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*Original Article*

# Relationship between cytokine gene polymorphisms and recurrent spontaneous abortion

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**Abstract:** Recurrent spontaneous abortion (RSA), defined as three or more sequential abortions before the twentieth week of gestation. Some studies have led to the awareness that immunological factors play an important role in establishing a successful pregnancy. The aim of present study was to investigate the relationship between RSA and polymorphisms of cytokine genes coding for TNF-α (-308 G→A, -238 G→A), TNF-β (+252 G→A) as Th1 or pro-inflammatory factors as well as IL-6 (-634 G→C, -174 G→C), IL-10 (-1082 A→G, -819 C→T, -592C→A) as Th2 cytokines in women with RSA compared with healthy women. A total of 284 women with RSA and 284 control women with at least two successful pregnancies and no history of abortion were included in the study. The polymerase chain reaction (PCR), allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) or PCR-RFLP (restriction fragment length polymorphism) methods were used for genotyping. In addition, the ELISA was conducted to investigate four cytokine serum levels in women with RSA and healthy women. Results showed that: TNF-α -308G/A, IL-6 -174 G/C and IL-10 -819 G/C polymorphisms showed statistically significant differences between the RSA patients and controls (P=0.008, P=0.0005 and P=0.03 separately). Levels of four cytokines in the serum showed that there were no significant differences in TNF-α and TNF-β between patients and control (P>0.05), while the level of IL-6 and IL-10 were lower than control group and the differences were statically significant (P<0.05). This study demonstrated a possible association between TNF-α -308, IL-6 -174 and IL-10 -819 promoter polymorphism and RSA.

**Keywords:** Recurrent spontaneous abortion, cytokine gene polymorphisms, tumor necrosis factor-α, tumor necrosis factor-β, interleukin-6, Interleukin-10

## Introduction

Recurrent spontaneous abortion (RSA), defined as three or more sequential abortions before the twentieth week of gestation, affects approximately one in 300 pregnancies [1, 2]. Precipitating elements include genetic, endocrine, anatomic, immunologic, and microbiologic factors; however, in almost 50% of the cases the etiology remains unexplained [3, 4]. Some studies have led to the awareness that immunological factors play an important role in establishing a successful pregnancy. Considerable evidence has accumulated indicating that cytokines play an important role in the maintenance of pregnancy by modulating the immune system [5].

Different studies have shown that the role of balance of Th1/Th2 immunity play a major role in reproductive phenomena [6], in which Th2-type dominant response has been associated with normal pregnancy, whereas the Th1-type response has been related to pregnancy failure [7].

In humans, tumor necrosis factor (TNF)-α and interferon-gamma (IFN-γ) inhibit embryonic and fetal development as well as the proliferation of human trophoblast lines [8]. It is also demonstrated that enhanced uterine expression of pro-inflammatory cytokines such as TNF, IFN-γ, interleukin (IL)-1β has been associated with embryo loss. Anti-inflammatory cytokines such as IL-6 and IL-10 considered essential for maintaining a normal pregnancy [9, 10].

The production of cytokines can be influenced by genetic polymorphisms, especially in the promoter regions and result in high, intermediate or low levels of cytokines [11]. And some stud-

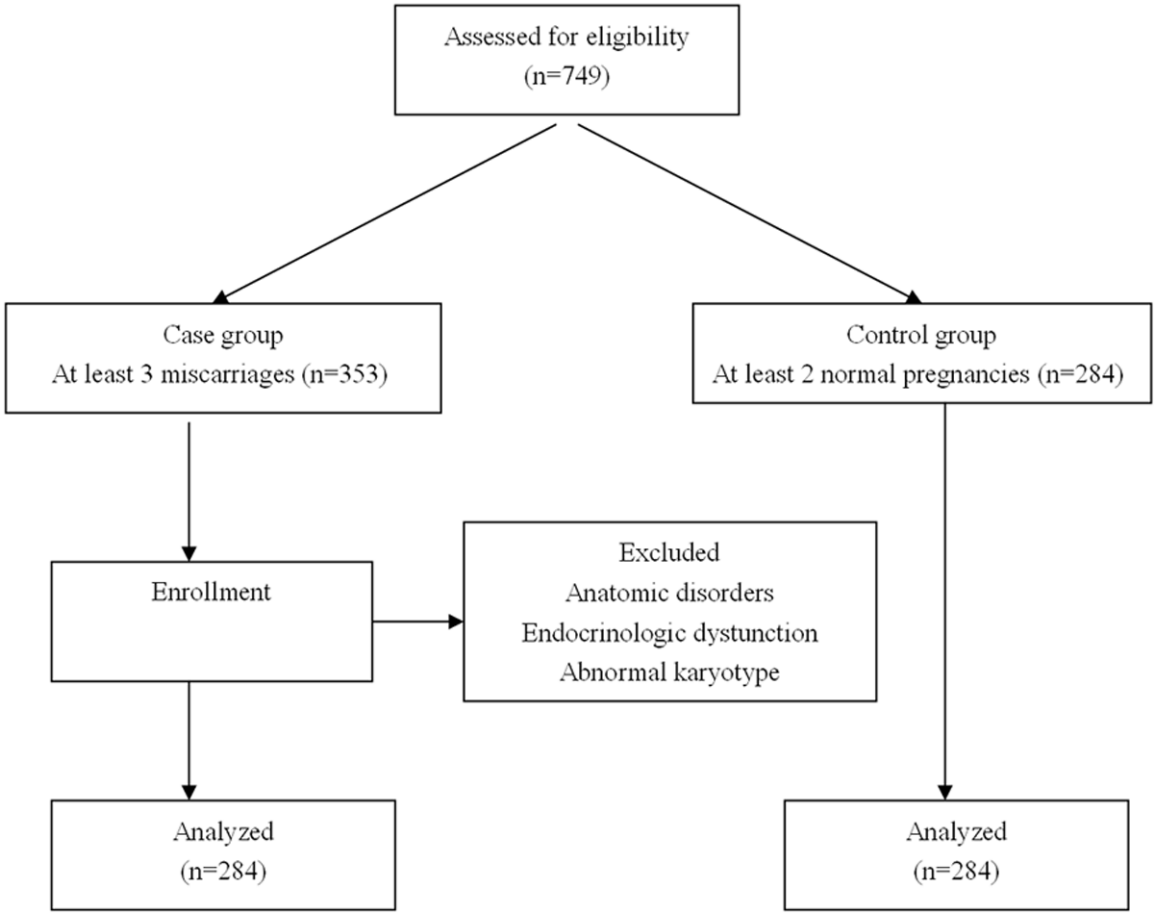


**Figure 1.** Flow chart for the enrollment of the patients and controls in the study.

**Table 1.** Primer sequences and methods used for detection of cytokine gene polymorphisms

| Locus | Primers | Method |
|---|---|---|
| TNF-α -308 | Forward: 5'-CAGGCCTCAGGACTCAACAC-3';<br>Reverse: 5'-AAGGAAGTTTTCCGCTGGTT-3' | PCR |
| TNF-α -238 | Forward: 5'-CAGGCCTCAGGACTCAACAC-3';<br>Reverse: 5'-AAGGAAGTTTTCCGCTGGTT-3' | PCR |
| TNF-β +252 | Forward: 5'-CCGTGCTTCGTGGTTTGGACT-3';<br>Reverse: 5'-AGAGGGGTGGATGCTTGGGTTC-3' | NcoI based RELP |
| IL-6 -634 | Forward: 5'-AGGCAAACCTCTGGCACA-3';<br>Reverse: 5'-TTCTAGCCTGTTAATCTGGTCAC-3' | PCR |
| IL-6 -174 | Forward: 5'-GGAGTCACACACTCCACCT-3';<br>Reverse: 5'-CTGATTGGAAACCTTATTAG-3' | NlaIII based RELP |
| IL-10 -1082 | Common primer: 5'-CAGCCCTTCCATTTTACTTTC-3';<br>G allele primer: 5'-TACTAAGGCTTCTTTGGGAG-3';<br>A allele primer: 5'-CTACTAAGGCTTCTTTGGGAA-3' | ASO-PCR |
| IL-10 -819 | Forward: 5'-TCATTCTATGTGCTGGAGATGG-3';<br>Reverse: 5'-TGGGGGAAGTGGGTAAGAGT-3' | MaeIII based RELP |
| IL-10 -592 | Forward: 5'-GGTGAGCACTACCTGACTAGC-3';<br>Reverse: 5'-CCTAGGTCACAGTGACGTGG-3' | RsaI based RELP |

PCR: polymerase chain reaction, RFLP: restriction fragment length polymorphism, ASO-PCR: allele-specific oligonucleotide polymerase chain reaction.

**Table 2.** Polymerase chain reaction (PCR) conditions for cytokine genes amplification

| Locus | PCR conditions | Reference |
|---|---|---|
| TNF-α -308 | 36 cycles: 95°C 30 s, 60°C 30 s, 72°C 1 min | [15] |
| TNF-α -238 | 36 cycles: 95°C 30 s, 60°C 30 s, 72°C 1 min | [15] |
| TNF-β +252 | 31 cycles: 94°C 30 s, 61°C 2.5 min, 72°C 30 s | [16] |
| IL-6 -634 | 36 cycles: 95°C 30 s, 60°C 30 s, 72°C 1 min | [15] |
| IL-6 -174 | 35 cycles: 95°C 1 min, 56°C 2 min, 72°C 1 min | [17] |
| IL-10 -1082 | 30 cycles: 94°C 30 s, 56°C 30 s, 72°C 30 s | [16] |
| IL-10 -819 | 30 cycles: 94°C 45 s, 60°C 30 s, 72°C 1 min | [16] |
| IL-10 -592 | 30 cycles: 94°C 1 min, 63°C 70 s, 72°C 1 min | [16] |

**Table 3.** Demographic and clinical characteristics of RSA patients in comparison with the control group

| Characteristic | RSA patients (n= ) | Controls (n= ) | *P*-value |
|---|---|---|---|
| Age, years | 34.09±0.76 | 34.57±0.95 | 0.6 |
| BMI, kg/m$^2$ | 28.65±3.86 | 28.85±3.42 | 0.7 |
| Previous pregnancy losses | None | 3.01±1.31 | 0.000 |
| Live birth | 1.85±0.72 | None | 0.000* |

RSA, recurrent spontaneous abortion; BMI, body mass index; Values are mean ± SD.

**Table 4.** Genotype and allele frequencies of polymorphism in TNF-α gene promoter region in RSA patients compared with the control group

| Genotype | Control | Patients | Control versus patients | | *P*-value |
|---|---|---|---|---|---|
| | n (%) | | OR | 95%CI | |
| TNF-α -308 | | | | | |
| GG | 205 (72.18) | 144 (50.7) | 0.39 | 0.19-0.82 | 0.011 |
| GA | 61 (21.48) | 105 (36.97) | 2.13 | 0.98-4.64 | 0.054 |
| AA | 18 (6.34) | 35 (12.32) | 2.14 | 0.61-7.5 | 0.223 |
| Total | 284 | 284 | | | |
| Allele | (Frequency) | | | | |
| G | 471 (0.829) | 393 (0.692) | | 0.25-0.83 | 0.008 |
| A | 97 (0.171) | 175 (0.308) | | 1.21-3.94 | |
| TNF-α -238 | | | | | |
| GG | 249 (87.68) | 240 (84.51) | 0.77 | 0.28-2.10 | 0.61 |
| GA | 35 (12.32) | 30 (10.56) | 0.86 | 0.29-2.53 | 0.78 |
| AA* | 0 (0) | 14 (4.93) | 0 | 0 | 0.24 |
| Total | 284 | 284 | | | |
| Allele | (Frequency) | | | | |
| G | 533 (0.938) | 510 (0.898) | | 0.24-1.48 | 0.255 |
| A | 35 (0.062) | 58 (0.102) | | 0.68-4.24 | |

OR: odds ratio; CI: confidence interval.

ies had reported that there was a correlation between such polymorphisms and the production levels of various cytokines important to pregnancy, including TNF-α and -β, IL-6 and IL-10 [11-14].

Considering the potential role of these cytokines in RSA and few studies on the relationship between these cytokine gene polymorphisms and susceptibility to RSA, the aim of this study is to investigate the association between RSA and the polymorphisms in Th1-type cytokine genes (TNF-α -308 G→A, -238 G→A; TNF-β +252 G→A) and Th2-type cytokine genes (IL-6 -634 G→C, -174 G→C; IL-10 -1082 A→G, -819 C→T, -592C→A) and analyze the blood levels of four cytokines in these women.

## Materials and methods

### *Subjects*

A total of 284 women aged 18-42 years with RSA who were referred to our center between January 2013 and December 2014 and 284 ethnically matched normal controls comprised our study population. Both patients and controls had a single partner during their reproductive age. And RSA cases were diagnosed clinically and serologically by a gynecologist. Exclusion criteria included anatomical abnormalities, previously known systemic disease, endocrine disorders, previous venous or arterial thrombosis or a family history of thromboembolism. Chromosomal abnormalities and Rh incompatibility were ruled out before inclusion in the study. As infection was linked with RSA, all subjects included were confirmed to be negative for the TORCH agents Toxoplasma gondii, rubella, cytomegalovirus (CMV), herpes simplex viruses (HSV-1 and HSV-2), varicella zoster virus (VZV) and human immunodeficiency virus (HIV-1 and HIV-2) by indirect enzyme-linked immunosorbent assay (ELISA). Transvaginal ultrasound was performed to confirm sponta-

**Table 5.** Genotype and allele frequencies of polymorphism in TNF-β gene promoter region in RSA patients compared with the control group

| Genotype | Control | Patients | Control versus patients | | *P*-value |
|---|---|---|---|---|---|
| | n (%) | | OR | 95%CI | |
| TNF-β -634 | | | | | |
| GG | 44 (15.49) | 40 (14.08) | 0.88 | 0.33-2.34 | 0.88 |
| GA | 100 (35.21) | 122 (42.96) | 1.38 | 0.68-2.80 | 0.37 |
| AA | 140 (49.30) | 122 (42.96) | 0.78 | 0.39-1.56 | 0.48 |
| Total | 284 | 284 | | | |
| Allele | (Frequency) | | | | |
| G | 188 (0.331) | 202 (0.356) | | 0.66-1.85 | 0.69 |
| A | 380 (0.669) | 366 (0.644) | | 0.54-1.51 | |

OR: odds ratio; CI: confidence interval.

**Table 6.** Genotype and allele frequencies of polymorphism in IL-6 gene promoter region in RSA patients compared with the control group

| Genotype | Control | Patients | Control versus patients | | *P*-value |
|---|---|---|---|---|---|
| | n (%) | | OR | 95%CI | |
| IL-6 -634 | | | | | |
| GG | 236 (83.1) | 231 (81.34) | 0.90 | 0.37-2.22 | 0.82 |
| GA | 35 (12.32) | 39 (13.73) | 1.15 | 0.41-3.18 | 0.80 |
| AA | 13 (4.58) | 14 (4.93) | 1.00 | 0.19-5.15 | 1.00 |
| Total | 284 | 284 | | | |
| Allele | (Frequency) | | | | |
| G | 507 (0.893) | 501 (0.882) | | 0.43-2.00 | 0.84 |
| A | 61 (0.107) | 67 (0.118) | | 0.50-2.34 | |
| IL-6 -174 | | | | | |
| GG | 211 (74.23) | 171 (60.21) | 1.85 | 1.24-2.78 | 0.0034 |
| CG | 63 (22.18) | 95 (33.45) | 2.41 | 1.02-5.67 | 0.0448 |
| CC | 10 (3.52) | 18 (6.34) | 1.93 | 1.31-2.83 | 0.0008 |
| Total | 284 | 284 | | | |
| Allele | (Frequency) | | | | |
| G | 485 (0.854) | 437 (0.769) | | 0.40-0.77 | 0.0005 |
| C | 83 (0.146) | 131 (0.231) | | 1.29-2.47 | |

OR: odds ratio; CI: confidence interval.

neous abortion. The flow chart for the enrolment of the patients and controls is presented in **Figure 1**. The study was performed based on an informed consent and was approved by UMF Carol Davila Bucharest Ethics Committee.

*Cytokine gene polymorphisms*

Five ml blood samples were taken from all women by venipuncture in ethylenediaminetetraacetic acid (EDTA) tubes for total genomic DNA extraction using the DNA Puregene purification kit. Concentrations of the samples were deter-mined with ultraviolet (UV) spectrometry by measuring an A260 absorbance. Purity was determined by an A260/A280 ratio. Samples were diluted to a standard concentration and stored at -20°C.

Genomic DNA was amplified by polymerase chain reaction (PCR) using TNF-α (-308 G/A, -238 G/A), TNF-β (+252 G/A), IL-6 (-634 G/C, -174 G/C) and IL-10 (-1082 A/G, -819 C/T and -592C/A) specific primers (**Table 1**). And PCR conditions for each gene polymorphism are shown in **Table 2**. Restriction enzyme digestions were employed to determine the TNF-α (-308 G/A, -238 G/A), TNF-β (+252 G/A), IL-6 (-634 G/C, -174 G/C) and IL-10 (-1082 A/G, -819 C/T and -592C/A) variants, respectively (**Table 1**) and the resulting fragments were analyzed on 2-2.5% agarose gels.

*ELISA*

Another 5 ml venous blood samples were taken from all participants in plain tubes and centrifuged then the plasma was stored at -80°C until measurement of TNF-α, TNF-β, IL-6 and IL-10 levels. And the plasma levels of four cytokines were measured by use of relevant ELISA kits (AssayPro, St. Charles, MO, USA) following the manufacturer's protocols.

*Statistical analysis*

Statistical analysis was performed by using SPSS version 20.0 software. Data were presented as median and range or mean standard deviation (S.D.). Data were analyzed using the $\chi^2$-test and Fisher's exact test where appropriate. All tests were performed two-tailed with a confidence interval (CI) of 95%. Differences at the level of $P<0.05$ were considered statistically significant.

## Results

The demographic data of the unexplained RSA patient and controls are presented in **Table 3**.

**Table 7.** Genotype and allele frequencies of polymorphism in IL-10 gene promoter region in RSA patients compared with the control group

| Genotype | Control | Patients | Control versus patients | | *P*-value |
|---|---|---|---|---|---|
| | n (%) | | OR | 95%CI | |
| IL-10 -1082 | | | | | |
| GG | 128 (45.07) | 96 (33.8) | 0.62 | 0.29-1.31 | 0.257 |
| AG | 107 (37.68) | 150 (52.82) | 1.86 | 0.89-3.91 | 0.13 |
| AA | 49 (17.25) | 38 (13.38) | 0.73 | 0.26-2.04 | 0.61 |
| Total | 284 | 284 | | | |
| Allele | (Frequency) | | | | |
| G | 363 (0.639) | 342 (0.602) | 0.84 | | 0.66 |
| A | 205 (0.361) | 226 (0.398) | 1.18 | | |
| IL-10 -819 | | | | | |
| TT | 13 (4.58) | 16 (5.63) | 1.25 | 0.27-5.82 | 1.00 |
| CT | 80 (28.17) | 152 (53.52) | 2.95 | 1.44-6.08 | 0.0046 |
| CC | 191 (67.25) | 115 (40.49) | 0.33 | 0.16-0.67 | 0.003 |
| Total | 284 | 284 | | | |
| Allele | (Frequency) | | | | |
| T | 106 (0.187) | 184 (0.324) | 2.09 | | 0.03 |
| C | 462 (0.813) | 382 (0.676) | 1.25 | | |
| IL-10 -592 | | | | | |
| CC | 155 (54.58) | 123 (43.31) | 0.64 | 0.32-1.26 | 0.22 |
| CA | 116 (40.85) | 136 (47.89) | 1.33 | 0.67-2.66 | 0.48 |
| AA | 13 (4.58) | 25 (8.80) | 1.93 | 0.46-8.09 | 0.49 |
| Total | 284 | 284 | | | |
| Allele | (Frequency) | | | | |
| C | 426 (0.75) | 382 (0.673) | 0.69 | | 0.27 |
| A | 142 (0.25) | 186 (0.327) | 1.48 | | |

OR: odds ratio; CI: confidence interval.

Age and body mass index (BMI) were matched in both groups (P>0.05), while, there were significant difference in the number of pregnancies and number of children and BMI in the 2 groups (P<0.05).

*Genotyping results*

*TNF-α polymorphism:* Results were shown in the **Table 4**. They presented the genotype and allele frequency of the G and A alleles of TNF-α -238 G/A and -308 G/A polymorphism in patients and controls. No significant differences (P=0.255) were identified in the genotype of allele frequencies of G and A alleles of -238G/A polymorphism, while the genotype and allele frequencies of -308G/A polymorphism showed a statistically significant difference when the RSA patients were compared to the controls (P<0.05).

*TNF-β polymorphism:* **Table 5** presented the genotype and allele frequency of TNF-β +252 G/A polymorphism in patients and controls. Most patient and control samples were homozygous for the wild type G allele. However, there were no significant differences in the genotype and allele frequencies between patients and controls.

*IL-6 polymorphism:* **Table 6** presented the genotype and allele frequencies of IL-6 polymorphism. No significant differences (P=0.84) were identified in the frequencies of -634 G/C polymorphism, while the genotype and allele frequencies of -174 G/C polymorphism showed a statistically significant difference when the RSA patients were compared to the controls (P<0.05).

*IL-10 polymorphism:* The frequencies of the genotypes/alleles 819 C/T, 592 C/A and 1082 A/G in IL10 gene in study, are presented in **Table 7**. No significant differences (P=0.66 and 0.27) were identified in the frequencies of -1082 G/A and -592 C/A polymorphism, while the genotype and allele frequencies of -819 G/C polymorphism showed a statistically significant difference when the RSA patients were compared to the controls (P<0.05).

*Results of ELISA*

Levels of four cytokines in the serum were shown in **Table 8**, in which no significant differences were identified in TNF-α and TNF-β between patients and control (P>0.05), while the level of IL-6 and IL-10 were lower than control group and the differences were statically significant (P<0.05).

## Discussion

A successful pregnancy depends on maintaining equilibrium between immunity mediated by Th1 cells and that mediated by Th2 cells [18]. On the other hand, associations between Th1 or Th2 cytokine gene polymorphisms and different genetic abilities to produce these cytokines have been shown using both in vivo and in vitro models [19]. The study investigates the

**Table 8.** Serum level of TNF-α, TNF-β, IL-6 and IL-10 in women with and without RSA

| Cytokines | Level in the serum (pg/mL) | |
|---|---|---|
| | Women with RSA n=284 | Control n=284 |
| TNF-α | 4.77±1.91 | 4.89±1.82 |
| TNF-β | 4.31±0.98 | 4.28±1.01 |
| IL-6 | 82.65±23.15* | 210.43±40.63 |
| IL-10 | 59.75±19.26* | 173.31±20.12 |

Values are mean ± SD; *Comparing with control group P<0.05.

association between TNF-α, TNF-β, IL-6 and IL-10 gene polymorphism with RSA. The results show that polymorphisms in the TNF-α -308, IL-6 -174 and IL-10 -819 are associated with RSA. But other polymorphisms have no association with RSA. The ELISA results also show that IL-6 and IL-10 levels are significantly lower in patients with RSA than controls.

Polymorphisms in TNF-α gene have been extensively investigated in cases of unexplained RSA. While the majority of these studies evaluated SNP at the -308 position, some assessed other functional TNF-α polymorphisms, such as -238 and -863 [20-22]. In our study, results show that TNF-α-308 polymorphism might relate with incidence of RSA. Although there are no significant differences in TNF-α serum level between patients with RSA and healthy people, effects of TNF-α-308 polymorphism to TNF-α serum level might counteract by other polymorphism.

Almost all of the studies that evaluated IL-6 polymorphism in RSA patients assessed the SNP at position -174 [20, 21, 23], which was accord with this study. And IL-6 serum level in RSA was lower than normal, it suggested that -174 polymorphism might be an important cause of RSA.

Interleukin-10 interacts with different factors and cells playing a central role in pregnancy. A meta-analysis suggested a possible relationship between IL10 GG (-1082) and RSA patients in comparison with the general pregnant population [20], and several studies showed IL-10 -592 polymorphism had relationship with RSA [16, 24], but this study found that IL-10 -819 polymorphism might play the effective roles in the incidence of RSA. Meantime, the IL-10 serum level was significantly lower in RSA than control. These results suggested that IL-10 might play a critical role in the normal pregnant.

In conclusion, it seems that TNF-α -308G/A, IL-6 -174 G/C and IL-10 -819 G/C polymorphisms might have relationship with the susceptibility of RSA. While further studies are needed that is comparisons of cytokine serum levels in their different polymorphisms.

## Disclosure of conflict of interest

None.

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