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Clinical and Molecular Spectrum of Tuberous Sclerosis Complex Patients: Identification of Three Novel Mutations

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ABSTRACT

Objective: Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous syndrome. TSC arises from mutations in either *TSC1*, at 9q34, or *TSC2*, at 16p13.3. Skin lesions, such as hypomelanotic macules, facial angiofibromas, shagreen patches, and ungual fibromas, are frequently seen in these patients. The present study aims to investigate clinical manifestations, molecular findings and phenotype-genotype correlations in 17 patients with TSC.

Materials and Methods: *TSC1* and *TSC2* molecular analyses were performed on a next-generation sequencing platform (Illumina MiSeq). Variant interpretation was made in accordance with the American College of Medical Genetics 2015 recommendations.

Results: Four patients carried a heterozygous mutation in *TSC1*, while the remaining seven carried mutations in *TSC2*. Three novel variants in *TSC2* were defined. Sequencing failed to detect a mutation in six patients. In only one of these patients, multiplex ligation-dependent probe amplification (MLPA®) could be performed, and a large deletion in the *TSC1* gene was detected. A wide spectrum of phenotypic features was noted throughout the study group. Dermatological findings were observed in almost all patients.

Conclusion: In this study, in addition to the three novel mutations reported herein, the spectrum of *TSC1* and *TSC2* gene mutations and their phenotypes were reported.

Keywords: Tuberous sclerosis complex, *TSC1*, *TSC2*, mTOR inhibitor

INTRODUCTION

Tuberous sclerosis complex (TSC) is a rare neurocutaneous disorder characterized by typical cutaneous findings and multiple hamartomatous lesions in different organs. The incidence of the disease has been reported as being 1 per 6,000 to 10,000 live births (1). Disease shows complete penetrance with variable expressivity. The phenotypic spectrum may vary between mild to severe even in patients from the same family.

Tuberous sclerosis complex arises from heterozygous mutations in *TSC1* or *TSC2* gene located on 9q34 and 16p13, respectively (2). Loss of function mutations in *TSC1* and *TSC2* may lead to activation of the rapamycin (mTOR) pathway, responsible for cellular proliferation and inhibition of cellular apoptosis. More than 400 unique *TSC1* mutations and 1300 unique *TSC2* mutations have been reported in The Human Gene Mutation Database (HGMD) (3).

Revised Diagnostic Criteria for Tuberous Sclerosis Complex was established by a consortium in 2012 (1). In cases that do not meet the clinical criteria, identification of a pathogenic mutation in the *TSC1* or *TSC2* gene is sufficient for diagnosis. Molecular diagnosis also helps to predict prognosis and is essential for proper genetic counseling.

In this study, we aimed to define the clinical and molecular features of patients with TSC and contribute to the genotype and phenotype correlation.

MATERIALS and METHODS

Study Group

Seventeen patients diagnosed to have TSC based on clinical diagnostic criteria were included in this study. All patients were evaluated by an expert clinical geneticist between 2015 and 2019. Demographic data, family history and clinical features were all obtained from hospital records. Cranial and abdominal imaging results were evaluated retrospectively.

This study was approved by the Ethical Committee of the Ege University Medical Faculty (dated: 15.04.2020, no. 20-4.1T/28), and samples from the patients were obtained in accordance with the Helsinki Declarations. Written

Table 1. Demographical findings and clinical features of the study group

Case no	Genotype	Age	Sex	Symptom on admission	Family history	Cutaneous findings	ID	TAND	Epilepsy	Semiology	CT	SEN	SEGA	Renal	Cardiac	Ophthalmological examination
1	*TSC1*	28 years	F	Abdominal pain, renal mass	Mother and sister (epilepsy)	Hypopigmented macules, FA, shagreen patch	–	–	–		+	+	–	Renal cell carcinoma	Normal	Normal
2	*TSC2*	7 years	M	CR, cutaneous lesions	No	Hypopigmented macules	Severe	+	+	Infantile spasm	+	+	-	AML	Rhabdomyoma	Normal
3	*TSC1*	13 years	F	Head ache, vomiting	No	Hypopigmented macules, shagreen patch	Mild	–	+	Focal non-motor (emotional)	–	–	+	AML	Normal	Normal
4	*TSC1*	28 years	M	Intractable epilepsy	No	Hypopigmented macules, FA shagreen patch	Mild	–	+	Generalized tonic-clonic	+	+	–	Normal	Normal	Normal
5	*TSC2*	5 years	F	Hypopigmented macules	No	Hypopigmented macules, FA	Mild	–	–		+	+	+	AML, renal cyst	Rhabdomyoma	Astrocytic hamartoma
6	NMI	21 months	M	Fetal CR	No	Hypopigmented macules	–	–	–		+	–	–	Normal	Rhabdomyoma	Normal
7	NMI	9 years	M	Seizure	Mother (PKD, hypopigmented macules)	Hypopigmented macules	Mild	–	+	Focal motor	+	+	+	PKD	Normal	Bilateral lens dislocation
8	*TSC2*	21 months	F	Fetal CR	No	Hypopigmented macules, FA	–	–	+	Infantile spasm, focal motor	+	+	–	Normal	Rhabdomyoma	Normal
9	*TSC1*	16 months	M	Fetal CR	No	Normal	–	–	–		–	+	–	Normal	Rhabdomyoma	Normal
10	NMI	15 months	F	Fetal CR	No	Hypopigmented macules	–	–	–		+	+	–	Normal	Rhabdomyoma	Normal
11	*TSC2*	20 months	F	Fetal CR	No	Hypopigmented macules, FA	Mild	–	+	Focal motor	+	+	–	Normal	Rhabdomyoma	Normal
12	*TSC1*	45 years	M	Seizure	No	Hypopigmented macules, ungual fibroma	Mild	+	+	Focal motor	+	–	–	AML, renal cyst	Normal	Normal
13	*TSC2*	27 months	M	Fetal CR	Mother (FA, hypopigmented macules, subungual nodule)	Hypopigmented macules, FA	Mild	–	+	Infantile spasm	+	+	–	Renal cyst	Rhabdomyoma	Normal

Table 1 (cont.). Demographical findings and clinical features of the study group

Case no	Genotype	Age	Sex	Symptom on admission	Family history	Cutaneous findings	ID	TAND	Epilepsy	Semiology	CT	SEN	SEGA	Renal	Cardiac	Ophthalmological examination
14	NMI	23 years	F	Hypopigmented macules	Mother (Hypopigmented macules, shagreen patch)	Hypopigmented macules, FA, shagreenpatch	–	–	–		–	–	–	Normal	Normal	Normal
15	*TSC2*	3 years	F	Fetal CR	No	Hypopigmented macules	–	–	+		–	+	–	AML	Rhabdomyoma	Normal
16	*TSC2*	11 years	F	Neonatal hyperbilirubinemia	No	Hypopigmented macules, FA, shagreen patch	Mild	–	+	Focal motor	+	+	–	AML, oncocytoma	Normal	Normal
17	NMI	22 months	F	Seizure	No	Hypopigmented macules	–	–	+	Infantile spasm, focal motor	+	+	–	Normal	Rhabdomyoma	Normal

AML: Angiomyolipoma; CR: Cardiac rhabdomyoma; F: Female; FA: Facial angiofibroma; M: Male; NMI: No mutation identified; PKD: Polycystic kidney disease

informed consent for genetic testing was obtained from all patients and/or their parents/guardians.

Molecular Analysis and Data Interpretation

Genomic DNA of the patients was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Kit (Qiagen, Germany). DNA quality and quantity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All coding regions and exon-intron boundaries of the *TSC1* and *TSC2* were amplified by PCR using specific primers. Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA) was used for target enrichment; in accordance with the manufacturer's instructions. Paired-end sequencing was performed on all samples using the Illumina MiSeq platform (Illumina Inc., San Diego, CA). Sequencing data were analyzed using the Integrative Genomics Viewer (IGV).

The impact of previously undefined *TSC1* and *TSC2* variants on the protein structure was evaluated using several in silico prediction tools, such as MutationTaster, REVEL, and SIFT (4–6). Conservation of residues across species was evaluated by GERP (7). Pathogenicity of the detected variants was classified in accordance with the American College of Medical Genetics (ACMG) guidelines (8). In one of the six patients in whom sequencing failed to detect a mutation, multiplex ligation-dependent probe amplification (MLPA®) was performed collaborating with another center. The diagnosis was depended on only clinical findings in the remaining five patients.

Statistical Analysis

Descriptive analysis was used to evaluate the data in this study. Statistical analyses were performed using SPSS v. 25.0 (IBM, Armonk, NY, USA).

RESULTS

Clinical Manifestations

Seventeen patients with TSC from unrelated families [10 (58.8%) females and seven males (41.2%)] enrolled in this study. The median age of the patients was five years (ranged between 15 months to 45 years). The majority of the patients (8, 47.8%) was presented during the fetal or neonatal period with cardiac rhabdomyosarcoma. In four patients, a positive family history with an affected parent and/or a sibling was noted. Clinical features of the study group are given in Table 1.

In nine patients (52.9%), a developmental delay or intellectual disability was observed, with eight of them mildly affected. Eleven patients (64.7%) had epilepsy. Different types of seizures included epileptic spasms, focal motor and generalized tonic-clonic were observed. Neuroimaging studies were performed in all patients. Cortical tuber, subependymal nodules and subependymal giant cell astrocytoma (SEGA) were detected in 11 (64.7%), 10 (58.8%) and three (17.6%) of the patients, respectively.

Cutaneous lesions, including hypomelanotic macules (94.1%), facial angiofibroma (47.1%), and shagreen patch (29.4%), were noted in almost all patients (Fig. 1a). Confetti skin lesion was observed in Case 15 (Fig. 1b). Cardiac and renal involvements were observed in 10 (58.8%) and 9 (52.9%) patients, respectively. Oph-

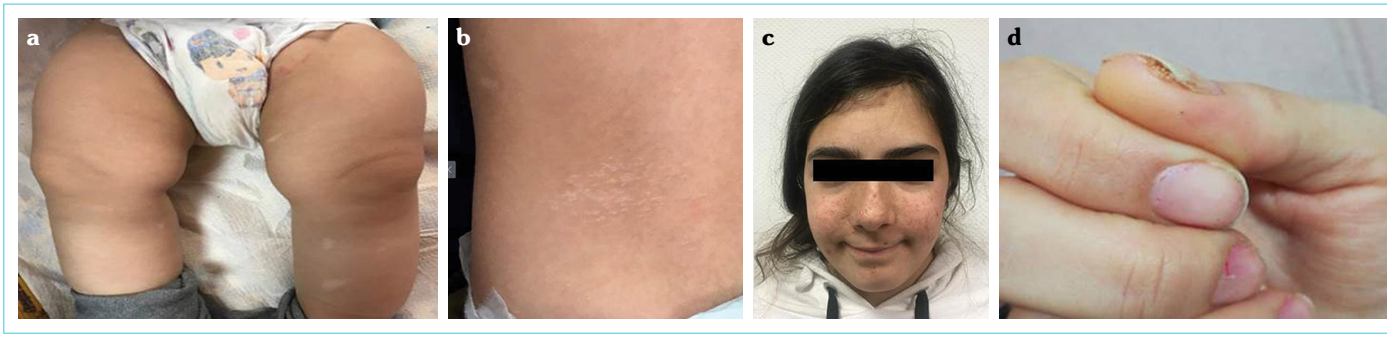

Figure 1. (a) Hypopigmented macule in Case 6. (b) Confetti skin lesions in Case 15. (c) Facial angiofibroma in Case 16. (d) Subungual fibroma in the mother of the Case 13

thalmological examination revealed astrocytic astrocytoma in one patient and bilateral lens dislocation in another patient.

Oral mTOR inhibitor therapy was administered to five patients. Case 3, a 14-year-old girl, was diagnosed to have TSC via hypomelanotic macules, SEGA and epilepsy at the age of nine years. She had focal emotional seizures with crying lasting for 3–4 seconds. Despite a combination therapy of levetiracetam and valproic acid (VPA), seizures continued 1–2 times a week. At the age of 11.5 years, an everolimus treatment with a dose of 10 mg per day was started. Following everolimus treatment, a marked decrease in seizure frequency and improvement in cognitive functions were observed. VPA dose was reduced by 40% in this patient. In four neonatal patients (Case 8, Case 9, Case 10 and Case 11), sirolimus was administered for cardiac rhabdomyomas leading arrhythmia and/or left ventricular outflow obstruction. Treatment was initiated at 2 to 13th days of life with a dose of 2 mg/m^2 per day. The median duration of treatment was six months (ranged between 2–16 months), and normalization of the electrocardiogram and prominent reduction of rhabdomyoma size were observed in all of them. Additionally, in Case 16, an 11-year -old girl, a topical sirolimus treatment was applied for severe facial angiofibromas (Fig. 1c), and a limitation in the extension of the lesions was observed within six weeks.

Molecular Findings

Sequence analysis of *TSC1* and *TSC2* genes established a molecular diagnosis in 11 (64.7%) of the study group. MLPA analysis could be performed in only one of the patients in whom a disease-causing variant could not be detected using sequence analysis, and a large deletion, including eight exons of the *TSC1* gene, was identified. Five different variants (two frameshift, two nonsense, and one large deletion) were detected in the *TSC1* gene and seven different variants (three frameshift, two splice site, two missense, and one nonsense) in the *TSC2* gene. Three variants in the *TSC2* gene have not been previously reported in public databases; however, they have been predicted as being disease-causing using in silico analysis. Molecular test results are given in Table 2.

In four cases, one of the parents was clinically diagnosed to have TSC (Fig. 1d). Unfortunately, molecular defects could not be detected by sequencing in two of these cases (Case 7 and 14). In one of the other two cases (Case 13), the affected mother was found to have a disease-causing variant. In the other case (Case 1), parents were not available for segregation analysis.

DISCUSSION

In this study, 17 unrelated patients with TSC followed by a single center were evaluated retrospectively. Tuberous sclerosis complex is a phenotypically heterogeneous autosomal dominant genetic disorder. Symptoms of the disease may occur at any age, and both sexes are equally affected (1). It is significant to recognize patients with mild symptoms and verify them with a molecular diagnosis, follow-up, plan treatment and identify risky individuals in the family.

Cutaneous lesions are the most common findings of TSC. They are seen in almost all patients, and patients are generally recognized via dermatological findings (9). Ding et al. (10) evaluated clinical and molecular features of 174 unrelated patients with TSC, and reported the frequency of hypomelanotic macules, facial angiofibromas and shagreen patches as being 95.40% (166/174), 43.68% (76/174), and 32.76% (57/174). In our study, almost all patients had cutaneous lesions, mostly hypomelanotic macules.

Neurological findings, including epilepsy, cognitive impairment, autism spectrum disorder, and CNS lesions, are also commonly seen in TSC patients, and they are the most significant factors affecting mortality and morbidity. Although infantile spasms are common, all types of seizures may occur with advancing age (11). The frequency of epilepsy in patients with TSC has been reported as being 53–85% in different studies (10, 12–14). The study conducted by Ding et al. (10) revealed that 85% of 174 patients with TSC had epilepsy with 65.54% of them presented epilepsy in the first year of life. Benova et al. (15) showed that the presence of severe epilepsy, a high number of dysplastic lesions on MRI, and abnormal background activity on EEG are predictors of intellectual disability in patients with TSC. Epilepsy has been noted in 64.7% of our study group (11 patients), and it is associated with neurocognitive dysfunction in 72.7% of them (8 patients).

Renal involvement is also a significant cause of mortality and morbidity in TSC. Renal lesions usually occur in the infantile period and increase with age, causing hypertension, hematuria or renal failure (16, 17). Kingswood et al. (17) investigated 2216 patients registered to the TuberOus SClerosis registry to increase disease Awareness (TOSCA) and found renal angiomyolipoma (AML) in 51.8% of 2065 patients who had renal imaging. In their study, the mean age at the time of diagnosis was 16.9 years. Renal AML was lower (35.3%) in our study. This may be because our study included younger patients, and they may show renal

Table 2. Molecular findings of the study group

Case no	*TSC1*		*TSC2*		Mutation type	Novelty	GERP score	ACMG classification
	Mutation (DNA)	Mutation (protein)	Mutation (DNA)	Mutation (protein)				
1	c.[286_290delGTCAT];[=]	p.[Val96LysfsTer9];[=]	Normal		Frameshift	Known	5.3499	Pathogenic
2	Normal		c.724dupA	p.[Thr242AsnfsTer96];[=]	Frameshift	Known	5.05	Pathogenic
3	del ex1-8		Normal		Large deletion	Known		Pathogenic
4	c.[982C>T];[=]	p.[Gln328Ter];[=]	Normal		Nonsense	Known	5.7899	Pathogenic
5	Normal		c.[4074delC];[=]	p.[Ile1359SerfsTer24];[=]	Frameshift	Novel	4.8499	Pathogenic
6	Normal		Normal					
7	Normal		Normal					
8	Normal		c.[1947-1G>C];[=]		Splice site	Known	5.4499	Pathogenic
9	c.[2227C>T];[=]	p.[Gln743Ter];[=]	Normal		Nonsense	Known	5.69	Pathogenic
10	Normal		Normal					
11	Normal		c.[4375C>T];[=]	p.[Arg1459Ter];[=]	Nonsense	Known	5.0199	Pathogenic
12	c.[2509_2512delAACA];[=]	p.[Asn837ValfsTer11];[=]	Normal		Frameshift	Known	5.6399	Pathogenic
13	Normal		c.[3593T>C];[=]	p.[Leu1198Pro];[=]	Missense	Novel	4.7399	VUS
14	Normal		Normal					
15	Normal		c.[2221-2A>G];[=]		Splice site	Known	5.46	Pathogenic
16	Normal		c.[5020_5023dupACCC];[=]	p.[Pro1675HisfsTer32];[=]	Frameshift	Novel	4.59	Pathogenic
17	Normal		Normal					

DNA: Deoxyribonucleic acid; GERP: Genomic Evolutionary Rate Profiling; ACMG: American College of Medical Genetics; VUS: variant of unknown significance

involvement later in life. Polycystic kidney disease (PKD) is a rare manifestation of TSC that arises from contiguous deletions of *TSC2* and PKD1 genes (18). The incidence of tuberous sclerosis/polycystic kidney disease contiguous gene syndrome (PKDTS, MIM #600273) has been reported to be approximately 2–5% of all TSC cases (17, 19). In the present study, Case 7 and his affected mother had a history of PKD. In addition to typical TSC findings (mild intellectual disability, epilepsy, hypopigmented macules), he had bilateral lens dislocation, to our knowledge, that has not been reported in patients with TSC to date. The sequence analysis of the patient failed to detect a mutation in *TSC1* or *TSC2* gene. A contiguous gene deletion leading PKDTS and lens dislocation may explain the phenotype in this patient.

Cardiac rhabdomyoma is one of the pathognomonic findings of TSC seen in newborn and childhood, with a frequency of 30–60% (10, 11, 19). They generally regress spontaneously; however, in some cases, it may cause cardiac failure or arrhythmias by resulting stenosis in the outflow tract (20, 21). In our study, the frequency of cardiac rhabdomyoma was similar to the literature. While spontaneous regression of cardiac rhabdomyoma was observed in the majority of patients, four patients required treatment.

Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved mTOR inhibitor treatment in patients with TSC with renal angiomyolipomas, SEGA and refractory partial-onset epilepsy. However, in recent years, the safety and efficacy of mTOR inhibitors in various clinical conditions associated with TSC have been evaluated (22). It has been shown that mTOR inhibitors provide a significant reduction in rhabdomyoma size in approximately four weeks. (21, 23, 24). Additionally, Nathan et al. (25) showed that systemic mTOR inhibitor treatment resulted in the reduction of facial angiofibroma size in 14 adults treated for lymphangioleiomyomatosis, and in three of them, no deterioration was observed in the lesions at a median of 14 months after cessation of treatment. Topical treatment is another option for cutaneous lesions. Several studies revealed that topical rapamycin and sirolimus provide improvement in facial angiofibromas (26–28). However, recurrence of the lesions following discontinuation of the medication has been observed. In our study, five patients received oral mTOR inhibitor treatment and one patient received topical mTOR treatment. Clinically, noticeable improvement

in disease symptoms was observed in all patients. Beside these therapeutic effects, mTOR inhibitors may lead to many adverse reactions, including non-infectious pneumonitis, rash, stomatitis and infections (29). Saffari et al. (22) evaluated the safety of the mTOR inhibitor treatment (everolimus) in 17 patients with TSC under the age of two years. They reported that everolimus is an efficient and safe agent with mild adverse events in the great majority of cases. No major side effect was noted in our patients who received mTOR inhibitors. In the literature, to our knowledge, there has been no study investigating relationships between genetic defects and treatment effects in TSC patients. However, it is expected that as the number of patients treated increases, the relationship between treatment and genotype will be better understood.

To our knowledge, three variants found in *TSC2* were identified for the first time in this study. Two of them were frameshift variants and classified as pathogenic in accordance with ACMG recommendations. The c.3593T>C substitution results in an amino acid change (leucine to proline) at 1198th codon of *TSC2* mRNA, a highly conserved amino acid during evolution. In silico analysis showed this substitution is deleterious on protein function. Additionally, the affected mother was a carrier of the same variant using segregation analysis. Therefore, this variant in the *TSC2* gene was considered to be disease-causing.

Previous studies showed that despite comprehensive molecular analysis, a genetic diagnosis could not be achieved in 10–25% of patients with TSC (11, 19). It has been considered that those no mutation identified (NMI) patients are either mosaic or have an intronic, mutation which is not covered by the technique used (Sanger sequencing or NGS). In our five patients (29.4%), a disease-causing variant could not be identified. MLPA analysis could not be performed in these patients due to MLPA analysis for TSC was not available in our center. Because of economic or technical reasons, it could be performed in another center for only one of the six patients without mutations by sequencing. The failure to exclude deletion/duplication mutations in these patients is a limitation of our study.

In TSC, genotype generally provides to predict the clinical picture. In the literature, *TSC2* mutations have been reported more frequently in both sporadic and familial cases and shown to be associated with poor prognosis (2, 11). However, some specific *TSC2* variants are associated with the mild phenotype (30). On the other hand, it has been reported that NMI patients show a milder phenotype than patients with a known pathogenic variant in *TSC2* (31). Peron et al. (32) had compared the phenotype of NMI patients with patients carrying a mutation in either the *TSC1* or *TSC2* gene. They showed that NMI patients were diagnosed at an older age, had more frequent normal cognition and less frequent epilepsy, subependymal nodules and giant cell astrocytomas than patients with *TSC2* pathogenic variants. However, they found no significant differences between NMI patients and *TSC1* mutation-positive patients, except for renal and pulmonary involvement. In our study, the patients carrying the *TSC2* variant were more frequent than either a patient carrying *TSC1* variants or patient with no mutation. However, the number of patients was not sufficient to compare the phenotype between the groups.

CONCLUSION

This study provides information about clinical and molecular features in patients with TSC from Turkey. Three novel *TSC2* variants detected in this study expands the mutation spectrum of the disease. Because TSC symptoms are treatable using mTOR inhibitors, early genetic diagnosis is essential for promoting appropriate management and decreasing the disease's harmful effects.

Ethics Committee Approval: The study was approved by the Ethical Committee of the Ege University Medical Faculty (date: 15.04.2020, number: 20-4.1T/28) and samples from the patients were obtained in accordance with the Helsinki Declarations.

Informed Consent: Written informed consent for genetic testing was obtained from all patients and/or their parents/guardians.

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Author Contributions: Concept – EI, TA, FÖ; Design – EI, AES, TA, FÖ; Supervision – HO, TA, ÖÇ, FÖ; Resource – HO, TA, SG, HT, EL, MK, NK, ÖÇ, FÖ; Materials – EI, TA, AES, DT, SG, HT, EL, MK, NK, ÖÇ, FÖ; Data Collection and/or Processing – EI, HO, TA, AES, DT, SG, HT, EL, MK, NK, ÖÇ, FÖ; Analysis and/or Interpretation – EI, AES, TA, HO; Literature Search – EI, AES, DT; Writing – EI, FÖ; Critical Reviews – EI, HO, TA, AES, DT, SG, HT, EL, MK, NK, ÖÇ, FÖ.

Conflict of Interest: The authors have no conflict of interest to declare.

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