INTERNATIONAL JOURNAL OF BIOASSAYS
ISSN: 2278-778X
CODEN: IJBNHY
ORIGINAL RESEARCH ARTICLE
OPEN ACCESS

Association of Tumor Necrosis Factor-alpha and Interleukin-6 Gene Polymorphisms with Preeclampsia

Madhavi Puppala[1]*, V. Lakshmi Kalpana[1], Anuradha A[1], M. Sushma[2], G. Sudhakar[1], Sunil Kumar Polipalli[3]

[1]Department of Human Genetics, Andhra University, Visakhapatnam, Andhra Pradesh, India.
[2]Department of Obstetrics & Gynaecology, King George Hospital, Visakhapatnam, Andhra Pradesh, India.
[3]Cytogeneticist, Pediatrics Research & Genetic Lab, Department of Pediatrics, Lok Nayak Hospital, New Delhi, India.

Received for publication: October 05, 2015; **Revised**: October 24, 2015; **Accepted**: November 27, 2015

Abstract: Preeclampsia is a pregnancy-specific syndrome that may be dangerous especially to the fetus. Different cytokines have been found to be elevated in women with preeclampsia and may have possible roles in the development of this disorder. Alleles of the interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) genes are associated with preeclampsia in several studies in different populations. The aim of the present study was to investigate the relationship between IL-6 (G174C) and TNF-α (G-308A) gene polymorphisms with preeclampsia in North Coastal Andhra Pradesh of India. The *TNFA* (-308 A/G) and IL6 (-674G/C) genotypes were determined in 100 preeclamptic women and 100 normal pregnant women as control group, using allele-specific oligonucleotides-polymerase chain reaction method. Data was analyzed using chi-square and Fisher's exact tests. TNF-α (G-308A) G/G genotype showed a significantly higher frequency among the preeclamptic group than the control group (odds ratio, 0.4603, 95% confidence interval, (0.2521- 0.8405); P = .005). G/A genotype also showed higher frequency among the preeclamptic group compared to control group (odds ratio, 2.508, 95% confidence interval, ((1.341-4.689); P = .001). IL-6 (G174C) genotype significantly higher frequency among the preeclamptic group than the control group (odds ratio, 0.4603, 95% confidence interval, (0.2521-0.8405); P = .005). The present study might suggest a role for TNF-α (G-308A) and -174 GC of IL-6 genotype in the development of preeclampsia; suggesting that they are of differing genetic predisposition/pathophysiology.

Key words: Gene Polymorphism; Interleukin-6; Preeclampsia; Tumor Necrosis Factor-α

Introduction

Pre-eclampsia(PE) belongs to a group of hypertensive disorders in pregnancy, most frequently encountered medical complication, causing considerable maternal and fetal morbidity and mortality (1). Preeclampsia affects approximately 5% of all pregnancies and in up to 10% to 20% of nulliparous women (2). The biological factors that determine the progression of PE to E are unknown. General endothelial cell activation is present in both conditions, and seems to be related to an impaired maternal immune response. (3)

Cytokines play a major role in a number of biological activities including proliferation, development, homeostasis, regeneration, repair and inflammation. (4) Synthesis profiles of cytokines can be considered as either T helper cell type 1 (Th1) responses, promoting cell-mediated immunity and interleukin-2 (IL-2) and interferon gamma (IFN-γ), or Th-2 responses, promoting humoral immunity (IL-4, IL-5, IL-6, IL-10) and Th-3 subset characterized by the transforming growth factor beta (TGF-ß). (5)

Cytokine gene polymorphisms have been associated with certain inflammatory and infectious diseases, including some obstetric disorders. (4-7) Polymorphisms likely to occur in regulatory regions of cytokine genes may not only increase susceptibility to some infectious diseases, but also influence the course and prognosis of the disease. (5) Many studies have evaluated the putative relevance of cytokine gene polymorphism in the pathogenesis of preeclampsia (6,7); however, the effect of genetic factors in the pathogenesis of preeclampsia remains unclear. (8-12) The results reported by different investigators may in part be controversial because of selection criteria. Genotype frequencies of SNPs and linkage disequilibrium patterns can differ among ethnic groups, leading to different results. (13)

The TNF-α gene is positioned within the highly polymorphic major histocompatibility complex (MHC) region on chromosome 6p21.3. TNF-α is essential in the instrumentation of the cytokine cascade, and it is a therapeutic target in many inflammatory diseases. (14) As pregnancy develops, high TNF-α concentration have been related to the development of preeclampsia. It embodies many polymorphisms including microsatellites and single nucleotide polymorphisms (SNPs). There are many SNPs within the TNF- α gene promoter. The -308 G/A SNP has been the most studied polymorphism. (15).

***Corresponding Author:**
Madhavi Puppala
Research Scholar,
Department of Human Genetics,
Andhra Univeristy, Visakhapatnam,
Andhra Pradesh-530003, India.

Interleukin-6 (IL-6) is a multifunctional pleiotropic cytokine with pro- and anti-inflammatory actions, plays an important role in the hemostatic and immune systems. (15) While, some studies found elevated IL-6 in PE, others found no association with the disease. (16-18) In humans, the IL-6 gene is located on short arm of chromosome 7 (7q21). It encodes for the proinflammatory cytokine, IL-6, secreted mainly by neutrophils, granulocytes, and macrophage. The IL-6 is the main stimulant of the acute phase response, it stimulates T lymphocytes, differentiation of B lymphocytes, and the production of C reactive protein (CRP). (19-21) Various polymorphisms in the promoter region of the IL-6 gene was reported to influence IL-6 transcription. (22-23).

Considering that cytokine gene polymorphism may influence disease susceptibility and severity our study was designed to investigate whether polymorphisms in genes, TNF-α promoter (-308 G>A), IL6 promoter (-174 G>C), are associated with PE in North coastal Andhra Pradesh, India.

Materials and Methods

The present study included 100 Preeclampsia Women and 100 Healthy Pregnant Women as controls who belongs to North Coastal Andhra Pradesh. The patients who attended the Out Patient Department (OPD) of Obstetrics and Gynecology Unit of King George Hospital and as well as the admitted in the antenatal and postnatal wards of the hospital were enrolled, using the predesigned questionnaire relevant information pertaining to Age, Weight, Height, BMI, Family History, Number of Pregnancies, Type of marriage, Blood Pressure, diabetes from the subjects and the patient information sheet and consent form were taken before collecting the blood samples. The inclusion and exclusion criteria for recruiting cases and controls were also postulated in order to have appropriate subjects for the present study. The study was approved by the institutional ethical Committee and the work has been performed at Department of Human Genetics, Andhra University.

Sample collection and analysis

We isolated DNA from a 3-mL whole blood sample of all the participants using the standard method of salting out. (24) Two polymorphisms were studied: the - 308G/A polymorphism in the promoter region of the TNF- α gene (rs1800629), and the - 174G/C (polymorphism in the 5' flanking region of the IL-6 gene (rs1800795).

Polymorphism of TNF-α gene (G-308A): The polymorphisms of TNF-α gene (G-308A) in the promoter region were screened by polymerase chain reaction (PCR)-based methods. We used the following primer sequences, for 1st round of PCR: common forward primer F: 5'-CTGCATCCCCGTCTTTCTCC -3', reverse primer R 1:5'-ATAGGTTTTGAGGGGCATCG-3' and the second round of PCR with common forward primer and reverse primer R2:5'-ATAGGTTTTGAGGGGCATCA-3'.

Amplification of fragments from the genomic region containing the SNP was performed in PCR assay. Each 25µl of PCR mixture contained 100ng of genomic DNA, 0.5U of Taq DNA polymerase (NEB), 1x PCR reaction buffer (NEB), 0.2mM each dNTP (Merck), 0.5µM each primer. Reactions were carried out in a thermal cycler (Biorad, USA) consistent with the following scheme: 95°C for 80 sec, 35 cycles at 95 for 60 sec, 55 °C for 80 sec, 72°C for 60 sec and 72 for 5 min. Aliquots of the PCR products were analyzed on 2% agarose gel stained with ethidium bromide to verify the proper amplification of the fragments.

Polymorphism of IL6 (174G/C): For IL- 6-174G/C SNP (rs 1800795), DNA was amplified using a common forward primer F: 5'-GAG CTT CTC TTT CGT TCC -3', reverse primer R1: 5'-CCT AGT TGT GTC TTG CC -3' and R2: 5'-CCC TAG TTG TGT CTT GCG -3'. A reagent control without DNA served as negative control. With the following PCR conditions, 25µl of PCR mixture contained 100ng of genomic DNA, 0.5U of Taq DNA polymerase (NEB), 1x PCR reaction buffer (NEB), 0.2 mM each dNTP (Merck), 0.5µM each primer. Reactions were carried out in a thermal cycler (Biorad, USA) consistent with the following scheme: 94°C for 3 min, 30 cycles at 94 for 30 sec, 54 °C for 1 min, 72°C for 1 min and 72 for 7 min. Aliquots of the PCR products were analyzed on 2 % agarose gel stained with ethidium bromide to verify the proper amplification of the fragments.

Statistical analysis:

Genotype distribution in the control and case groups were compared with values predicted by Hardy-Weinberg equilibrium analyses were performed using Fisher's exact and chi square tests. The results were considered to be significant when the *p*-value was less than 0.05. Odd ratios (OR) and their 95% confidence intervals were used to measure the strength of association between IL-6 and TNF-α gene polymorphism and preeclampsia.

Results

In this study a total of 100 women with preeclampsia and 100 normal controls were analyzed for carrying TNF-α (G-308A) and IL- 6-174 C/G polymorphisms, the frequencies of TNF-α and IL-6 genotypes are summarized in Table 1 & 2. As shown in the table, TNF-α (G-308A) G/G genotype showed a significantly higher

frequency among the preeclamptic group than the control group (odds ratio, 0.4603, 95% confidence interval, (0.2521- 0.8405); *P* = .005). G/A genotype also showed higher frequency among the preeclamptic group compared to control group (odds ratio, 2.508, 95% confidence interval, ((1.341-4.689); *P* = .001).

Table 1: Genotypic frequencies of TNF-α (308G>A) and IL-6 (G174C) in Preeclampsia Patients and Controls.

Genotypes	Preeclampsia Patients		Controls		ODDS RATIO (IC 95%)	p value
	n (100)	Frequency	n (100)	Frequency		
TNF-α (308G>A)						
G/G	58	60.84 %	75	80.1%	0.4603 (0.2521- 0.8405)	0.005*
G/A	40	34.32 %	21	18.8%	2.508 (1.341-4.689)	0.001*
A/A	2	4.84 %	4	1.1%	0.4898 (0.08767-2.737)	0.20 NS
IL-6 (G174C)						
G/G	68	63.2%	78	73.96%	0.5994 (0.3183-1.128)	0.055*
G/C	23	32.6%	16	24.08%	1.568 (0.7718- 3.186)	0.106 NS
C/C	09	4.2%	06	1.96%	1.549 (0.5302- 4.528)	0.210 NS

n = number of individuals; p value = probability value of the statistical test,
* = significant (p value <0.05), NS= not significant

Table 2. Allelic frequencies of TNF-α (308G>A) in Preeclampsia Patients and Controls**.**

1.	Preeclampsia Patients		Controls		p value
	n (100)	Frequency	n (100)	Frequency	
		TNF-α (308G>A)			
G	156	78%	171	85.5%	
A	44	22%	29	14.5%	0.052*
		IL-6 (G174C)			
G	159	79.55%	172	86%	0.085NS
C	41	20.5%	28	14%	

n = number of alleles, p value = probability value of the statistical test, * = significant (p value <0.05; NS =not significant

The distribution of IL-6 (-174 GC) G/G genotype showed a significantly higher frequency among the preeclamptic group than the control group (odds ratio, 0.5994, 95% confidence interval, (0.3183, 1.128); *P* = .005).

Discussion

Hypertension is one of the most common medical conditions complicating pregnancy, with significant implications on maternal and perinatal morbidity and mortality. However, the etiology of this complex health problem remains undetermined. Impaired maternal immune tolerance plays a major role in the pathogenesis of PE. In addition, the role of genetic predisposition is well-recognized but not yet defined. (25,26)

Several genetic polymorphisms in cytokines have already been shown by Rinehart *et al.,* in pre-eclampsia. Increased expression of TNF-α, IL-6 was demonstrated in preeclamptic patients' placentas. This event may be due to decreased oxygenation of placenta and may be the result of endothelial dysfunction observed in preeclampsia. (27) Both TNF-α (-308), and IL-6 (-174) genes are associated with different signs of metabolic syndrome, (27,28) which has also been implicated in the pathogenesis of PE.

The results of earlier studies on the effects of the TNFa (-308) polymorphism on the risk of PE are controversial. Saarela *et al., (*29) found that the mutant A allele was associated with susceptibility to PE in Finish women, but there were no statistically significant differences in the genotype distributions between PE and control patients.

The results of study of Mirahmadian M *et al.,* also showed that TNF- α (G308A) A allele and-238 G allele frequencies are significantly elevated in preeclamptic patients compared to those of the control group. (30) In another study, Vural P *et al.,* showed no notable differences were observed in allele or genotype frequencies for TNF- α (G308A) and IL-6 (-174) genes between preeclamptic patients and controls. (31)

The polymorphism of TNF- α (G308A) gene at the position of G308A in the promoter region was reported to be associated with elevated TNF- α levels and a number of infectious and metabolic diseases. (32,33) Chen YP *et al.,* showed that maternal A allele of TNF- α (G308A) promoter region at position -308 could play a role in the alteration of blood pressure and might augment urinary protein excretion during pregnancy. They concluded that TNF- α (G308A) promoter region at position-308 might play an important role in the development of both gestational hypertension and preeclampsia. (34) Galbraith *et al.,* demonstrated an association between the two polymorphisms in the promoter region of the TNF- α (G308A) gene and preeclampsia. (35)

In the present study our data support a role for polymorphism of IL-6 gene at position 174C for G/G genotype the risk of pre-eclampsia and severity was in corroboration with previous studies associated with insulin resistance and dyslipidemia. (19,20). The polymorphism of TNF- α gene at the position of G308A in the promoter region was reported to be associated with elevated TNF- α levels and a number of infectious and metabolic diseases. (34,36) Our study also showed an association between the promoter region of the TNF- α gene and preeclampsia vs controls as

previous study by Haggert CL *et al.,* examined cytokine genotypes among 150 primiparous preeclamptic women. They showed that preeclamptic white women had upregulated TNF-α –308 A/A genotype. (35)

The differences between populations suggest that ethnicity plays an important role in susceptibility to pre-eclampsia. Our study group was small and has a high frequency of consanguineous marriage. Thus, we expect that our group gene pool is homogenous; and the allele's frequencies are low. Therefore, the small sample size recruited in the current study reflects the genotype and allele frequency.

The conflicting outcomes of preeclampsia genetic association studies may be attributed to differences in genetic background and gene environment interactions among various populations. Therefore, the present results cannot be considered contradictory to some of the previous studies as there is considerable ethnic variability in each of the studied polymorphic loci. The present data add to the importance of ethnic as well as intra-regional variability in such studies concerning multifactorial disorders including preeclampsia.

Our findings regarding the two investigated polymorphisms and their associations with preeclampsia can be cautiously concluded that: TNF-α (G-308A) and IL-6 (G174C) polymorphism can be considered as a marker of susceptibility to preeclampsia in North Coastal Andhra Pradesh women, Southern India.

References

1. Mihu D, Sabau L, Costin N, Ciortea R, Oancea M, Malutan A. Evaluation of Leukocytes and Neutrophils Markers of Inflammatory Syndrom in Preeclampsia. Applied Medical Informatics 27 (2010): 27:15-22.

2. Faas M. M, Schuiling G. A, Linton E. A, Sargent IL, Redman C. W. Activation of peripheral leukocytes in rat pregnancy and experimental preeclampsia. Am Journal Obstet Gynecol 182: 2 (2000):351-7.

3. Bidwell J, Keen L, Gallagher G, Kimberly R, Huizinga T, McDermott M. F, Oksenberg J, McNicholl J, Pociot F, Hardt C, D'Alfonso S. Cytokine gene polymorphism in human disease: on line databases. Genes Immun 1 (2001) :3–19.

4. Daher S, Shulzhenko N, Morgun A, Mattar R, Rampim G. F, Camano L, DeLima M. G. Associations between cytokine gene polymorphisms and recurrent pregnancy loss. J Reprod Immunol 58 (2003)69–77.

5. Speer E. M, Gentile D. A, Zeevi A, Pillage G, Huo D, Skoner D. P. Role of single nucleotide polymorphisms of cytokine genes in spontaneous preterm delivery. Hum Immunol 67 (2006): 915-923.

6. Choi Y. K, Kwak-kim J. Cytokine gene polymorphisms in recurrent spontaneous abortions: a comprehensive review. Am J Reprod Immunol 60 (2008): 91–110.

7. Daher S, Sass N, Oliveira L. G, Mattar R. Cytokine genotyping in preeclampsia. Am J Reprod Immunol 55 (2006):130–135.

8. Canto-Cetina T, Canizales-Quinteros S, Me´ndez R, Patricia C. Analysis of C-850T and G-308A polymorphisms of the tumor necrosis factor-a gene in maya-mestizo women with preeclampsia. Hypertens Pregnancy 26 (2007) :283–291.

9. Stonek F, Hafner E, Metzenbauer M, Katharina S, Stu¨mpflen I, Schneeberger C, Zeisler H, Husslein P, Philipp K. Absence of an association of tumor necrosis factor (TNF)- alpha G308A, interleukin-6 (IL-6) G174C and interleukin-10 (IL-10) G1082A polymorphism in women with preeclampsia. Am J Reprod Immunol 77 (2008): 85–90.

10. Mirahmadian M, Kalantar F, Heidari G, Safadarin L, Mansouri R, Amirzargar AA. Association of tumor necrosis factor-alfa and interleukin-10 gene polymorphisms in Iranian patients with pre-eclampsia. Am J Reprod Immunol 60 (2008): 179–185.

11. Molvarec A, Jermendy A, Kova´cs M, Proha´szka Z, Rigo´ Jr J. Toll-like receptor 4 gene polymorphisms and preeclampsia: lack of association in a Caucasian population. Hypertens Res 31 (2008): 859–864.

12. Vatten L. J, Skjaerven R. Is pre-eclampsia more than one disease? BJOG 111:4 (2004): 298-302.

13. Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. Am J Obstet Gynecol 183: 1 (2000): S1-S22.

14. Benyo D. F, Adatisa P, Conrad K. P. Levels of inflammatory cytokines in normal term and preeclamptic placentas and their regulation by oxygen. Journal Soc Gynecol Investig (2000); (Suppl 7).

15. Hahn A. B, Kasten-Jolly J. C, Constantino D. M, *et al.,* TNFalpha, IL-6, IFN-gamma, and IL-10 gene expression polymorphisms and the IL-4 receptor alpha-chain variant Q576R: effects on renal allograft outcome. Transplantation. 72 (2001):660-5.

16. Awad M. R, El-Gamel A, Hasleton P, Turner D. M, Sinnott P. J, Hutchinson I. V. Genotypic variation in the transforming growth factor-beta1 gene: association with transforming growth factor-beta1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. Transplantation. 66 (1998): 1014-20.

17. Jacob C. O, Fronek Z, Lewis G. D, Koo M, Hansen J. A, McDevitt H. O. Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor alpha: relevance to genetic predisposition to systemic lupus erythematosus. Proc Natl Acad Sci U S A. 87 (1990):1233-7.

18. Brinkman B. M, Giphart M. J, Verhoef A, *et al.,* Tumor necrosis factor alpha-308 gene variants in relation to major histocompatibility complex alleles and Felty's syndrome. Hum Immunol. 41 (1994): 259-66.

19. Mayer F. R, Messer G, Knabe W, Mempel W, Meurer M, Kolb H. J, Holler E. High response of TNF secretion in vivo in patients undergoing BMT may be associated with the 308 bp TNF-α gene enhancer polymorphism [abstract]. Bone Marrow Transplant. 17 (1996): s101.

20. Azzawi M, Hasleton P. S, Turner D. M, *et al.,* Tumor necrosis factor-alpha gene polymorphism and death due to acute cellular rejection in a subgroup of heart transplant recipients. Hum Immunol. 62 (2001)140-2.

21. 21.Laresgoiti-Servitje E, Gomez-Lopez N, Olson D. M. An immunological insight into the origins of pre-eclampsia. *Hum Reprod Update* 16 *(*2010): 510-524.

22. Laresgoiti-Servitje E, Gomez-Lopez N. The pathophysiology of preeclampsia involves altered levels of angiogenic factors promoted by hypoxia and autoantibody-mediated mechanisms. *Biol Reprod (*2012) 87: 36.

23. Arosio B, Trabattoni D, Galimberti L, Bucciarelli P, Fasano F, Calabresi C, *et al.*, Interleukin-10 and interleukin-6 gene polymorphisms as risk factors for Alzheimer's disease. *Neurobiol Aging* 25*(*2004) :1009-1015.

24. Miller S. A, Dykes D. D, Polesky H. F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:3 (1988): 1215.

25. Skjaerven R, Vatten L. J, Wilcox A. J, Ronning T, Irgens L. M, Lie R. T. Recurrence of pre-eclampsia across generations: exploring fetal and maternal genetic components in a population based cohort. *BMJ* 331: *(*2005):877-881.

26. Chappell S, Morgan L. Searching for genetic clues to the causes of pre-eclampsia. *Clin Sci (Lond)* 110 *(2006)*: 443-458.

27. Rinehart B. K, Terrone D. A, Lagoo-Deenadayalan S, Barber W. H, Hale E. A, Martin J. N Jr, *et al.,* Expression of the placental cytokines tumor necrosis factor alpha, interleukin 1beta, and interleukin 10 is increased in preeclampsia. Am Journal Obstet Gynecol 181:4 (1999): 915-20.

28. Mayer F. R, Messer G, Knabe W, Mempel W, Meurer M, Kolb H. J, Holler E. High response of TNF secretion in vivo in patients undergoing BMT may be associated with the 308 bp TNF-α gene enhancer polymorphism [abstract]. Bone Marrow Transplant. 17 (1996): s101.

29. Fujii D, Brissenden J. E, Derynck R, Francke U. Transforming growth factor beta gene maps to human chromosome 19 long arm and to mouse chromosome 7. Somat Cell Mol Genet. 12(1986):281-8.

30. 30.Mirahmadian M, Kalantar F, Heidari G, Safdarian L, Mansouri R, Amirzargar A. A. Association of Tumor Necrosis Factor-Alpha and Interleukin-10 Gene Polymorphisms in Iranian Patients with Pre-eclampsia. Am J Reprod Immunol 60:2 (2008):179-85.

31. Vural P, Degirmencioglu, Saral N. Y, Demirkan A, Akgul C, Yildirim G, *et al.,* Tumor necrosis factor a, interleukin- 6 and interleukin-10 polymorphisms in preeclampsia.j J Obstet Gynaecol Res 36:1 (2010):64-71.

32. 32.Abraham L. J, Kroeger K. M. Impact of the −308 TNF alpha promotor polymorphism on the transcriptional regulation of the TNF alpha gene: relevance to disease. J Leukoc Biol 66:4 (1999):562-6.

33. Pihlajamäki J, Ylinen M, Karhapää P, Vauhkonen I, Laakso M. The effect on the −308A allele of the TNF alpha gene on insulin action is dependent on obesity. Obestet Res 11:7(2003):912-7.

34. Galbraith G. M, Pandey J. P. Tumor necrosis factor alpha (TNF-a) gene polymorphism in alopecia areata. Hum Genet 96:4(1995):433-6.

35. Haggerty C. L, Ferrell R. E, Hubel C. A, Markovic N, Harger G, Ness R. B. Association between allelic variants in cytokine genes and preeclampsia. Am Journal Obstet Gynecol 193:1(2005):209-15.

36. Tosun M. G, Celik H, Avci B, Yavuz E, Alper T, Malatyalio E. Maternal and umbilical serum levels of interleukin-6, interleukin-8, and tumor necrosis factor-a in normal pregnancies and in pregnancies complicated by preeclampsia. J Matern Fetal Neonatal Med 23:8(2010):880-6.

Cite this article as:
Madhavi Puppala, V. Lakshmi Kalpana, Anuradha A, M. Sushma, G. Sudhakar, Sunil Kumar Polipalli. Association of Tumor Necrosis Factor-alpha and Interleukin-6 Gene Polymorphisms with Preeclampsia. *International Journal of Bioassays* 5.2 (2016): 4744-4747.

Source of support: Nil
Conflict of interest: None Declared