



RESEARCH COMMUNICATION

A novel mutation in *USF1* gene is associated with familial combined hyperlipidemia

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Abstract

Background: Familial combined hyperlipidemia or FCHL is one of the most common genetic causes of hyperlipidemia and is associated with elevation of cholesterol, triglycerides or both, and increased serum apolipoprotein B (apoB). Linkage analysis and next generation sequencing have been successfully used for identifying rare genetic variants that have moderate-to-large effects.

Methods: We characterized a large pedigree from a proband identified following recruitment into the MASHAD study, in northeast Iran, with FCHL accompanied by early-onset coronary artery disease. We used linkage analysis for several candidate regions in previous studies such as 1q21-23, 11q23, and 8p, and then whole-exome sequencing to identify the disease-associated gene in this family.

Results: We identified a novel variant in the *USF1* gene, leading to a substitution of a tryptophan for arginine at position 196. Arg196Trp co-segregated in all the affected family members in this pedigree with clinical syndrome and was not found in any unaffected family members of this pedigree, or in unrelated controls.

Conclusions: We speculate that this mutation [Arg196Trp] in the *USF1* gene might be associated with FCHL and early-onset coronary heart disease in this family. However, the substantial mechanism requires further investigation. These findings indicate that *USF1* plays an important role in the biological pathways associated with lipid metabolism.

KEYWORDS

cardiovascular disease, cholesterol, familial combined hyperlipidemia, FCHL, triglycerides

Abbreviations: apoB, Apolipoprotein B100; CAD, Cardiovascular disease; CHD, Coronary artery diseases; FCHL, Familial combined hyperlipidaemia; HDL-C, High-density lipoprotein cholesterol; IDL, Intermediate-density lipoprotein; LDL, Low-density lipoprotein; MI, Myocardial infarction; NGS, Next-generation sequencing; VLDL, Very low density lipoprotein; WES, Whole-exome sequencing.

1 | INTRODUCTION

Familial combined Hyperlipidemia (FCHL) is the most common genetic and metabolic form of hyperlipidemia with prevalence of 0.5% to 2% worldwide and about 10% of these patients suffer from cardiovascular disease (CAD). This number has increased by approximately 11.3% in young survivors of myocardial infarction (MI) and by 40% among all the people who survived an MI.¹⁻⁴ Based on previous studies about 3.5 million people in Europe and 7.2 million people in the United States are affected by this disorder.^{5,6} Biochemically, FCHL is associated with increased levels of triglyceride, total cholesterol, or both, and increased levels of VLDL, LDL, decreased HDL, and increased apo B level.^{1,7} Based on a combination of past and recent definitions, FCHL is now been recognized as a common metabolic disorder and is characterized in a family by an increased serum triglyceride and LDL in two members of a family accompanied by increased risk of premature coronary artery disease.^{4,8} FCHL is genetically complex; in addition to the role of environmental factors, the FCHL is genetically heterogeneous.^{9,10}

Three important genetic loci for FCHL have been identified: 1q21-23, apolipoprotein A-I/C-III/A-IV cluster on chromosome 11 and lipoprotein lipase (LPL) gene on 8p that in several different studies had significant LOD scores with FCHL. Pajukanta et al. reported a linkage between FCHL and chromosome 1q markers in Finnish families.^{11,12}

Next-generation sequencing (NGS) has had an important role in identifying the genes and rare variants in a huge number of diseases with moderate-to-large effects.¹³ NGS can be applied for identification of rare mutations in families with specific extreme phenotypes with high-speed, high-power, and low-cost.^{14,15} The linkage analysis technique has been used for many years as the first method of gene discovery and genetic mapping of Mendelian and complex traits which

have a familial aggregation.¹⁶ Linkage analysis can be used in conjunction with NGS filtering approaches as an important and powerful technique for discovering of some genes involved in diseases etiology.^{17,18} In this study, linkage analysis and whole-exome sequencing were used to investigate the members of a large family, with a familial pattern of FCHL associated with coronary artery disease.

2 | MATERIALS AND METHODS

2.1 | Study population

The protocol of this study was approved by the local ethics committee of Mashhad University of Medical Sciences and all study participants in present study signed consent form. We identified a family with a recurring familial pattern of FCHL from a cohort as name MASHAD study in northeast Iran, Mashhad, who had an unusual constellation of juvenile-onset FCHL accompanied by an early-onset coronary artery disease and elevated fasting serum triglyceride and cholesterol levels that this was in accordance with FCHL diagnosis based on 2016 ESC/EAS Guidelines for the Management of Dyslipidemias. This large pedigree was notable for having several affected members with FCHL with what appeared to be an autosomal dominant inheritance pattern. Also there were several people in this pedigree who did not have these traits (Figure 1). In this family, affected members had a familial clustering and could trace their descent from a common ancestor that suggested that the affected family members had an autosomal dominant pattern. After evaluation of these family members, we collected clinical and laboratory data for all members of this family who were older than 30 years of age. Also we collected 5 mL of blood contain EDTA anticoagulant and then, genomic DNA was extracted. For biochemical analysis, we

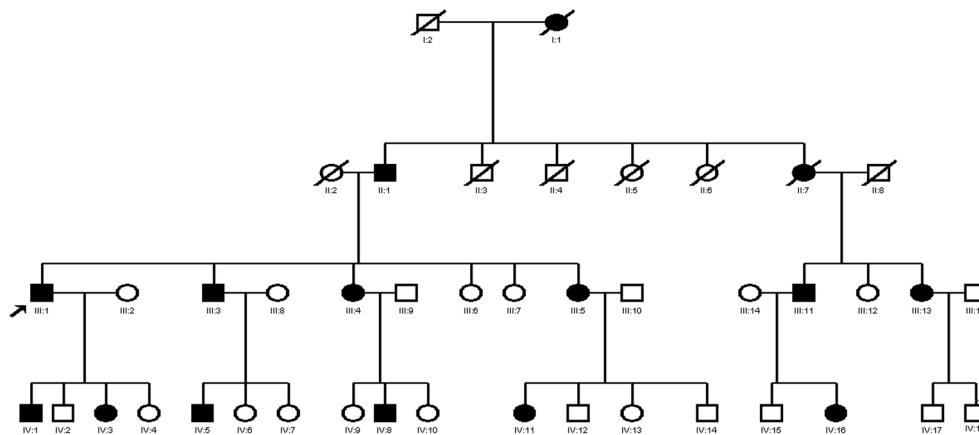


FIGURE 1 Pedigree of a family with FCHL associated with early-onset coronary artery disease. Circles indicate female family members, and squares male family members; slashes indicate that the family member is deceased. Family members with FCHL are indicated by solid symbols, and those without FCHL indicated by open symbols

TABLE 1 Characteristics of primers for PCR and sanger sequencing

Primers (5' -> 3')	Product size (bp)	Features associated with this product
Forward primer AGACACCACACCTAGCTACCA	284	Upstream stimulatory factor 1 isoform 1
Reverse primer GCCACAAGTCCCAGGGTAAA		

collected 5 mL of blood without anticoagulant and after 30 min we separated serum with centrifuge at 5000 RPM for 10 min at room temperature. Also 5 mL of blood contain EDTA was obtained, and genomic DNA was extracted. Also we collected 5 cc of blood from 100 healthy people over 60 years of age for later studies.

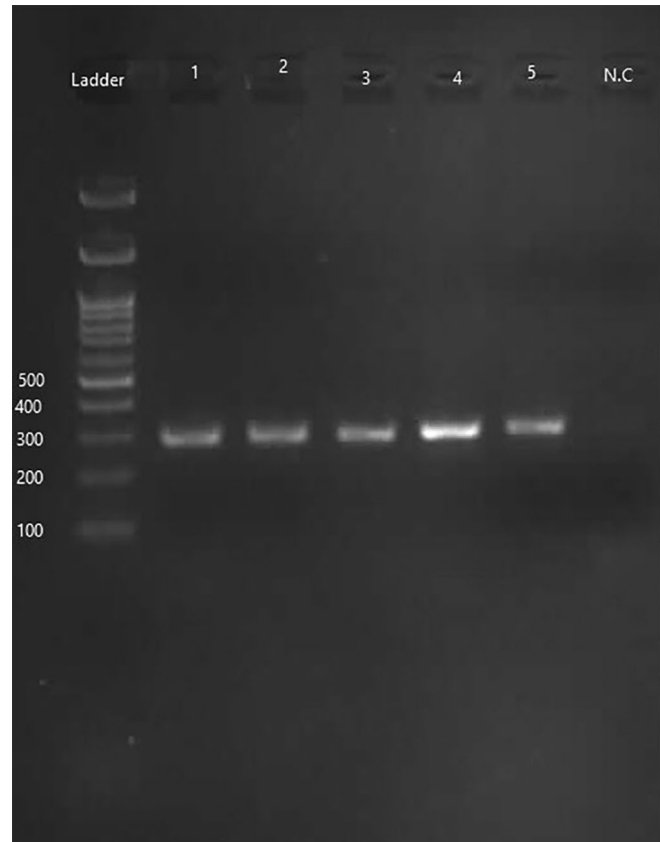
2.2 | STR markers genotyping and linkage analysis

DNA samples from 25 living family members were used for linkage studies, including 12 family member's diagnosed for FCHL disease and 13 unaffected family members. We performed a parametric analysis of linkage with STR markers for 1q21-23, 11q21, and 8P using samples obtained from affected members. Primer sequences are present in UCSC genome browser. A panel of two different STR markers were genotyped for 1q21-23 locus on chromosome 1 including D1S104 and D1S1677. We used the D11S4127 marker for apolipoprotein A-I/C-III/A-IV on chromosome 11 and D8S282 marker for LPL gene that is a candidate gene for FCHL based on the past studies. Subjects were considered to be affected or unknown to ignore the reduced penetrance effect. Polymerase chain reactions (PCR) were used under standard conditions optimized for each four markers. Details of conditions and concentrations reactions can be provided by the authors on request. We calculated the LOD score for each marker using the formula below: where NR = number of nonrecombinants and R = number of recombinants.

$$\text{LOD score} : \log_{10} \left[\frac{((R/(R+NR))^R) * ((1 - (R/(R+NR)))^{NR})}{0.5^{(NR+R)}} \right]$$

2.3 | Whole exome sequencing

DNA from the index patient from this family was sequenced for mutations by using the WES technique. Whole exome sequencing was used to enrich all exons of protein-coding regions as well as some important other genomic regions. NGS was performed using an Illumina Sequencer to sequence approximately 100 million reads. In total, this platform sequenced >95% of the targeted regions with an acceptable sensitivity of >99%. In this approach,

**FIGURE 2** PCR products with 284 bp length. N.C; Negative control, 1, 2, 3, 4, and 5 are the PCR products for sanger sequencing

duplications, micro-insertion/deletions, and point mutations can be detected. After this, we used bioinformatics analysis for sequencing results by using international databases and standard bioinformatics software then, filtration of raw data were performed, to remove common variants that are exist in reference genomes. We also applied filters against some published data in databases and those variants with novelty that had a deleterious effect on proteins, tissue expression were selected for more investigation.

2.4 | Variant validation studies with sanger sequencing

After WES annotation, we found a missense mutation in exon 8 *USF1* gene, which is reported to be an important gene for susceptibility to FCHL in previous studies.^{19,20} We confirmed this

TABLE 2 The clinical characteristics of the affected and unaffected family members

Groups	ID No.	Age of Onset		FBG (mg/dL)	Mean (±SD)	Chol (mg/dL)	Mean (±SD)	LDL-C (mg/dL)	Mean (±SD)	TG (mg/dL)	Mean (±SD)	ApoB (mg/dL)	Mean (±SD)	Smoking	HTN	
		CHD	CHD													
Affected	II2	45	45	79	86.7 (7.2)	305	291.5 (16.9)	270	250.5 (16.8)	201	195.3 (41.92)	130	132.4 (10.34)	No	Yes	
	III1	35	35	83		301		250		189		151		No	Yes	
	III3	41	41	99		295		253		202		125		No	Yes	
	III5	42	42	92		289		239		115		132		No	No	
	III9	39	39	92		296		256		180		128		No	Yes	
	III11	52	52	88		300		258		168		134		No	Yes	
	III13	49	49	79		285		240		221		150		No	Yes	
	IV1	35	35	85		312		271		198		139		No	Yes	
	IV3	37	37	88		261		218		214		120		No	yes	
	IV5	51	51	95		308		271		183		122		No	yes	
	IV9	38	38	74		300		261		162		120		No	Yes	
	IV11	31	31	91		275		243		205		141		No	Yes	
	IV16	37	37	81		262		226		301		129		No	Yes	
	Non-affected	III7	-	-	73	87.2 (9.2)	168	181.7 (9.4)	118	126.5 (5.4)	99	103.4 (18.5)	68	88.4 (19.2)	No	No
		III8	-	-	110		175		120		101		78		No	No
		III12	-	-	76		177		121		111		99		No	No
IV2		-	-	84		185		122		118		111		No	No	
IV4		-	-	91		168		120		85		101		No	No	
IV6		-	-	94		183		132		121		98		No	No	
IV7		-	-	90		175		126		74		59		No	No	
IV8		-	-	88		180		128		81		65		No	No	
IV10		-	-	83		186		129		94		109		No	No	
IV12		-	-	79		196		130		124		118		No	No	
IV13		-	-	87		172		125		80		85		No	No	
IV14		-	-	92		197		126		98		89		No	No	
IV15	-	-	78		182		131		106		96		No	No		
IV17	-	-	88		195		136		131		93		No	No		
IV18	-	-	95		186		133		128		57		No	No		

Abbreviations: Chol, cholesterol; FBG, fasting blood glucose; HTN, hypertension; LDL-C, low density lipoprotein cholesterol; TG, triglyceride.

variant in the index patient by PCR technology and sanger sequencing technique (PCR primers and their characteristics are shown in Table 1). Also we carried out segregation studies in other members of this family who were not applied for WES and PCR was used for sequencing target regions especially the variants of interest (Figure 2). The PCR was performed with mastermix solutions contain PCR buffer, MgCL₂, deoxyribonucleotide triphosphates, Taq DNA polymerase, and 10 pmol of each primer, 100 ng genomic DNA in a volume of 25 μ L. We set up thermocycler for an initial denaturation at 95°C for 5 min in Step 1 followed by 35 cycles in Step 2 for an denaturation at 95°C for 1 min, annealing at 59°C for 45 sec, an extension at 72°C for 1 min and a final extension in Step 3 at 72°C for 10 min. Then the products of PCR were run and separate on 1% agarose gel and visualized with green viewer. The amplified products were sequenced by sanger sequencing technique, and data were analyzed with the help of Snap gene software. In addition, we tested 100 healthy people in over 60 years of age for this variant from general population of this area.

3 | RESULTS

3.1 | Study population

The clinically characterized members of this family included 13 affected family members with FCHL. All 13 affected family members had CAD at a mean (\pm SE) age of 40 ± 5 years. All the affected family members had a serum total cholesterol >240 mg/dL, LDL>160 mg/dL, TG>120 mg/dL, and apoB >120 mg/dL and based on 2016 ESC/EAS Guidelines for the Management of Dyslipidaemias they met the standard definition of a metabolic syndrome called FCHL (Table 2).

3.2 | Linkage study

We carried out a parametric analysis of linkage with STR markers for 1q21-23, 11q21, and 8P using genotypes obtained from affected and non-affected members of this pedigree. The condition was considered as an autosomal dominant trait. Analysis of linkage showed significant evidence for of 1q21-23 linked to FCHL patients. The maximum LOD score was 3.05 and other loci showed a LOD score less than 1.0. Linkage analysis for 1q21-23 in this pedigree represented a founder mutation.

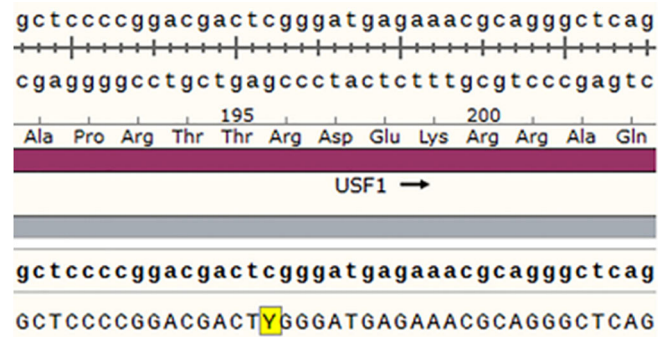


FIGURE 3 DNA sequence of a segment flanking p. Arg196Trp in *USF1* gene from an affected member. A single base substitution changes a cytosine to thymine, leading to the substitution of arginine (R) for tryptophan (W) at codon 196. Y denotes a heterozygote C \rightarrow T nucleotide substitution

No individual in this family was homozygous for this locus. The PCR conditions for STR markers are shown in Table 3.

3.3 | Whole-exome sequencing and validation using sanger sequencing

With filtration raw data from WES in the index patient, removing common SNPs and other variants that were found in databases as nonpathogenic, we identified a rare protein-altering variants in the index patient in *USF1* gene on chromosome 1 in 1q21-23 locus, which was linked to FCHL in all affected members of this family. This mutation led to a substitution of tryptophan for arginine at position 196 of *USF1* (p. Arg196Trp) (Figure 3). Segregation genotyping showed a cosegregation of this variant in all affected members. Also these variant was not found in healthy members of this pedigree.

The Arginine 196 of the *USF1* gene is highly conserved between orthologues and paralogues in some species including Ptrogodytes, Mmulatta, Fcatus, Mmusculus, Drerio, and humans. (Figure 4). We genotyped 100 ethnically healthy control from matched Iranians for Arg196Trp variant, and this mutation was absent in all samples. This variant was not found in databases such as ExAC, gnomAD, and iranome. Several programs such as Mutation taster, SIFT, PROVEAN, FATHMM, DANN, and FATHMM-MKL predicted the variant to be damaging (Table 4).

TABLE 3 Characteristics of STR markers used for linkage analysis in 1q21-23

STRs names	Forward primer sequence (5' - > 3')	Reverse primer sequence (5' - > 3')	PCR product range (bp)
D1S104	ATCCTGCCCTTATGGAGTGC	CCCACTCCTCTGTCATTGTA	152–162
D1S1677	AGTCAGCTTGATTGATCCAG	CTTAGTGTGACAGGAAGGACG	188–208
D11S4127	ATGAGAAGTGCCATCCAGC	ACTATGCCCACTGTGTGTGC	87–103
D8S	GGGCACAGGCATGTGT	GGCTGCATTCTGAAAGGTTA	260–272

Human	Y S P K S E A P R T T R D E K R R A Q H N E
Ptroglydotes	Y S P K S E A P R T T R D E K R R A Q H N E
Mmulatta	Y S P K S E A P R T T R D E K R R A Q H N E
Fcatus	X X X K S E A P R T T R D E K R R A Q H N E
Mmusculus	Y S P K S E A P R T T R D E K R R A Q H N E
Trubripes	Y I A K Q E A P R G S R D E K R R A Q H N E
Drerio	Y N A K S E G P R T S R D E K R R A Q H N E
Dmelanogaster	- S S K L E A Y K K - R D D K R R A T H N E

FIGURE 4 An amino acid sequence of *USF1* gene in position 196 arginine is completely conserved in different species

TABLE 4 Some features of R196W mutation *USF1* gene in prediction software's

#	Prediction software	Status
1	Mutation taster	Disease causing
2	DANN	Damaging
3	MetalR	Tolerated
4	SIFT	Damaging
5	FATHMM	Damaging
6	FATHMM-MKL	Damaging
7	PROVEAN	Damaging
8	DANN	Damaging

4 | DISCUSSION

FCHL is one of the most common type of hyperlipidemia worldwide with increased risk of premature CHD and due to increased VLDL and LDL levels affected persons have an elevation of both cholesterol and triglycerides in the blood.

The present study showed an association between a non-conservative mutation in the *USF1* gene and FCHL patients associated with early-onset CHD in a family with an informative pedigree. The *USF1* locus on 1q21-23 has been linked to FCHL and CHD in some previous studies.^{11,21,22}

USF1 is a transcription factor belong to the bHLH-Zip class, and the domain affected here is important for DNA binding and dimerization. The functional protein forms homodimers, or heterodimers with *USF2* and through binding at distal E-box elements, activates the transcription of some target genes.^{23,24} Thus some posttranslational modifications such as phosphorylation in *USF1* can change transactivation activity.²⁵ This gene can also play a role in chromatin barrier insulator function and protect euchromatin regions from heterochromatin-induced gene silencing.²⁶ Reduced expression of *USF1* gene may lead to increased production and reduced metabolism of lipoproteins and plasma lipids because *USF1* is involved in regulation of several genes of glucose and lipid metabolism.²⁵ Whole-genome ChIP analysis identified 2,518 binding sites for *USF1*

in HepG2 cells related to chromatin context, which were strongly recorelated with expression level of some target genes and this suggested key roles for *USF1* gene in transcription activation.²⁴

We have found that FCHL is linked to 1q21-23 and this is confirmed in some previous linkage analysis such as studies done by Pajukanta et al.,^{11,19} Huertas-Vazquez,²⁷ and Pei et al.²¹ investigated FCHL families from nonisolated regions in China and Germany to investigate some evidence for linkage to a chromosome 1q locus and they observed a linkage between FCHL patients and 1q21-q23 locus.²¹ Pajukanta et al. also carried out a linkage analysis using several markers for 10 chromosomal regions and their results strongly supported that a FCHL gene is located on chromosome 1q.¹¹ It seemed that FCHL linkage to 1q21-23 is due to that 1q21-23 locus has several genes associated with lipid metabolism and usually based on recombination roles, and it cosegregates in affected members of a pedigree if the genes in this locus are causes of the diseases.

We carried out WES for one patient of this pedigree and data analysis with more focus on 1q21-23 region showed a missense mutation in exon 8 of *USF1* gene (p. Arg196Trp).

Initially, Pajukanta et al. showed that FCHL is linked to the *USF1* gene and they observed that FCHL is associated with a common haplotype contain noncoding SNPs within the *USF1* gene.¹⁹ It was also shown that individuals with the risky allele have a lack of insulin-induced increase of *USF1* expression in fat tissue and skeletal muscle. The *USF1* gene has an important role in adipose tissue metabolism and through mediating the glucose-regulated expression of hormone-sensitive lipase (HSL) influences de novo lipogenesis by HSL, which is an important enzyme in the regulation of lipid source in adipose tissue.^{28,29} In addition, it was also reported that *USF-1* deficiency alleviates inflammation, boosts cholesterol efflux from macrophage-foam cells and prevents cholesterol accumulation in macrophages.³⁰ *USF1* also has an important role in transcription of fatty acid synthase, which is involved in the synthesis of fatty acids and can play an important role in the transcription of several apolipoproteins such as APOCIII, APOAII, and APOE, hepatic lipase, angiotensinogen, glucokinase, and

ABCA1.^{29,31} To date, 121 SNPs in the *USF1* gene have been identified in the dbSNP database and only the rs4126997 T>C causes a nonsynchronous mutation but data on functional studies and allele frequency are not available for this SNP. rs2073658 A>G in intron 7 and rs3737787 C>T in the 3'-UTR are two functional SNP of *USF1* gene that are in linkage disequilibrium, which the minor allele is accompanied with normal expression of *USF1* in fat tissues and human muscles but there is a loss of insulin-induced upregulation of *USF1* mRNA and related target genes of *USF1* gene and as a result insulin-mediated anti-lipolytic activity reduces.^{32–34}

As *USF1* gene has an important role in the regulation of numerous genes involved in the metabolism of lipids and glucose, non-responsive *USF1* expression can adversely affect the metabolism of lipids and lipoproteins resulting in increased plasma lipids, thus *USF1* is an ideal candidate gene for involvement with clinical features of FCHL and hyperlipidemia and some problems associated with CHD such as hypertension and we can say given that *USF1* gene affects the complex lipid phenotype of FCHL. Arg196Trp mutation *USF1* gene might be associated with FCHL and early-onset coronary heart disease in this family and can be a cause for related high triglycerides, ApoB, and total cholesterol. Because the *USF1* gene product is a transcription factor known to regulate the expression of genes involved in lipid metabolism it is plausible, though unproven, that genetic variation such as Arg196Trp variant in *USF1* expression or function could cause the phenotype of FCHL. Also, since this mutation was observed in the heterozygous state, it can be said that this mutation can be a gain of function mutation but for further confirmation it is necessary to undertake a functional study for this variant and can be later confirmed in controlled studies in animal models.

5 | CONCLUSIONS

We have confirmed that 1q21-23 locus is linked to FCHL in an Iranian pedigree, and a new mutation in *USF1* gene, which is within this locus may be related with some clinical characteristics, which is specified by elevation cholesterol, triglyceride, CAD. Therefore, our results indicate that *USF1* gene plays an important role in some biologic pathways associated with lipid metabolism.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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