ORIGINAL ARTICLE

Evaluation of common COL2A1 gene variants in Iranian patients suspected with Stickler syndrome type I

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Background: Stickler syndrome, also known as hereditary progressive arthro-ophthalmopathy, is a connective tissue disorder that arises from mutations in multiple genes, each with distinct hereditary patterns. Stickler syndrome type I, which is inherited in an autosomal dominant manner, is specifically linked to mutations in the *COL2A1* gene. The objective of this study is to investigate the prevalence of common variants of the *COL2A1* gene among individuals suspected of having Stickler syndrome type I. **Materials and Methods:** Twenty-six Iranian patients suspected of having Stickler syndrome type I referring to Al-Zahra Hospital of Isfahan were employed in this cross-sectional study. The DNA was extracted from the patient's peripheral blood samples, and the selected exons of the *COL2A1* gene were amplified by polymerase chain reaction. Subsequently, the purified amplicons were subjected to Sanger sequencing to identify common variants associated with Stickler syndrome type I. **Results:** All patients exhibit cleft abnormalities (palate, lip, and alveolar), 84.6% of patients exhibit ocular abnormalities, 53.8% of patients exhibit hearing abnormalities, and 34.6% of patients exhibit skeletal abnormalities. As the data displays, the highest phenotype presentation prevalence rate was related to cleft lip and palate, while hemiparesis was the lowest clinical finding among the patients. Molecular analysis which conducted to screen the *COL2A1* gene of patients, identified two different variants, including a novel nonsense variant, (c.1030C>T), consistent with dominantly inherited Stickler syndrome type I, also synonymous mutation (c.213C>T) affecting in exon 2, which have been reported in database. **Conclusion:** Genetic analysis of Twenty-six unrelated families with Stickler syndrome type I disorder discovered one novel pathogenic variant in the *COL2A1* gene in a patient with Stickler syndrome type I. Genetic analysis is helpful for the diagnosis of this clinically variable and genetically heterogeneous disorder.

Key words: *COL2A1* gene, hereditary arthro-ophthalmopathy, Stickler syndrome type I, Stickler syndrome vitreous type I

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INTRODUCTION

Cleft lip and palate are among the most common congenital anomalies.[1] Depending on the severity of the condition, these can be classified as either unilateral or bilateral.[2] In addition, cleft lip and palate can be categorized into syndromic and nonsyndromic types based on the presence of associated developmental defects or organ dysfunctions.[1] In Iran, according to a review study from 2000 to 2016, the prevalence of cleft lip and palate has been ranged from 0.78 to 2.14 per 1000 live births.[3] A more recent review conducted

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in 2020 about cleft lip and palate reported an overall incidence rate of 1 in every 600–800 live births.[4] One of the most common causes of cleft lip and palate is Stickler syndrome, first described by Gunnar Stickler *et al*.[5] It is a hereditary progressive arthro-ophthalmopathy syndrome, recognized as the most prevalent hereditary cause of retinal detachment in children.[6,7] The incidence rate of Stickler syndrome is approximately 1 in 10,000 newborns,[8] with a prevalence rate of 1 in 7500–9000 newborns.[9] It is not only genetically but also phenotypically heterogeneous syndrome causing abnormal collagen formation, that leads to the most common causes of connective tissue dysplasia. STL1 syndrome can affect such various organs as the eyes, ears, joints, and orofacial regions.[10,11]

There are six different types of Stickler syndrome, each caused by mutations in specific genes: *COL2A1*, *COL11A1*, *COL11A2*, *COL9A1*, *COL9A2*, and *COL9A3*.[12,13] These mutations could present either autosomal dominant or autosomal recessive inheritance patterns. The autosomal dominant forms are normally caused by mutation in the *COL2A1*, *COL11A1*, and *COL11A2* genes, whereas the autosomal recessive forms are caused by mutation in the *COL9A1*, *COL9A2*, and *COL9A3* genes, which are responsible for the formation of collagen types II, XI, and IX, respectively.[14-17] The aforementioned genetic mutations can result in the abnormal formation of collagen, which in turn gives rise to a subgroup of STL1, called nonsyndromic ocular Stickler.[18]

The clinical manifestations of STL1 include a range of abnormalities such as myopia, membranous congenital vitreous anomaly leading to retinal detachment, presenile cataract, midface hypoplasia, cleft palate, Pierre Robin sequence, early-onset arthritis, mild spondyloepiphyseal dysplasia, and sensorineural deafness.[18-21]

Čopíková *et al*. studied patients with Stickler syndrome in the Czech Republic to evaluate their clinical manifestations and correlated variants using whole exome sequencing of the *COL2A1* and *COL11A1* genes, confirmed by Sanger sequencing.[22] Likewise, Choi *et al.* also evaluated the clinical manifestations of patients affected by Stickler syndrome and employed whole exome sequencing, gene panel analysis, and Sanger sequencing to identify all variants in the *COL2A1* and *COL11A1* genes.[7]

Considering the significance of Stickler syndrome as a main cause of cleft palate and lip, and the lack of research on *COL2A1* and its variants in Iran, this study aimed to address this research gap. By investigating common variants of the *COL2A1* gene in patients suspected of having STL1, the study intended to enhance the understanding of the genetic factors contributing to the syndrome and contribute prevention strategies for future generations.

METHODS

This cross-sectional study was carried out on Iranian patients in 2022, with the goal of identifying common *COL2A1* gene variants in individuals suspected of having STL1, in accordance with the STROBE guidelines.

Researchers collected a 5 ml blood sample containing ethylenediaminetetraacetic acid (EDTA) in a sterile tube. DNA was subsequently extracted from the peripheral blood using the salting-out method. For the polymerase chain reaction (PCR), oligonucleotide primers were designed using Primer3Plus and Oligo 7 software (version 7.54, Molecular Biology Insights Inc., Cascade, CO, USA) to amplify the common exons of the *COL2A1* gene and their corresponding introns [Table 1a]. Furthermore, Primer-BLAST was employed to ensure the specificity of the oligonucleotide primers by analyzing their sequences.

PCR amplification was performed in a 25 µl reaction mixture, which was subjected to the specified temperatures using an Applied Biosystems thermocycler, as presented in Table 1b. PCR products were subjected to electrophoresis on an agarose gel (2%) using Tris–Borate–EDTA buffer. In addition, to ensure the absence of contamination, a negative control was included in each experimental cycle. The size of the bands was determined by comparison with the ladder, and the band images were analyzed using the Gel Doc system alongside the ladder.

Participants

A total of 26 participants, suspected to be affected by STL1 syndrome, were selected using the census method from 3925 medical records of patients with cleft lip and palate at the cleft lip and palate clinic of Isfahan University of Medical Sciences.

Inclusion criteria

Patient selection was based on the following inclusion criteria:

1. Orofacial abnormalities: Cleft palate (open cleft, submucous cleft palate, bifid uvula), cleft lip and gum, cleft lip, and palate
2. Facial abnormalities: Malar hypoplasia, midface retrusion, broad or flat nasal bridge, scooped out facial appearance, micrognathia, retrognathia
3. Ocular abnormalities: Vitreous changes, retinal abnormalities, retinal detachment, myopia, astigmatism, strabismus, poor vision, early cataract, hyperopia, nystagmus, lateral eye deviation, and retinal pigment epithelium inclination
4. Hearing abnormalities: Sensorineural deafness, hearing loss, cochlear implant, eardrum rupture, otitis media with otosclerosis, recurrent otitis media, middle ear bone joint laxity, and recurrent otitis externa

Table 1a: Sequence of oligonucleotide primers

Exon number	Forward primer sequence	Reverse primer sequence	Length of the PCR product (bp)
2	CCCAGCCTACATTCTTCAGC	GTGGCCTTTCCTTTCTACCC	478
17	AAGCCCATTACTGCCTTCTG	CCCCTTTCCAGTAGACATCA	363
23	AAGGCCCAGATACAGCTTCA	AACACGGACCACAAGGACTC	421
42	ACTTCCCGCATTTTCTCCTT	ATCCTCTCTCACCACGTTGC	469
44	GGGTGCTTACCACTTGCACT	CCAAGTTTCCCTCCTCCTTC	373
51	AGGGGCACTTTCACACAATC	TACAGGGACAAGGGATGAGG	697

PCR=Polymerase chain reaction

Table 1b: The temperature and time settings of the polymerase chain reaction cycles

Step	Temperature	Time	Number of cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1 min	
Annealing			
Exon 2	62°C	1 min	30
Exon 17	61°C		
Exon 23	63°C		
Exon 42	60°C		
Exon 44	60°C		
Exon 51	62°C		
Extension	72°C	1 min	
Final extension	72°C	7 min	1

5. Skeletal abnormalities: Joint laxity or hypermobility, joint weakness or motor limitation, ligamentous laxity, early arthritis, hemiplegia, hemiparesis, and hypotonia

Exclusion criteria

The exclusion criteria consisted of two main factors:

1. The presence of environmental factors or teratogens could affect the mother and fetus health during pregnancy and result in the disease
2. Patients with other disorder criteria, including Pierre Robin syndrome, Van der Woude syndrome, and Treacher Collins syndrome.

Based on the ethical license issued by the Research Ethics Committee of Isfahan University of Medical Sciences, patient data were reviewed to identify individuals suspected of having Stickler syndrome. A total of 26 participants from various cities across Iran were recruited for the study. After providing their consent, each participant submitted a 5-cc peripheral blood sample, which was sent to the genetic laboratory at Al-Zahra Hospital in Isfahan for further analysis.

Data analysis

To determine the sequence of the desired fragment, PCR products were analyzed by Sanger sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Foster City, California, USA) ABI 3730XL platform (Applied Biosystems™). The sequencing results were then analyzed using Chromas 2.6.6 and NCBI Blast. Subsequently, *in silico* tools such as MutationTaster and the database available at https://www.ncbi.nlm.nih.gov/snp/were employed to investigate the impact of nucleotide changes on the proteins.

RESULTS

The study analyzed 26 patients, consisting of 20 men and 6 women, from various families who were suspected of having STL1. The average age of the participants was 13.06 years, with a standard deviation of 6.14 years. The ages ranged from 2 months to 33 years.

Peripheral blood samples were collected from all subjects, and genomic DNA was extracted using the salting-out protocol. The quality and quantity of the DNA samples were assessed using a NanoDrop 2000 spectrophotometer and agarose gel electrophoresis. All enriched DNA samples underwent sequencing of the coding exons 2, 17, 23, 42, 44, and 51 of the *COL2A1* gene. The DNA analysis revealed no pathogenic variants in any of the exons, except for exon 17. However, nucleotide changes were detected in introns 17, 22, 44, 51, and exon 2.

All suspicious variants in candidate exons were sorted using bioinformatics tools based on their position in exon, intron, and splice sites. The type of variant, amino acid substitution in missense variants, and their proximity to the splice site are listed in respective tables [Table 2a]. To determine the novelty of the variants, the ClinVar database and MutationTaster were explored for the candidate variants to check whether they have been previously reported in publications or as clinical evidence.

According to the findings, identified variants included:

Heterozygous synonymous mutation (c.213C>T) in one patient [Figure 1a]; Heterozygous hot spot mutation (c.1030C>T) in one patient [Figure 1b]; and polymorphisms (g. 17929_17930insC) in one patient; (g. 26675C>T) in nine patients, (g. 17938_17939insC) in three patients; (g. 29275T>C) in one patient; (g. 14845_14846insG) in one patient; (g. 29281T>G) in 11 patients; and (g. 29377G>A) in four patients.

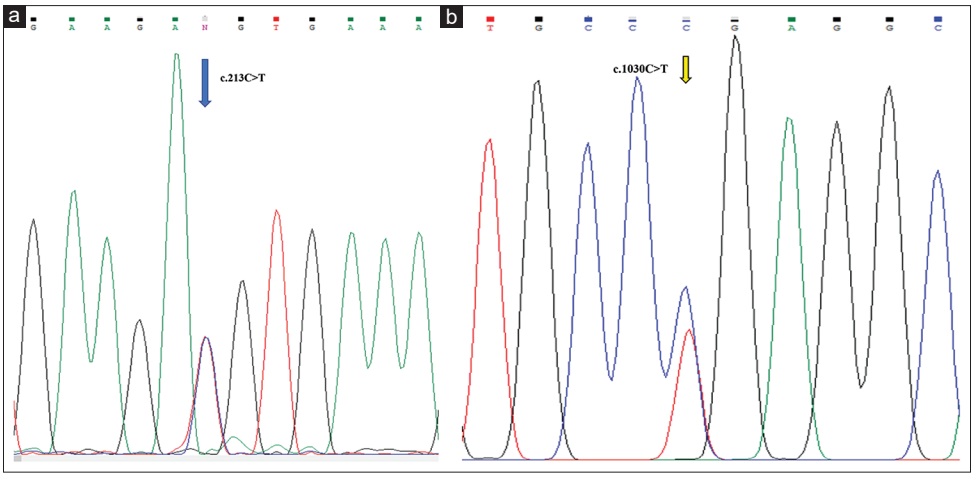

Figure 1: (a) Sequencing results of Sanger, Synonymous Mutation. The blue arrow indicates the substituted nucleotide (c.213C>T) as heterozygous in exon 2, in one family, (b) Sequencing results of Sanger, Hotspot Mutation. The yellow arrow indicates the substituted nucleotide (c.1030C>T) as heterozygous in exon 17 in one family

Table 2a: Identification of variants of exons 2, 17 and introns 17, 22, 44, and 51 of the gene *COL2A1* in patients suspected to have Stickler syndrome type I

Mutation	Site of mutation	Type of mutation	Amino acid change	Effect on protein	Reference
c.213C>T	Exon 2	Synonymous	p.Asp71=	-	-
c.1030C>T	Exon 17	Nonsense	p.Arg344*	NMD, protein features, amino acid sequence changed, splice site changes	[19,30-33]
g.17938_17939insC	Intron 22	Insertion	-	-	-
g.17929_17930insC	Intron 22	Polymorphism	-	-	-
g.26675C>T	Intron 44	Polymorphism	-	-	-
g.29275T>C	Intron 51	Polymorphism	-	-	-
g.29281T>G	Intron 51	Polymorphism	-	-	-
g.29377G>A	Intron 51	Polymorphism	-	-	-
g.14845_14846insG	Intron 17	Polymorphism	-	-	-

NMD=Nonsense-mediated decay

Pedigree was prepared using the online tool at https://pedigree.progenygenetics.com/for both family members with variant [Figure 2a and b].

As the data displays, the highest phenotype presentation prevalence rate was related to cleft lip and palate, while hemiparesis was the lowest clinical finding among the patients.

As the patients were selected from the Cleft Lip and Palate Clinic of Isfahan University of Medical Sciences, they were suspected of having STL_1 exhibited the syndromic form of the disease.

DISCUSSION

Stickler syndrome was initially discovered by Dr. Gunnar B. Stickler in 1956.[5] Since then, numerous pathogenic mutations have been identified, with at least 405 mutations reported for this gene in the HGMD database and previous studies.[23] Among the different types of Stickler syndrome, Type I is the most common, accounting for approximately 80% to 90% of all reported cases.[20,24]

Stickler syndrome overlaps clinical manifestations with such other syndromes as Donnai–Barrow syndrome and facio-oculo-acoustico-renal syndrome. Due to this similarity, accurate diagnosis through clinical examinations is unlikely, and genetic tests are required.[25] Early and accurate diagnosis of Stickler syndrome plays a vital role in managing the condition and preventing its recurrence in future generations.

In this study, all patients primarily presented with cleft lip and palate, while a smaller group exhibited hemiparesis. Multiple studies have documented a wide range of abnormalities associated with Stickler syndrome. For example, Huang *et al*. observed that myopia was the most common vision-related abnormality, while glaucoma had the lowest prevalence among patients with Stickler syndrome.[9] In another study, Čopíková *et al*. found that most patients suffered from myopia, notably high myopia, while congenital bilateral cataracts were less common.[22] Furthermore, Choi *et al*. stated that orofacial abnormalities were most prevalent, with hearing abnormalities being less frequent among patients.[7] Given that the *COL2A1* gene comprises 54 exons, the researchers in this study focused

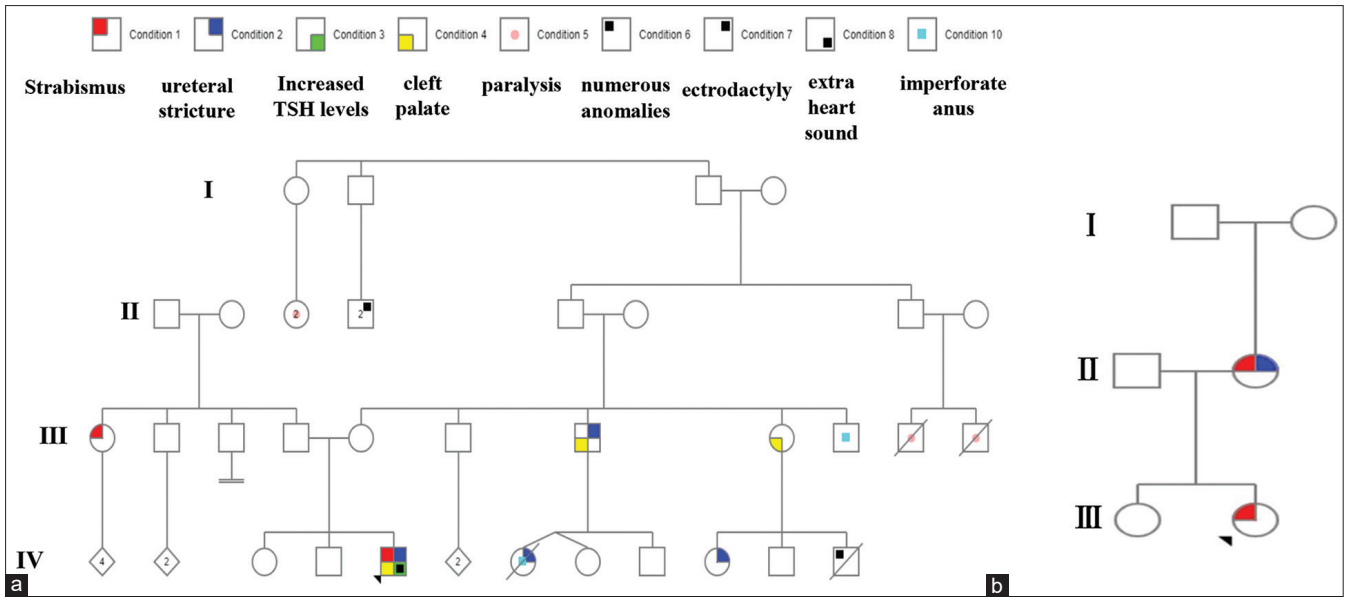

Figure 2: (a) The family pedigree with the synonymous mutation (c.213C>T) include 35 individuals spanning four generations. In the pedigree, circles represent females, and squares denote males. Each color corresponds to a specific clinical feature: red denotes strabismus, blue indicates ureteral stricture, green highlights increased TSH levels, yellow signifies cleft palate, pink denotes paralysis, black-shaded quadrants represent three phenotypes (numerous anomalies, ectrodactyly, and an extra heart sound), and light blue symbolizes imperforate anus, (b) The family pedigree with the hotspot mutation (c.1030C>T) includes of 6 individuals spanning three generations. In the pedigree, circles represent females, and squares denote males. Each color signifies a specific clinical feature: red indicates cleft palate, while blue represents retinal pigmentosa. Individual (III: 2) has been confirmed to have Stickler syndrome I

their analysis on the most frequently occurring exons, specifically exons 2, 17, 23, 42, 44, and 51, to investigate any nucleotide changes that may have occurred.

In this study, the researchers identified a synonymous variant, c.213C>T, in exon 2. Notably, this type of mutation does not affect the protein, and the individual does not show clinical manifestations of the disease. In addition, pathogenic variants in exon 2 were reported in other studies conducted by McAlinden *et al*.[26] and Sun *et al*.[27] Interestingly, a study by Yoon *et al*. in South Korea indicated that pathogenic variants occurred in exons other than exon 2 for patients who only had eye complications. However, this finding contradicts previous reports suggesting that patients with only eye complications typically have mutations in exon 2.[28]

Further, researchers of the present study observed a pathogenic variant, c.1030C>T, in exon 17. This finding is aligned with several previous studies that have also identified the same variant in exon 17. Particularly, Wang *et al*. in 2019,[29] Barat-Houari *et al*. in 2016,[30] Richards *et al*. in 2010,[31] and Hoornaer *et al*. in 2010[19] reported the c.1030C>T pathogenic variant in exon 17. In addition, Zhang *et al*. in 2020[32] also identified the c.1030C>T variant as a hotspot mutation in exon 17.

A review study by Zhang *et al.* (2020)[32] revealed that hotspot mutations in the *COL2A1* gene have been linked to Stickler syndrome type I [Table 2b]. Notably, the c.1030C>T mutation was also identified in the present study. This mutation changes the codon for the amino acid arginine to a stop codon, which can result in the production of a nonfunctional truncated protein or the elimination of the shortened mRNA during the nonsense-mediated decay process that consequently, type II collagen is not expressed, leading to the clinical manifestations of the disease in affected individuals.

In the present study, no pathogenic variant was detected in exon 23. However, this result is in contrast with previous research where pathogenic variants were found in exon 23. Specifically, Kondo *et al*.,[15] Wang *et al*.,[29] Besides, Rose *et al*.,[33] and Liberfarb *et al*.[34] reported the presence of pathogenic variants within this exon.

Moreover, no pathogenic variants were detected in exon 42 which contrasts with the results reported by multiple previous studies. Richards *et al*.,[31] Choi *et al*.,[7] Hoornaert *et al*.,[19] and Richards *et al*.[35] all identified pathogenic variants in exon 42. Moreover, no pathogenic variant was found in exon 44 which is in contrast to several other studies that identified such pathogenic variants within the same exon. Choi *et al*.,[7] Hoornaert *et al*. (2010),[19] and Huang *et al*.[9] all reported pathogenic variants in exon 44.

While the current study did not identify any pathogenic variants in exon 51 of the *COL2A1* gene, several other studies have reported the presence of such pathogenic variants in the same exon. These studies include those conducted by Zechi-Ceide *et al*.,[36] Huang *et al*.,[9] Hoornaert *et al*.,[19] and Choi *et al*.[7]

Table 2b: Hot spots of the *COL2A1* mutations

Variants	Mutation effect	Protein variants	Disorder
c.625C>T	Nonsense	p.Arg209*	STL 1
c.1030C>T	Nonsense	p.Arg344*	STL 1
c.1597C>T	Nonsense	p.Arg533*	STL 1
c.1693C>T	Missense	p.Arg565Cys	STL 1
c.1957C>T	Nonsense	p.Arg653*	STL 1
c.2101C>T	Nonsense	p.Arg701*	STL 1
c.2353C>T	Nonsense	p.Arg785*	STL 1
c.2710C>T	Missense	p.Arg904Cys	STL 1
c.2794C>T	Nonsense	p.Arg932*	STL 1
c.3106C>T	Nonsense	p.Arg1036*	STL 1
c.3138delT	Frameshift	p.Gly1047Alafs*83	STL 1

STL 1=Stickler syndrome type I

The discrepancy between the current study's findings and those of previous studies may be attributable to a number of factors. These include differences in sample size, ethnicity, race, the presence of a mutation in other exons, and the age group of the participants, as well as the use of next-generation sequencing (NGS). Moreover, additional funding is required to facilitate further investigation into other exons.

Notwithstanding the aforementioned discrepancies, the findings of the study remain of value in advancing our understanding with regard to the prevention of hereditary diseases, the promotion of genetic counseling before marriage, and the encouragement of prenatal testing.

CONCLUSION

A total of 26 patients with suspected Stickler syndrome type I were included in the study. The analysis demonstrated that a single individual was carrying a pathogenic variant (c.1030C>T). The identification of this hotspot mutation underscores the potential utility of preimplantation genetic diagnosis within the family as a strategy for preventing the recurrence of the disease in subsequent generations. In cases where pathogenic mutations were not identified, NGS is recommended for genetic analysis, given the considerable size of the *COL2A1* gene.

Genetic counseling is of pivotal importance in the prevention of similar cases, providing guidance on family planning decisions and the promotion of prenatal testing. A diagnosis of a *COL2A1* gene mutation can markedly improve the management of the disease for affected individuals.

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Conflicts of interest

There are no conflicts of interest.

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