durazzo M, Musso G. Polymorphism in microsomal triglyceride transfer protein: A link between liver disease and atherogenic postprandial lipid profile in NASH? *Hepatology* 2007;45:1097-107.
24. Stewart SF, Leathart JB, Chen Y, Daly AK, Rolla R, Vay D. Valine-alanine manganese superoxide dismutase polymorphism is not associated with alcohol-induced oxidative stress or liver fibrosis. *Hepatology* 2002;36:1355-60.
25. Marra F. NASH: Are genes blowing the hits? *J Hepatol* 2004;40:853-6.

Source of Support: Nil, **Conflict of Interest:** None declared.

Author Help: Reference checking facility

The manuscript system (www.journalonweb.com) allows the authors to check and verify the accuracy and style of references. The tool checks the references with PubMed as per a predefined style. Authors are encouraged to use this facility, before submitting articles to the journal.

- The style as well as bibliographic elements should be 100% accurate, to help get the references verified from the system. Even a single spelling error or addition of issue number/month of publication will lead to an error when verifying the reference.
- Example of a correct style
Sheahan P, O'leary G, Lee G, Fitzgibbon J. Cystic cervical metastases: Incidence and diagnosis using fine needle aspiration biopsy. *Otolaryngol Head Neck Surg* 2002;127:294-8.
- Only the references from journals indexed in PubMed will be checked.
- Enter each reference in new line, without a serial number.
- Add up to a maximum of 15 references at a time.
- If the reference is correct for its bibliographic elements and punctuations, it will be shown as CORRECT and a link to the correct article in PubMed will be given.
- If any of the bibliographic elements are missing, incorrect or extra (such as issue number), it will be shown as INCORRECT and link to possible articles in PubMed will be given.