ORIGINAL ARTICLE

A significant decrease in the gene expression of interleukin-17 following the administration of synbiotic in patients with allergic rhinitis who underwent immunotherapy: A placebo-controlled clinical trial

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Background: Allergic Rhinitis (AR) is the most common allergic disease worldwide. The present study, evaluated effects of synbiotic on gene expression of interferon-gamma (IFN-γ), interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-17 (IL-17), transforming growth factor beta (TGF-β), and forkhead box P3 (FoxP3) in AR patients who received concomitant immunotherapy in a placebo-controlled clinical trial. **Materials and Methods:** Twenty AR patients were randomized in synbiotic and placebo groups and received cluster immunotherapy for 2 months. RNA was extracted from peripheral PBMCs, then the cDNA synthesized. Subsequently, SYBR Green real-time Reverse transcription polymerase chain reaction technique was employed for studying the expression of mentioned genes. In addition, SNOT-22 and mini-Rhinoconjunctivitis Quality of Life Questionnaire questionnaires were completed by patients. Data were analyzed before and also 2 and 6 months after intervention. **Results:** Clinical symptoms and quality of life were improved with immunotherapy, but there was no significant difference between the placebo and synbiotic groups. Gene expression of IFN-γ, TGF-β, and FoxP3 was increased whereas the gene expression of IL-4 and IL-10 decreased, but not significant. Interestingly, the gene expression of IL-17 in the synbiotic group was significantly decreased versus placebo after 2 months (P = 0.001) and also at the end of intervention (P = 0.0001) comparing with the time zero. **Conclusion:** Significant reduction in the IL-17 gene expression following administration of synbiotic versus placebo shows the importance of synbiotic in control of the immunopathogenesis of AR. Further studies with more samples are recommended. In addition, evaluating the effects of synbiotic in patients who do not undergo immunotherapy is suggested to get a better conclusion.

Key words: Allergic rhinitis, forkhead box P3 transcription factor, immunotherapy, interferon-gamma, interleukin-10, interleukin-17, interleukin-4, synbiotic, transforming growth factor beta

How to cite this article: Dehnavi S, Azad FJ, Hoseini RF, Moazzen N, Tavakkol-Afshari J, Nikpoor AR, *et al.* A significant decrease in the gene expression of interleukin-17 following the administration of synbiotic in patients with allergic rhinitis who underwent immunotherapy: A placebo-controlled clinical trial. J Res Med Sci 2019;24:51.

INTRODUCTION

Allergic diseases have become one of the most important health problems worldwide, and it is thought that 20%–30% of western populations suffer from at least one form of them.[1] Allergic Rhinitis (AR) as the most common allergic diseases has an ascending prevalence worldwide[2,3] that annually billion dollars paid for the treatment of physical and mental problems and disabilities of such patients that imposes a heavy burden on health services.[4,5] General principles of treatment are education and awareness, the avoidance of allergen,

Access this article online

Quick Response Code:

Website: www.jmsjournal.net

DOI: 10.4103/jrms.JRMS_543_18

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Received: 02-08-2018; **Revised:** 29-01-2019; **Accepted:** 12-03-2019

appropriate medicine use, and finally the immunotherapy application.[6-8] Given that complete allergen avoidance is not possible and administrations of current medicines are restricted because of their side effects and also decreased effectiveness following continuous consumption. Therefore, trying to find ways to increase immunotherapy effects and choosing alternative treatments are still one of the issues of interest. Based on hygiene hypothesis and evidence, probiotics or nonpathogenic commensal microorganisms that coexist with the living being, especially human, are beneficial and can be considered as a complementary therapy for allergic diseases. Definition of the Food and Agriculture Organization of the United Nations and the World Health Organization for probiotics is "Live microorganisms that when administrated in sufficient quantities confer a health benefit on the host." Prebiotics are nutrition elements that selectively induce growth and activity of limited numbers of the colon residing bacteria and is consist of indigestible carbohydrates such as inulin, fructooligosaccharides (FOS), galactooligosaccharides, and lactose. The mixture of probiotics and prebiotic called Synbiotic.[9]

The aim of this study was to evaluate the effectiveness of synbiotic on gene expression of interferon-gamma (IFN-γ), interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-17 (IL-17), transforming growth factor beta (TGF-β), and forkhead box P3 (FoxP3) when it administrated simultaneously with immunotherapy in a placebo-controlled, randomized, double-blind clinical trial in AR patients in the city of Mashhad, in North-Eastern Iran.

MATERIALS AND METHODS

Characterization of patients

Twenty AR patients enrolled at the Mashhad University of Medical Sciences, Allergy ward of Ghaem Hospital, Mashhad, North-Eastern Iran. Each participant signed a written informed consent. Patients were considered for the study based on clinical criteria, positive prick test for aeroallergens and established perennial AR. Exclusion criteria comprised pregnancy, history of autoimmune or immunodeficiency disorders, systemic corticosteroids or immunosuppressive medicine usage, suffering from allergic asthma, malnutrition, also infection and antibiotic intake during enrolment.

Study design

The study was designed as a placebo-controlled, randomized, double-blind clinical trial that the protocol was approved by the Ethics Committee of Mashhad University of Medical Sciences. Registration ID in Iranian Registry of Clinical Trail (IRCT) was IRCT2017061223235N11. The patient recruitment occurred from August 2015 to June 2016. The study was designed for 20 patients, ten in the synbiotic group who received synbiotic capsules from "ZistTakhmir" company (www.zisttakhmir.com) and each synbiotic capsule consisted of *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Streptococcus thermophilus* as probiotics and FOS as prebiotic. In addition, ten patients underwent immunotherapy and received placebo. Both groups took the synbiotic and placebo for 2 months. Cluster immunotherapy was performed based on a protocol in Middleton's Allergy book[10] for 2 months in both groups. Clinical symptoms and quality of life evaluated by SNOT-22[11] and mini- Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ)[12] standard questionnaires in time points 0, 2, and 6 months. Meanwhile, 3cc of venous blood was taken in EDTA for RNA extraction from PBMCs and synthesis of cDNA.

Gene expression assessment for interferon-gamma, interleukin-4, interleukin-10, interleukin-17, transforming growth factor beta, and forkhead box P3

RNA was extracted with GeneJET Whole Blood RNA Purification Mini kit (Made by Slovenian Thermo scientific company, Cat No, K0716#), then cDNA synthesis performed by RevertAid First Strand cDNA Synthesis Kit (Thermo scientific company).

The expression of mentioned genes was evaluated by SYBR Green real-time reverse transcription polymerase chain reaction (RT-PCR) technique. Primers designated by Beacon Designer software (7.9 version) and Primer NCBI online software and ordered by Pishgam company Tehran, Iran (www.pishgambc.com). Specificity of primers and product reaction confirmed by sequencing through Bioneer Company in South Korea. Sequences of primers are shown in Table 1.

Master mix provided by Takara Company (Japan [www.takara-bio.com]). The real-time RT-PCR amplifications were performed based on the following condition: for GAPDH, IFN-γ, IL-4, IL-10, TGF-β genes, primary denaturation was started at 95°C for 10 min and followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s and an extension at 72°C for 20 s. The reaction was conducted at the total volume of 10 μL which consisted of 0.4 μL forward primer (10 pmol/μL), 0.4 μL reverse primer (10 pmol/μL), 0.2 μL distilled water, 5 μL master mix, and 4 μL cDNA. For IL-17 and FoxP3 genes, primary denaturation was set up at 95°C for 2 min and followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and an extension at 72°C for 20 s. The reaction was prepared at the total volume of 10 μL which consisted of 0.25 μL forward primer (10 pmol/μL), 0.25 μL reverse primer (10 pmol/μL), 2.5 μL distilled water, 5 μL master mix, and 2 μL cDNA.

Table 1: Sequence of primers for SYBR Green real time-polymerase chain reaction assay

	Forward primer	Reverse primer
GAPDH	5'-CAC TAG GCG CTC ACT GTT CTC-3'	5'-CCA ATA CGA CCA AAT CCG TTG AC-3'
IFN-γ	5'-GAG TGT GGA GAC CAT CAA GGA AG-3'	5'-TGC TTT GCG TTG GAC ATT CAA GTC-3'
IL-4	5'-CCG TAA CAG ACA TCT TTG CTG CC-3'	5'-GAG TGT CCT TCT CAT GGT GGC T-3'
IL-10	5'-TCT CCG AGA TGC CTT CAG CAG A-3'	5'-TCA GAC AAG GCT TGG CAA CCC A-3'
IL-17	5'-CGG ACT GTG ATG GTC AAC CTG A-3'	5'-GCA CTT TGC CTC CCA GAT CAC A-3'
TGF-β	5'-TAC CTG AAC CCG TGT TGC TCT C-3'	5'-GTT GCT GAG GTA TCG CCA GGA A-3'
FoxP3	5'-GGC ACA ATG TCT CCT CCA GAG A-3'	5'-CAG ATG AAG CCT TGG TCA GTG C-3'

GAPDH=Glyceraldehyde-3-phosphate dehydrogenase; IFN-γ=Interferon-gamma; IL=Interleukin; TGF-β=Transforming growth factor beta; FoxP3=Forkhead box P3; SYBR=SYBR Green™

Statistics

Data of gene expression analyzed based on the $2^{-\Delta\Delta Ct}$ method,[13] and GAPDH chose as the house-keeping gene.[14] Statistical analysis such as descriptive statistics, Chi-square, unpaired *t*-test, and one way ANOVA was performed by SPSS software version 16 (SPSS Inc., Chicago, IL, USA) and the $P < 0.05$ was statistically significant.

RESULTS

Twenty AR patients randomized in Immunotherapy + Synbiotic (First group) and Immunotherapy + Placebo (Second group). Despite, lack of any side effects, three patients (two in the synbiotic and one in the placebo group) stopped treatment, and 17 patients (ten males and seven females) finished study procedure. The mean duration of the disease was 7.5 ± 2.3. The mean age of patients was 24 ± 12.82 with a range of 9–53 years. The age distribution was assessed by Kolmogorov–Smirnov test, and it was normal in both groups. Furthermore, the age difference was not significant between the two groups based on the *t*-test ($P = 0.4$). Demographic data were summarized in Table 2.

Table 2: Demographic data of patients with allergic rhinitis

Characteristics	Synbiotic group (*n*=8)	Placebo group (*n*=9)
Sex		
Male	6	4
Female	2	5
Age (year)	21.12±14.29	26.55±11.58

Table 3: Data of standard questionnaires for Sinonasal Outcome Test-22 and mini-Rhinoconjunctivitis Quality of Life Questionnaire

	SEM±mean		
	Before intervention	2 months after intervention	6 months after intervention
SNOT-22			
Synbiotic	44.6±7	16.4±3.6	11.4±3.5
Placebo	43±8.6	19.5±4.9	13.7±3.7
Mini-RQLQ			
Synbiotic	51.4±6.3	16.9±3.7	16±2.5
Placebo	40±6.9	21.3±5.9	16.1±2.9

Scores for severity of disease and quality of life obtained from SNOT22 and mini-RQLQ standard questioners data, respectively. RQLQ=Rhinoconjunctivitis quality of life questionnaire; SEM=Standard error of mean; SNOT-22=Sinonasal Outcome Test-22

The analysis of SNOT-22 standard questionnaire showed a significant reduction in clinical symptoms in both groups with $P = 0.0002$ and $P = 0.005$ for the synbiotic the placebo group, respectively. However, in the comparison between groups, the reduction was higher in the synbiotic group, but not statistically significant ($P = 0.9$). Based on the mini-RQLQ standard questionnaire analysis, quality of life was significantly improved in synbiotic ($P = 0.0001$) and placebo ($P = 0.014$) groups. Although the quality of life showed better improvement in synbiotic group, the difference was not significant ($P = 0.9$). The data of standard questionnaires for SNOT-22 and mini-RQLQ were summarized in Table 3.

The analysis of gene expression showed no significant difference between the two groups for IFN-γ ($P = 0.2$), IL-4 ($P = 0.7$), IL-10 ($P = 0.72$), TGF-β ($P = 0.07$), and FoxP3 ($P = 0.6$) when comparison performed before and 2 months after intervention. However, gene expression of IL-17 in synbiotic group was significantly reduced compared with placebo group, when analyzed before and 2 months after the intervention ($P = 0.001$) [Figure 1].

Similar analysis was performed in 2–6 months interval and showed no significant difference between the two groups with regard to IFN-γ ($P = 0.7$), IL-4 ($P = 0.2$), IL-10 ($P = 0.21$), TGF-β ($P = 0.43$) FoxP3 ($P = 0.16$), and IL-17 genes ($P = 0.48$) [Figure 2].

The analysis of gene expression in 0–6 months interval and comparison between the groups; showed no significant difference with regard to IFN-γ ($P = 0.6$), IL-4 ($P = 0.3$), IL-10 ($P = 0.1$), TGF-β ($P = 0.24$), and FoxP3 genes ($P = 0.73$), whereas the gene expression of IL-17 in synbiotic group was significantly decreased compared with placebo group ($P = 0.0001$) [Figure 3].

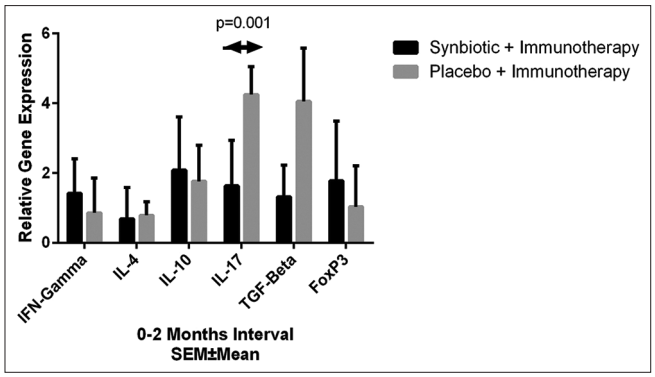

Figure 1: Gene expression of IFN-gamma, IL-4, IL-10, IL-17, TGF-beta, and FoxP3 in patients with allergic rhinitis who received synbiotic or placebo in combination with immunotherapy in time point zero and 2 months following the intervention

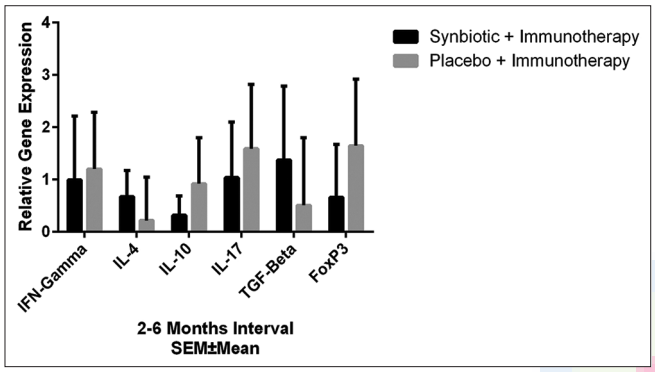

Figure 2: Gene expression of IFN-gamma, IL-4, IL-10, IL-17, TGF-beta, and FoxP3 in patients with allergic rhinitis who received synbiotic or placebo in combination with immunotherapy in time point 2 and 6 months following the intervention

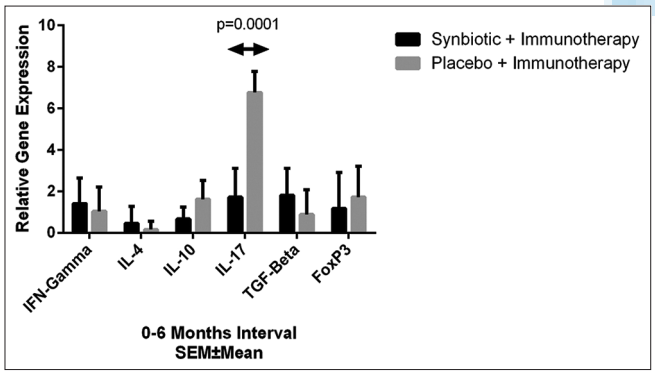

Figure 3: Gene expression of IFN-gamma, IL-4, IL-10, IL-17, TGF-beta, and FoxP3 in patients with allergic rhinitis who received synbiotic or placebo in combination with immunotherapy in time point zero and 6 months following the intervention

DISCUSSION

Annoying symptoms in AR patients are due to the disturbance in protective physiologic functions of the immune system.[15] Based on the evidence, probiotics are one of the alternative treatments for allergic diseases especially AR.[9] The examination of gene expression profile is a powerful tool to diagnosis and differentiate diseases in human; also can clarify the progression or regression and to screening treatment responses. For the first time, in the present study, the expression of related genes to the T-cell subsets was evaluated in patients with AR who received placebo or synbiotic in combination with immunotherapy.

The evaluation of mini-RQLQ and SNOT-22 revealed that quality of life and clinical symptoms of all patients improved significantly regardless of taking synbiotic or placebo. Although the improvement was more in synbiotic group comparing to the placebo, the difference was not significant. Our results are similar to Lin *et al*. and Nembrini *et al*. studies that they also reported significant improvement in the quality of life and clinical symptoms. Lin *et al*. evaluated the effects of *Lactobacillus paracasei* (HF. A00232) in 6–13-year-old children with perennial AR in a 12-week treatment. They measured clinical symptoms and quality of life by nasal total symptom score, eye total symptom score, pediatric RQLQ standard questionnaires following the probiotic administration.[16] In addition, Nembrini *et al*. evaluated modulatory effects of *L. paracasei* NCC2461 on AR patients and measured clinical symptoms and quality of life by total nasal symptom score, total ocular symptom score, and mini-RQLQ standard questionnaires.[7] On the contrary, Nagata *et al*. who evaluated *Lactobacillus plantarum* No. 14 effects on seasonal AR in Japanese students[17] and Costa *et al*. that evaluated efficacy of *L. paracasei* LP-33 in the same disease[18] showed that clinical symptoms improved significantly in the intervention group versus placebo. Meanwhile, Singh *et al*. that evaluated the immunemodulatory effect of *B. lactis* NCC2818 in patients with seasonal AR revealed that nasal symptoms were improved significantly in the synbiotic group.[19]

Previous studies have shown that Th1/Th2 imbalance and subsequently IL-4 and IL-13 secretion by Th2 cells leads to class switching in B lymphocyte and produce allergen-specific IgE in AR patients.[20] Moreover, several modulatory effects considered for probiotics such as inhibition of Th2 differentiation through dendritic cells and also suppression of IL-4, IL-5, and IL-13 cytokines secretion.[21] In this study, gene expression of IL-4 considered as the Th2 main cytokine and our results showed a reduction during the intervention. Although it was more in the synbiotic group in the first 2 months, the differences were not significant. This part of our results was in concordance with Lin *et al*. and Ivory *et al*. studies that evaluated IL-4 cytokine level in serum, nasal mucosal secretions, and supernatants of PBMCs cultures.[16,22] However, significant reduction of IL-5 and IL-13 in studies of Singh *et al*. and IL-13 in Chen *et al*. as Th2 cytokines were seen in synbiotic group versus placebo.[19,23] In addition, Nagata *et al*. reported a significant increase in Th1 population and also in Th1/Th2

ratio following treatment with *Lactobacillus plantarum* No. 14.[17,24]

On the other hand, several studies showed an important role of Th1 and its cytokine, IFN-γ; in the cellular immune responses.[25] Our results showed that the gene expression of IFN-γ increased in the first 2 months of treatment in the synbiotic group, whereas it was decreased in the placebo group. Although the gene expression of IFN-γ was lower in synbiotic receivers, it was not significant. Our results were similar to findings of Ivory *et al*.,[22] who reported that oral administration of *L. casei* significantly increased the level of IFN-γ cytokine production in cultured PBMCs. On the contrary, a significant decrease of IFN-γ as the main cytokine of Th1 subset was shown in Lin *et al*. who evaluated effects of *L. paracasei* (HF. A00232) on serum level of IFN-γ,[16] Chen *et al*. who studied the influence of L. gassei A5 on IFN-γ secretion by PBMCs,[23] Singh *et al*. who assessed IFN-γ secretion of stimulated blood lymphocytes in the presence of *B. lactis* NCC2818[19] and also Lopez *et al*. who detected IFN-γ secretion by T-cells following treatment by *B. bifidum* CMG13195. All these results were not in accordance with our data, too. Notably, in Perrin and colleagues' study, serum level of IFN-γ was shown to be not influenced by *L. paracasei* NCC2461.[24] Controversial results in different studies may be related to differences in treatment protocols, the period of synbiotic intake and also various strains of probiotics and methods to evaluate serum cytokine level or supernatants of cell cultures and gene expression.

Contrary to common belief that AR is merely a result of the imbalance between Th1/Th2 subsets, recently proofs imply on Th17 subset role in AR pathogenesis and these cells were found in nasal mucus of AR patients. Furthermore, the severity of clinical symptoms increases along with serum level of IL-17.[26] In our study, gene expression of IL-17 cytokine in 0–2 and 0–6-month intervals, was significantly decreased in the synbiotic group versus placebo. Similar findings showed in Zhang *et al*. that oral administration of *Enterococcus faecalis* FK-23 as in the murine model of allergy showed a significant reduction in the percentage of Th17 CD4+ cells.[27] Moreover, Owaga *et al*. showed that administration of *Lactobacillus* Gasseri in the murine model of allergic asthma lead to significant reduction of IL-17 serum level in bronchoalveolar liquid and also suppressed significantly IL-17 secretion in splenocytes.[28] These findings are in concordance with other studies that show probiotics lead to directly and indirectly downregulating and suppress Th17 subset and responses and cytokine secretion related to this cytokine.[29,30]

On the other hand, modulatory effects of Treg in the immune system have shown in previous studies regarding allergic disease. It should be noted that suppressive phenotype of regulatory T cells is dependent on gene expression of the FoxP3 transcription factor that plays a critical role in the differentiation of Treg CD4+ CD25+.[31] In our study, gene expression of FoxP3 increased in both groups. Although the gene expression of FoxP3 was more in synbiotic group, the difference was not significant. Our results are in agreement with Lopez *et al*. that used *B. bifidum*[32] and Chen *et al*. which used L. gasseri A5[23] as probiotics and reported that treatment with probiotics could trigger Treg CD4+ CD25+ FoxP3+ differentiation, but not significant.

Secretion of IL-10 and TGF-β as the major cytokines of Treg subset showed important modulatory effects. In our study, gene expression of IL-10 in 2 months and TGF-β in 6 months of intervention was not significantly increased in the synbiotic group in comparison with placebo receivers. Our results were in concordance with Lin *et al*. that used *L. paracasei* (HF. A00232) in 6–13-year-old children with perennial AR,[16] Ivory *et al*. who assessed effects of *L. casei* in seasonal AR patients[22] and Chen *et al*. who performed the same trial on children with AR by the administration of L. gassei A5.[23] We should note that all these three studies measured TGF-β and IL-10 in the serum of patients whereas our data was according to the gene expression. On the contrary, the serum level of IL-10 in Perrin *et al*. study which used *L. paracasei* NCC2461[24] and also nasal secretion level of this cytokine in Lopez *et al*. study which used *B. bifidum*,[32] was increased significantly due to synbiotic treatment and was in disagreement with our results.

CONCLUSION

Significant reduction in the gene expression of IL-17 as inflammatory cytokines in the synbiotic receivers compared with placebo in our study shows modulatory effects of synbiotic on immunopathogenesis of AR. To achieve a better conclusion regarding the effects of synbiotics, we suggest carry on more researches with a larger sample size and including of a group of patients who receive synbiotic without immunotherapy, extending the follow-up period and also taking advantages of more trial on various strains of synbiotics.

Acknowledgments

These data have been extracted from the thesis of Sajad Dehnavi, MSc student in clinical immunology, Mashhad University of Medical Sciences, Mashhad, Iran. Special acknowledgment should be dedicated to patients with allergic rhinitis, who helped us with blood collection and participation in the clinical trial. We should also acknowledge Allergy Research Center, Mashhad University of Medical Sciences, Mashhad, Iran, for financial help. The grant code for this research was 941403, approval code

of the ethics committee was IR.MUMS.fm.REC.1394.618 and the Registration ID in Iranian Registry of Clinical Trail (IRCT) was IRCT2017061223235N11.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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