Original Article

The Correlation Between Serum Level and Genotypes of IL-10 Gene and their Effects on Endometrial Thickness of Infertile Iraqi Females Undergoing Intrauterine Insemination

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Abstract

Background: Through the first trimester of pregnancy, women's interleukin-10 (IL-10) levels rapidly rise and remain fairly high. Deficiency of this interleukin may be related to adverse pregnancy outcomes like recurrent miscarriages or preeclampsia. Because interleukin-10 is a major immunosuppressant of many infections, its levels have been investigated in several infections of pregnant and non-pregnant females. **Objective:** The current investigation was designed to assess the serum level of IL-10 in females who underwent Intrauterine Insemination as well as the interleukin-10 gene polymorphism that may be associated with adverse effects and the effect of these variants at the level of Endometrial thickness (ET). **Materials and Methods:** The current study included 100 samples from infertile women who underwent intrauterine insemination, enzyme-linked immunosorbent assay (ELISA) technique was used to evaluate the level of interleukin-10, ultrasound was to evaluate ET, DNA extraction, and determination of polymorphism of interleukin-10 (rs180087) by high-resolution melting technique, and confirmation of the result by sequencing technique. **Results:** According to the current findings, there were no statistically significant differences between pregnant and non-pregnant women in the levels of interleukin (P = 0.3), the thickness of the endometrium (P = 0.9), or the genotype of polymorphism (P value = 0.6, 0.5, and 0.7) for CT, TT, and T allele, respectively, but there was a highly significant difference in the levels of IL-10 according to the polymorphism distribution of the IL-10 gene because the genotype of the interleukin-10 gene had an impact on the serum level because it was high in people with the Mutant and heterozygous genotypes, respectively, with a P value of (0.01). **Conclusion:** In conclusion, the mutated genotype of the interleukin-10 gene is related to elevate interleukin-10 levels, with no effect of serum interleukin-10 level on ET possibly due to the small sample size.

Keywords: Endometrial thickness, Interleukin-10, intrauterine insemination, recurrent pregnancy loss, repeated implementation failure

Introduction

Reproductive medicine has always faced its biggest challenges with the infertility issue. The proper reason for infertility is a failure to establish a clinical pregnancy following 12 months of regular or unprotected sexual activity.[1] The primary reasons for infertility vary depending on the country. There are several reasons for infertility about 30% are caused by female factors, 30% by male factors, 25% by undetermined reasons, and 15% by origins that mix both male and female.[2] Despite being used for many years to treat infertility, intrauterine insemination is still debatable. It can be done alone or in conjunction with ovarian stimulation. Initially, this treatment was stopped due to the possibility of pelvic infections and the occasionally severe reaction of the uterus to the insemination of raw semen.[3]

Access this article online

Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_1363_23

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Submission: 07-Sep-2023 **Accepted:** 11-Dec-2023 **Published:** 30-Apr-2026

How to cite this article: Hammood SK, Taha Al-Obaidi M, Ibrahem S. The correlation between serum level and genotypes of IL-10 gene and their effects on endometrial thickness of infertile Iraqi females undergoing intrauterine insemination. Med J Babylon 2026;23:480-6.

Numerous cells produce the interleukin-10 anti-inflammatory cytokine including T regulatory, Th2 lymphocytes, and activated macrophages. Because of its anti-inflammatory effects and function in maintaining immunological homeostasis, IL-10 is an important immune system regulator. It is also a potent suppressor of antigen-presenting cells and lymphocytes.[4,5]

The IL-10 is critical for controlling the host's immune response to prevent host injury and infections and preserving healthy tissue homeostasis. In addition to mediating follicle development, fertilization, embryo development, and implantation, ovarian interleukins also play a role in angiogenesis and corpus luteum formation in conjunction with levels of VEGF.[6]

However, it appears that the most important immunosuppressive agent mediating the regulatory actions of B cells, which are comparable to those of T cells, is IL-10. By promoting immunologic tolerance, the B cells that create IL-10 are essential for the preservation of the pregnancy. Some studies have shown that individuals with repeated pregnancy loss (RPL) had decreased levels of a subset of the B cells that produce IL-10.[7]

Endometrial receptivity including both thickness and vascularity is an influence on predicting the success of pregnancy in women. With the use of a uterine scoring system, embryo transfers will only be carried out in uteri that are in good condition, and cycles with bad uterine scores will be postponed or canceled.[8] The endometrium pattern and thickness are not thought to be a highest issue for the estimation the implantation of embryo by some researchers, whereas others do not. The level of endometrial development as assessed by ultrasound must first be established in order to decide the optimal time to do Doppler ultrasonography as a noninvasive procedure to evaluate uterine receptivity and make the best estimations. When administering human chorionic gonadotropin (HCG), a Doppler ultrasound should be done to get the best sensitivity and specificity.[9,10] ET, vascularization index, endometrial volume, Flow index, and vascularization flow index is measured by ultrasound, leukemia inhibitory factor (LIF), Vascular endothelial growth factor (VEGF), and tumor necrosis factor-alpha (*TNF-α)* levels in the uterine fluid were totally higher significant in pregnant females as compared with non-pregnant females.[11,12]

Due to the essential character of IL-10 in repeated implementation failure and RPL, and the limited studies on the role of IL-10 in infertile women, the goal of the current study was to examine the correlation between IL-10 serum levels and genetic variations of this gene in infertile women who had undergone intrauterine insemination.

Material and Methods

A period from September 2021 to March 2023, a total of 100 infertile women who intended conception at the infertility clinic of the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies/Al-Nahrain University participated in the study. The infertile female in the current study was in aged 18–39 with infertility (primary or secondary). Exclusion criteria include female had no anatomical abnormalities of the uterus and tubes.

Blood samples collection

Venous blood samples were taken from each female using a disposable syringe (Becton, Dickinson and Company, Franklin Lakes, New Jersy, USA) from the median cubical vein, and divided into two parts. The first one was in an anticoagulant-containing tube for genotyping and the other part was then centrifuged at 3500 rpm within 10 min after being allowed to clot in gel-containing tubes for 30 min and to isolate the serum.

ELISA technique

Serum was collected from each woman included in the study as described earlier and the serum was quantitively analyzed for LIF using enzyme-linked immunosorbent assay (ELISA) technology using a kit supplied by Shanghai YL Biotech Co., Ltd. (Shanghai, China) using BioKit machine (USA) according to the supplier instruction.[13] Figure 1 shows the standard curve of IL-10.

Endometrial thickness (ET) by ultrasound ($Y = 0.00158x$, $r^2 = 0.91$)

Monitoring of ovarian response to medication by transvaginal ultrasound was performed in the female infertility clinic using Voluson P8 (GE Health Care Technology, Shanghai, China) with a transvaginal probe (RIC5-9-RS) Probe (2.9–9.7 MHz) to optimize image quality. In this study, a vaginal scan in the early follicular phase second or third day of the patient's cycle has been used to identify antral follicle count and/or the presence of PCO ovary findings or any pathology like ovarian cyst before starting any induction of the ovulation program to be used, in addition to checking of endometrial lining for thickness, texture and any pathology that may interrupt the cavity antral follicle count and the largest follicle, and follow up on the day of trigger measuring the ET. The pattern, No. and size of mature follicles (follicles) and Doppler study for the sub-endometrial blood flow was measured. Figure 2 shows an example of Doppler study.

DNA extraction

Five milliliters of blood from venous located peripherally were taken from each person on the day of the IUI and placed in an EDTA tube. DNA was extracted and purified from whole blood using the QIAamp DNA Blood Mini Kit with Cat. No. (51104), Qiagen, Hilden, Germany.[14]

Conc.	OD
320	2.6665
160	2.2305
80	1.7535
40	0.8745
20	0.3075
10	0.0405

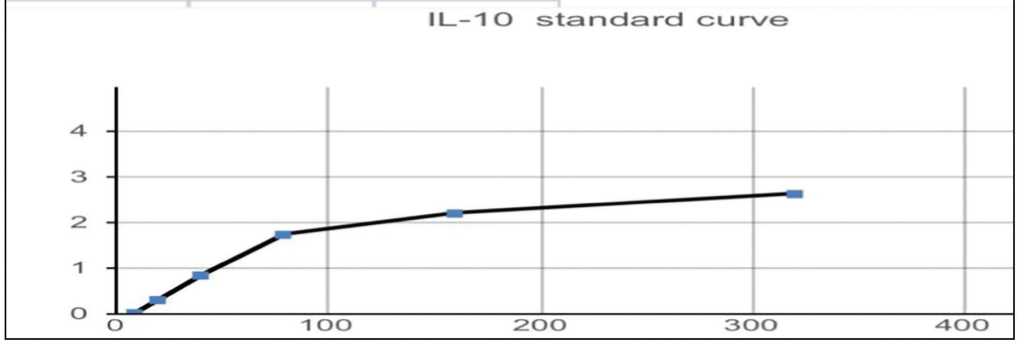

Figure 1: Concentration and optical density of IL-10 standards and the standard curve

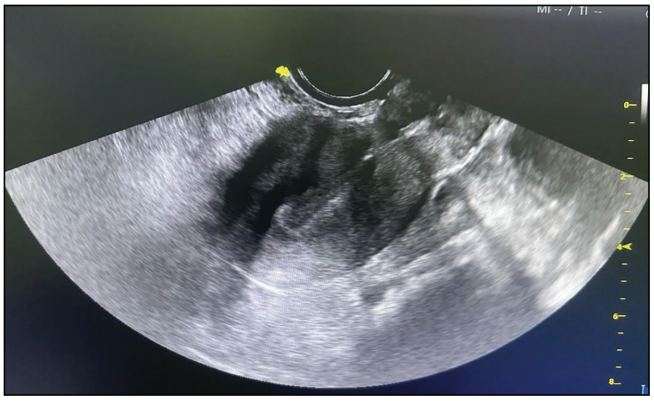

Figure 2: Ultrasonography of the uterus showing the pattern and thickness of the endometrial

DNA quality and quantity assessment

After DNA was eluted a Nabi UV/Vis NANO spectrophotometer (MicroDigital Co, Gyeonggi-do, Republic of Korea) was used to measure the samples' concentration and purity. Concentration has a measurement range of (2–1500 ng/μL) while the purity is in range (1.7–1.9) according to the A260/280 absorbance ratio. Table 1 shows the primer used in the current study.

Polymerase Chain Reaction (PCR)

The final volume of the PCRs reaction was 25 μL involving 10 μL of PCR master mix (Maxime PCR PreMix kit) with Cat. No. (25025), iNtRON Biotechnology Company, Gyeonggi-do, Republic of Korea), 20 ng of DNA, 20 pmol (2 μL) of both forward and reverse primer and the reaction was brought up to the final volume with nuclease-free water. Multi Gene Opti Max Gradient Thermal Cycler (Labnet Company, Edison, New Jersy, USA) was used in accordance with the program available in Table 2.

DNA Sequence and high-resolution melting (HRM)

Samples will be sent to the Macrogen Company in Seoul, Korea, for direct sequencing, the gold standard in the genotyping industry. HRM is a brand-new post-PCR analytical method for detecting genetic variability in nucleic acid sequences.

Statistical analysis

To estimate the associations between several factors such as SNP and pregnancy by computing the odds ratio (OR) and confidence of the intervals (95% CIs). Using the exact Fisher test, the association's significance was determined. The unpaired *t* test (also known as the Student *t* test) was used to examine quantitative (numerical) parameters. Software from Technelysium Pty Ltd Company, South Brisbane, Australia, Chromas 2.6.6, was used to view and evaluate the sequencing findings.

Ethical approval

The investigation was worked out accordingly with the Declaration of Helsinki's ethical precepts sample was prepared with the patient's verbal and diagnostic consent before being taken. On July 3, 2021, in line with document number 7662, a local ethics committee reviewed and approved the study protocol, subject information, and permission form.

Results

The current study included measuring serum level of the IL-10 gene, Table 3 shows the result of IL-10 level where the mean ± SD for each of them was (55.55 ± 14.74) and (58.30 ± 12.68) among the groups of this study including

Table 1: The primer Used in the current study

Gene	SNP (rs)	Sequences 5′--- 3′	Product size	Tm (C°)	Source
IL-10	rs180087	5′- TCATTCTATGTGC TGGAGATG -3′	209(bp)	64	[15]
		5′- TGGGGGAAG TGGGTAAGAGT -3′		66	

Table 2: Thermal profile of conventional PCR to amplify the region of (rs929271) of LIF gene

Steps of Reaction	Tm (°C)	Time	Number of the cycles
Denaturation-1	94	5 min	1
Denaturation-2	94	45 s	40
Annealing	62		
Extension-1	72		
Extension-2	72	7 min	1

Table 3: Mean and the standard deviation of IL-10 in females with negative and positive pregnancies respectively

Serum level of IL-10	Mean ± SD		*P* value
IL-10	Negative pregnancy 55.55 ± 14.74	Positive pregnancy 58.30 ± 12.68	0.3 N.S

negative and positive pregnancy females respectively, where the *P* value was 0.3 which shows that there are no statistical changes between the two groups, females with pregnancy or not.

Through a *P* value of 0.01, the results of the genotype distribution of the IL-10 gene and the serum level of the IL-10 gene showed that there were highly statistically significant differences among the three genotypes of the gene, with the mean ± SD of the common CC for IL-10 being approximately (59.46 ± 10.93). According to CT genotypes, the serum level of IL-10 was (54.55 ± 16.07), but it was (48.01 ± 17.08) for TT genotypes as shown in Table 4.

Table 5 shows the results of the ultrasound examination to evaluate the parameter in the 2 days of the cycle including the ET parameter amongst pregnant and non-pregnant females, where the mean ± SD was for each of them, and at a *P* value of 0.09, this demonstrated that there were no differences between the groups of the current study. The results of the ultrasound parameters included ET there were no significant differences between the two groups of the current study with a *P* value of 0.9.

Table 6 shows outcomes of the distribution of the genotype of the IL-10 gene with some ultrasound parameters, which included ET and there were no significant differences between the ET parameter for all genotypes of the target gene, whether wild type, heterozygous, or mutant with a *P* value (0.5). Figure 3 depicts the results.

Table 7 shows the results of the distribution of genotypes for the IL-10 gene with ultrasound parameters at the

Table 4: Serum level of IL-10 in accordance to IL-10 gene distribution

IL-10 level (ng/mL)	Genotypes of IL-10 gene		
	CC	CT	TT
Mean	59.64	54.55	48.01
SD	10.93	16.07	17.08
P value	0.01*		

Table 5: The mean and standard deviation of ET in groups of the current study on the 2 days of the cycle and the day of the trigger

ET	Mean ± SD		*P* value
	Negative	Positive	
Endometrial thickness(mm) at 2 days of the cycle	3.86 ± 0.29	3.73 ± 0.36	0.09 N.S
Endometrial thickness(mm) on the day of the trigger	7.84 ± 0.81	7.83 ± 0.78	0.9 N.S

Table 6: Ultrasound parameter at cycle Day 2 according to IL-10 gene distribution

Endometrial thickness	Genotypes of IL-10 gene		
	CC	CT	TT
Mean	3.84	3.78	3.87
SD	0.30	0.36	0.27
P value	0.5 N.S		

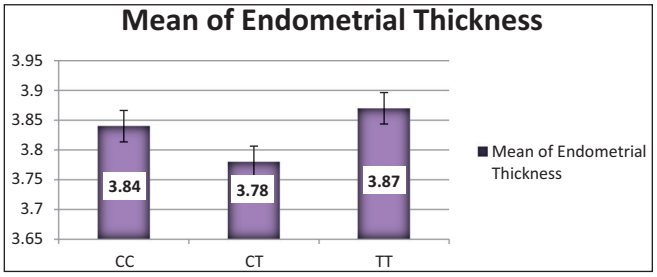

Figure 3: Ultrasound parameter on the next day of the cycle according to the genetic distribution of the IL-10 gene

day of trigger that included ET where the mean ± SD of the CC wild type genotype was (7.78 ± 0.7), While it was (8.00 ± 1.02) and for the heterozygous genotype CT, the mutant genotype TT was about (7.00 ± 0.4). Figure 4 depicts the results.

The results of the IL-10 gene genotype revealed that the wild type was most prevalent in both groups, with

percentages of 53.8% and 52.7% for pregnant and non-pregnant women, respectively, while the heterozygous genotype was CT with a percentage of 26.9% and 33.8%, which did not demonstrate any differences between the groups of the current investigations (*P* value = 0.6), while TT is the mutant type, which was 19.3% and 13.5% for women whose pregnancy was positive compared to women whose pregnancy was negative. Also, both alleles, whether dominant or mutant, showed no significant differences among the two groups. Table 8 and Figure 5 show the results.

The polymorphism of the IL-10 gene in the current study was—819 C/T (rs1800871), located in the promoter region (2KB upstream variant), and their location on the chromosome was GRCh37.p13 chr 1.

Discussion

Interleukin-10 (IL-10), a crucial immune-modulatory cytokine in the Th2 immune response, has anti-inflammatory and important vaso-protective effects and may facilitate effective placentation throughout a healthy pregnancy.[16] Preeclampsia has been linked to IL-10 dysregulation, which was explained by an increase in IL-10 levels in women who had abortions. This relationship highlights the importance of IL-10 interleukins, which function as messengers between immune cells and act to protect the body, (IL-10) also known as the inflammatory station, is one of the cytokines that promotes the development of infections and initiates the inflammatory process. The role of interleukins, whose purpose is to facilitate communication between immune cells and whose action is focused on bodily defense and their role in IUI process are not fully understood until now.[17]

The pregnancy process is a distinct and well-choreographed physiological procedure that includes changes in hormone levels, molecular and cellular activities at the maternal-fetal interface, and delicate interaction of inflammatory as well as anti-inflammatory microenvironment. IL-10 is a cytokine that is, safe to use during pregnancy and is essential for preserving immunological tolerance.[18] Dysregulation of IL-10 is related not only to cancer, inflammatory diseases, autoimmune atopic diseases, and other diseases, but also to adverse pregnancy complications such as preterm birth, miscarriage, fetal growth restriction, and preeclampsia.[19]

According to several investigations, interleukin-10 (IL-10) is essential for a healthy pregnancy, and low IL-10 levels are linked to pregnancy problems. While pathologic pregnancies are linked to lower levels of IL-10, healthy

Table 7: Ultrasound parameter on the day of a trigger according to IL-10 gene distribution

Endometrial thickness	Genotypes of IL-10 gene		
	CC	CT	TT
Mean	7.78	8.00	7.70
SD	0.71	1.03	0.46
P value	0.3 N.S		

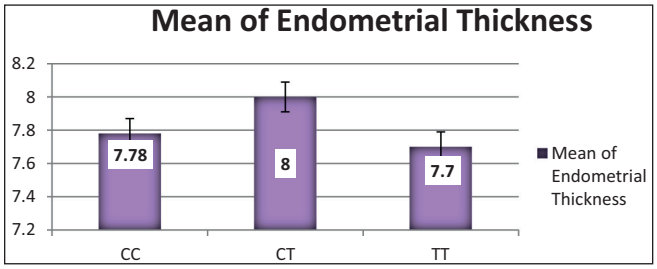

Figure 4: Ultrasound parameter on the day of a trigger according to the genetic distribution of the IL-10 gene

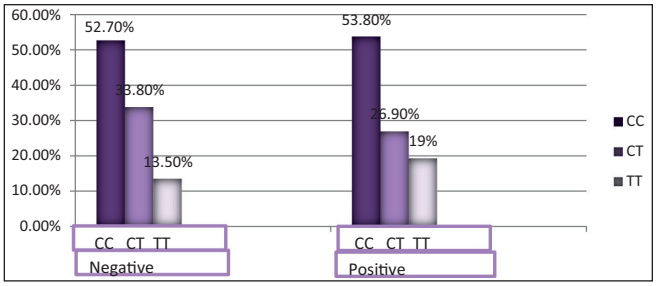

Figure 5: Genotypes of IL-10 gene percentage in both groups of the current study that include females with positive and negative pregnancy

Table 8: Genotypes and allele frequency of IL-10 gene

Genotype of IL-10	Pregnancy		*P* value	OR	CI 95%
	Negative (74)	Positive (26)			
CC	39 (52.7%)	14 (53.8%)	–	1.00	(Reference)
CT	25 (33.8%)	7 (26.9%)	0.6	1.2	0.45 - 3.61
TT	10 (13.5%)	5 (19.3%)	0.5	0.7	0.20 - 2.46
Total	74	26	–	–	–
Allele Frequency					
C	0.7 (103)	0.7 (35)	–	1.00	(Reference)
T	0.3 (45)	0.3 (17)	0.7	0.8	0.45–1.77

pregnancies are linked to greater levels.[20,21] According to the previous study, conducted by Talaat *et al*.[22], found that individuals with PCOS had significantly lower mean plasma levels of IL-10 than controls P value < 0.001 at 313.43 ± 30.1 and 4914.36 ± 303.7, respectively.

The current results agree with a previous study in wild and heterozygous genotypes but differ in the case of mutant type according to the study by Talaat *et al.*[22] which investigated that the distribution of 819 CT and CC genotypes amongst patients (PCOS patients) and controls did not change significantly from that of controls. Contrarily, PCOS patients' TT genotype frequency was considerably higher (P 0.05) than that of controls, suggesting that this genotype may be a risk factor for the condition.

The previous study by Juo *et al*.[23] displayed that the rs1800871 variant was significantly related to the disease, but that the mutant type did not affect disease in the population with endometriosis disease.

In our study, the results of the ultrasound examination showed no statics significant variance amongst pregnant and non-pregnant females regarding ET, due to stimulation that makes the endometrium within the desirable thickness. Our finding is consistent with the earlier study, which indicated that the mean midcycle thickness of the endometrium in pregnant women was 11.35, whereas it was 10.8 in non-pregnant cases with a P value of 0.62.[9] Regarding the thickness of the uterine lining in the middle of the cycle, previous studies showed agreement with this study like the study by Check *et al*.[24] and Kolibianakis *et al*.[25], as their findings were that there was no relationship through female with positive and negative pregnancy, as the study showed that there were similar pregnancy rates in patients with an ET (<6 and >6 mm) who were treated with clomiphene citrate.

When comparing the ultrasound imaging characteristics of the two study groups (pregnant and non-pregnant) for endometrial receptivity, a difference in the echogenicity of the dominant line in the endometrium was detected only, while another study showed that the echogenicity of the central line of the endometrium was more pronounced in pregnant women, which gave support to the results of the study by Zheng *et al.*[26]. Although the pregnant group's endometrial functional layer's echogenicity was more consistent, it did not reach statistical significance. In the same study, ET, volume, absence, presence, direction of endometrial peristalsis, or endometrial blood flow, among other variables, did not differ substantially between the two groups.[26] According to the study of the ultrasound results of the current issue, the thickness of the uterine lining was not significantly different between the two study groups. On the other hand, it is not believed that the pattern and thickness of the uterine lining are important for predicting the embryo implantation process, as well as for determining the best timing for performing a Doppler examination and ultrasound to evaluate the receptivity of the uterus as a non-surgical procedure while concerning the fetus, it has been assumed by some researchers that there is a relationship between the extent of development of the endometrial determined through ultrasound and the likelihood of the embryo implantation procedure. On the same day of the HCG injection, the patient must perform a Doppler ultrasound to obtain the best specificity and sensitivity.[9] There was the absence of significant difference revealed in the general endometrial vascularization parameters measured on the same day of transferring embryo.[27] Following previous studies, the expression of HOXA10 and IL-10 was studied, such as the study conducted by Wang *et al*.,[28], in women with adenomyosis to evaluate its effects on implantation. They discovered the expression of the endometrial receptivity marker protein HOXA10 and the anti-inflammatory cytokine IL-10 in comparing women with adenomyosis. With healthy controls, the HOXA10 protein level in the endometrium was significantly lower (by approximately 50%) in the first group (P < 0.001). Compared with normal controls, the endometrial of women with adenomyosis had 40% lower expression of IL-10. Also, the levels of HOXA10 and IL-10 protein showed a positive relationship, which was surprising.

Conclusions

The correlation between the IL-10 genotype and the serum level of IL-10 showed a highly significant difference, where the TT genotype was the highest level compared with the wild type, which means that the high level of interleukin may be linked with the risk of not getting pregnant after IUI. Also, there were no significant differences between the ET levels of the three IL-10 genotypes. Perhaps the small size of the sample or the cytokine secretion in different tissues may lead to a little expression in ET. It recommended that this polymorphism be studied in a larger sample size to confirm this result.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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