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Variants of the DMD gene in 18 patients, clinical severity prediction, genotype-phenotype correlation, and potential therapies

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Abstract
Background: Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked recessive rare diseases caused by various mutations in the DMD gene which induce quantity reduction or absence of the dystrophin protein.
Aims: To present variants of the DMD gene, predict clinical severity, and discuss genotype-phenotype correlation as well as potential therapies.
Material and Methods: Data was collected from patients of Mother Teresa University Hospital Center, Tirana, Albania, whose diagnosis were confirmed through molecular examinations.
Results: In this study, 18 male patients were enrolled. Gross mutations were detected in 11 patients, while point mutations were detected in 7 patients. The detected variants were pathogenic in 13 patients and likely pathogenic in 5 patients. The age at clinical onset was approximately 2 years old in 10 patients, 3 years old in 6 patients, 9 years old in 1 patient, and 14 years old in 1 patient. Age at the loss of ambulation was approximately 10 years in 3 patients, 11 years in 3 patients, 12 years in 2 patients, and 14 years old in 1 patient, while 9 patients are still ambulant. Signs and symptoms included muscular, skeletal, nervous, cardiovascular, and respiratory systems. Altogether, 16 patients were diagnosed with DMD and 2 patients were diagnosed with BMD.
Conclusion: The genetic analysis cannot predict the course and severity of the dystrophinopathy. Genetic test results must always be evaluated in the context of clinical findings, family history, and other data, yet they are crucial for therapy customization.

Keywords: Dystrophinopathy, Duchenne muscular dystrophy, Becker muscular dystrophy, dystrophin, DMD gene

Introduction
Dystrophinopathies are a spectrum of irreversible, progressive muscular dystrophies caused by the total absence or decrease of dystrophin protein [1]. Dystrophin is a cytoplasmic protein encoded by the largest gene in the human genome called the dystrophin gene (DMD gene) located in the short arm of the X chromosome, on the Xp21.2 locus [1, 2]; it is expressed in several isoforms in skeletal and cardiac muscles, as well as brain and retina, in smaller amounts [3]. The DMD gene is 2.4 megabase pairs and consists of 79 exons, 7 promoters, and 78 introns [1, 4]. The muscle dystrophin isoform (Dp427m) is a protein of 427 kDa molecular weight, 150 nm in length, with 3,685 amino acids. It protects the sarcolemma from deformability, thus contributing to muscle stiffness. Its four main domains are as follows: (i) amino-terminal (N-terminal) actin-binding domain; (ii) central rod domain; (iii) cysteine-rich domain; (iv) carboxy-terminal (C-terminal) domain; each domain is coded by a specific coding sequence (Figure 1) [5].

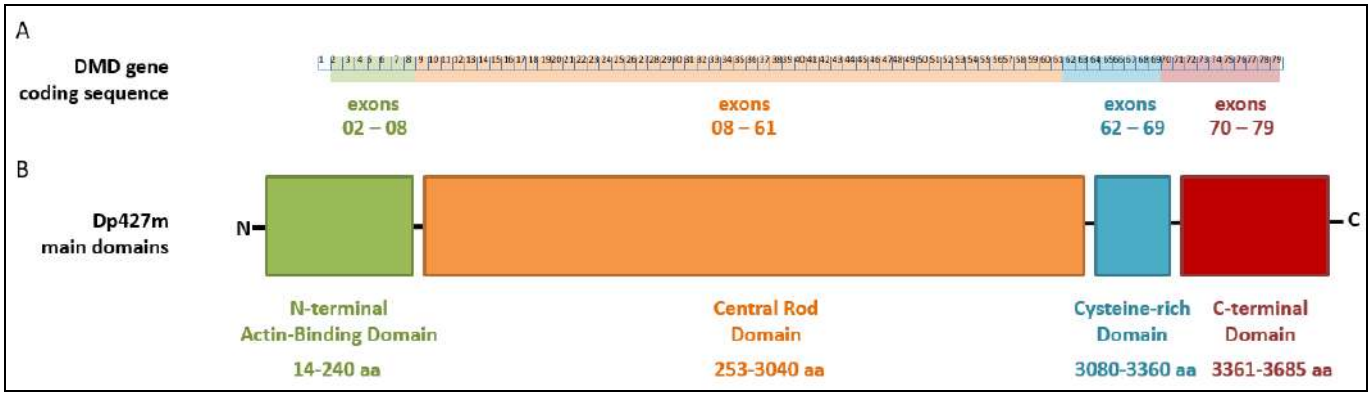

Fig 1: Schematic presentation of DMD gene exonic sequence (A) and Dp427m main domains (B)

Dystrophinopathies are inherited as allelic X-linked recessive disorders. Of the entire spectrum, Duchenne muscular dystrophy (DMD) is the most common in males yet the most severe, whereas Becker muscular dystrophy (BMD) presents a milder phenotype. The worldwide prevalence is approximately 1 per 3,500 to 5,000 live male births for DMD, and approximately 1 per 20,000 live male births for BMD, irrespective of race or ethnicity [6].

Variants in the DMD gene are responsible for dystrophinopathies. The variant rate is high due to the large size of the gene; thus 1 out of 3 is a de novo mutation. Large deletions and duplications are the most frequent mutations (75%), while the remaining are small mutations (25%) or seldom deep intronic variants. Mutations can occur everywhere in the gene, but some exonic hotspots are known, like deletions between exons 45-55 and duplications in the region of exons 2-10 [6]. Variants in the DMD gene lead to dystrophin absence in DMD, and reduction of amount or activity impairment in BMD [7].

Whether a mutation will result in DMD or BMD phenotype is theoretically predicted by the reading-frame rule: "Adjacent exons that can maintain an open reading frame (ORF) in the spliced mRNA despite a deletion event would give rise to the less severe BMD phenotype and predict the production of a lower molecular weight, semi-functional dystrophin protein. Adjacent exons that cannot maintain an ORF because of frame shifted triplet codons would give rise to the more severe DMD phenotype due to the production of a truncated, nonfunctional dystrophin protein" [8].

Patients with DMD are usually diagnosed by the age of 5 years, create wheelchair dependency before the age of 13, and die from either or both cardiac and respiratory failure by the age of 25. Meanwhile, BMD has a later clinical onset; patients are capable of walking unassisted until the age of 16 years or later and commonly expect to live a normal lifespan [9]. Improvements in multidisciplinary care have extended life expectancy; thus, many DMD patients live up to their 40s [10].

Material and Methods

Study Design

This is a retrospective, descriptive study.

Data collection

We recorded the demographics, clinical presentation, and laboratory test results of patients hospitalized and treated in Mother Teresa University Hospital Center, Tirana, Albania, whose diagnosis was confirmed by molecular analysis through methods like whole exome sequencing (WES), multiplex ligation-dependent probe amplification (MPLA), targeted sequencing, or amplicon-based next-generation sequencing.

Results and Discussion

In this study were enrolled 18 male patients from 9 to 26 years old. Patients #1 and #2 as well as patients #12 and #13 are brothers. Patients #8 and #9 are not consanguine, despite sharing the same variant.

Genotype

The diagnosis was confirmed with whole exome sequencing (WES) in 2 patients, multiplex ligation-dependent probe amplification (MPLA) method in 9 patients, and targeted sequencing in 3 patients, and amplicon-based next-generation sequencing in 4 patients. DNA samples were isolated from peripheral blood in filter cards. The tests identified the following variants (Table 1, 2): (i) gross mutations in 11 patients: deletion encompassing a single exon in 1 patient, deletion encompassing multiple exons in 9 patients, duplication encompassing multiple exons in 1 patient; (ii) point mutations in 7 patients: nonsense deletion in 3 patients, substitution (stop gain) in 1 patient and splice site mutations (2 substitutions and 1 insertion) in 3 patients. All the variants were hemizygous. Out of 10 large deletions, 7 were found in the hotspot of exons 45-55. The status of the variant is known only in 8 patients; it was inherited from a carrier mother in 5 patients, and in the other 3 it was a de novo mutation (Table 2). The mutations were detected in the intronic sequence of 3 patients, and in the exonic sequence of 15 others; 3 in the coding sequence of the actin-binding domain and 12 in the coding sequence of the central rode domain. According to the standards and guidelines for the interpretation of sequence variants of the American College of Medical Genetics and Genomics (AMCG) and the Association for Molecular Pathology (AMP), the detected variants belong to the first two classes: (i) pathogenic in 13 patients; (ii) likely pathogenic in 5 patients (Table 2). In Table 2 is also displayed each variant's coordinates after the Human Genome Variation Society (HGVS) nomenclature; the coordinates of patients #1 to #11 were provided online by the Leiden Muscular Dystrophy Pages (LMDP, www.dmd.nl/), which also predicted if each variant causes a shift of the reading frame, while the variant coordinates of patients #12 to #18 were provided by the laboratory reports.

Phenotype

The age at clinical onset was approximately 2 years in 10 patients, 3 years in 6 patients, 9 years in 1 patient, and 14

years in 1 patient. Age at the loss of ambulation was approximately 10 years in 3 patients, 11 years in 3 patients, 12 years in 2 patients, and 14 years old in 1 patient, while 9 patients are still ambulant (Table 1).

Table 1: Summary of patients' variants, age at onset, and age at the loss of ambulation

Variants	No. of patients	Approximate age at clinical onset	No. of patients
Gross mutations:	**11/18**	2 years	10/18
single-exonic deletion	1/18	3 years	6/18
multiple-exonic deletion	9/18	9 years	1/18
multiple-exonic duplication	1/18	14 years	1/18
		Approximate age at the loss of ambulation	**No. of patients**
Point mutations:	**7/18**	10 years	3/18
nonsense deletion	3/18	11 years	3/18
stop gain substitution	1/18	12 years	2/18
splice site	3/18	14 years	1/18
		still ambulant	9/18

Clinical findings were as follows (Table 2): (i) *Muscular system*: proximal hypotonia in 18 patients, muscular atrophy in 16 patients, gastrocnemius muscle pseudohypertrophy in 16, Gower sign in 16 patients; (ii) *Skeletal system*: hyperlordosis and scoliosis in 14 patients, only hyperlordosis in 2 patients, scapular winging in 2 patients; (iii) *Nervous system*: motor delay in 16 patients, hyporeflexia in 3 patients, gait unbalance and disturbances in 16 patients, only difficulty in climbing stairs in 2 patients; (iv) *Connective tissue*: joint contractures in 8 patients; (v) *Cardiovascular*: arrhythmia in 1 patient, right ventricular hypertrophy in 1 patient; (vi) *Respiratory*: restrictive ventilatory defect in 1 patient; (vii) *Blood biochemistry test*: elevated serum creatine kinase in 18 patients; (viii) *Immunohistochemistry test* (performed in 2 patients): dystrophin and gamma-sarcoglycan deficiency in 2 patients.

Genotype-phenotype correlation

According to Leiden Muscular Dystrophy Pages (LMDP), patients' #1 and #2 identical variants (brothers) are predicted to not shift the reading frame, thus presenting a BMD phenotype. Actually, our patients' clinical presentations are that of DMD, supported also by the evidence in the medical literature. We believe that is because the mutation impairs the actin-binding domain of dystrophin protein. Both of them were diagnosed with DMD.

As stated in LMDP, patient #3 large deletion is an in-frame variant; hence, according to the reading-frame rule, the patient's phenotype should have been that of BMD. In reality, our patient's clinical presentation was more severe. Also, this variant is evidenced as DMD in the medical literature. It is possible that in-frame changes like this significant exon deletion, are too substantial to generate functional dystrophin. A sample of muscle tissue was collected, and an immunohistochemistry test revealed a decrease in dystrophin and gamma-sarcoglycan. Gamma-sarcoglycan is a dystrophin-associated protein, a component of the dystrophin-glycoprotein complex (DGC), encoded in skeletal and cardiac muscle, the reduction or lack of which causes sarcolemma abnormalities independently of dystrophin protein [32]. Despite evidence in the literature claiming that the other components of DGC are either missing or mislocalized in the absence of dystrophin [33], further investigation is required to exclude a possible variant in the gamma-sarcoglycan gene. We diagnosed this patient with DMD.

Similar to the preceding case, the multi-exon deletion in patient #5 is predicted to be in-frame as stated in the LMDP database, which theoretically leads to a BMD phenotype, indicated also by the medical literature. In fact, our patient's phenotype was significantly more severe. Gamma-sarcoglycan was also found to be reduced in this case. We diagnosed this patient with DMD.

The variants in patients #8 and #9 were documented as both DMD and BMD by different authors; however, based on their phenotype, we diagnosed them with DMD.

Searching in all the databases provided in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) revealed that variants found in patients #12, #13, and #14 were not described before, but based on their phenotype, we diagnosed them with DMD. The identical variant of patients #12 and #13 (brothers) causes a shift in the reading frame starting at codon 461; the new reading frame ends in a premature stop codon 20 positions downstream. Likewise, in patient #14, the variation causes a shift in the reading frame starting at codon 1725; thus, the new reading frame ends 16 places downstream in a premature stop codon.

The variant in patient #16 is projected to disrupt the highly conserved acceptor splice site of exon 21. The patient had a BMD presentation, supported also by the literature.

The variant in patient #17 is expected to disrupt the highly conserved donor splice site of exon 50. It was not listed in any database, yet we diagnosed our patient with DMD based on his phenotype.

The variant causes the aberrant insertion of a 67 base pair pseudoexon in the mature transcript within intron 62 in patient #18, resulting in the occurrence of a high-quality donor splice site. It has previously been reported as a mutation causing BMD, corresponding to our patient's clinical presentation.

There was a correlation between the phenotypic prediction, diagnosis evidenced in the literature, and the actual clinical presentation in patients #4, #6, #7, #10, #11, and #15; they were diagnosed with DMD.

Collectedly, 16 patients were diagnosed with Duchenne muscular dystrophy and 2 patients were diagnosed with Becker muscular dystrophy.

Therapeutic approaches

Currently, there is no known cure for Duchenne and Becker muscular dystrophies. Multidisciplinary (neuromuscular, orthopedic, cardiac, respiratory, psychological) care is essential, especially in the latter stages of the disease;

however, treatment with glucocorticoids (anti-inflammatory drugs) such as prednisone and deflazacort and cardiac-protective blood pressure medication have been described to be beneficial, regardless of mutation location, if started at an early stage of the disease, resulting in the alteration of the disease course [34]. Also, there is an ongoing debate over phosphodiesterase type 5 (PDE5) inhibitors, FDA-approved for erectile dysfunction and pulmonary hypertension, which are believed to improve muscle blood flow in patients with dystrophinopathy [35].

Novel, variant-specific therapies are currently being investigated. Large deletions should be evaluated for antisense oligonucleotide (AOs)-mediated exon-skipping technology, a potential treatment approach for correcting and restoring the production of dystrophin, thus making muscular dystrophy less severe. Furthermore, due to its capacity to avoid the premature stop codon and to operate on nearly any region of the DMD gene regardless of the variant location, read-through therapy seems to be a promising approach to nonsense mutations [36]. Studies suggest that exon skipping therapy can also be applied to nonsense mutations by skipping the exon that contains the mutation while maintaining the reading frame [37].

More specifically, according to the information provided in DMD Open-access Variant Explorer (DOVE, www.dmd.nl/DOVE/), patients #1, #2, #3, #5, #15, #16, #17, and #18 are theoretically amenable to exon skipping therapy. Patient #4 is amenable to exon 44 skipping or exon 46 skipping. Patient #6 is amenable to exon 51 skipping with Eteplirsen (Exondys 51, FDA approved), patients #7, #8, and #9 are amenable to exon 45 skipping with Casimersen (Amondys 45, FDA approved) and patient #10 is amenable to exon 49 skipping or exon 50 skipping. Exon skipping therapy has not been clinically tested yet for the variants of patients #11 to #14. Read-through therapy with Ataluren (Translarna, EMA approved) is eligible only for the variant of patient #15.

Table 2: Summary of patients' data

Patient No. Age/ Onset/ Wheelchair (y)	Phenotype	Description of variant type & location	Variation coordinates / Amino acid change/ [Frame & protein domain]	Predicted condition & evidence	Diagnosis	Status of variant	Classification ACMG	Test
1 14 / 2 / ambulant	Clinical: motor delay, muscle weakness, muscle atrophy, Gower sign, calf muscle pseudohypertrophy, unstable gait, scapular winging. Rx: hyperlordosis, scoliosis. Lab: elevated serum CK.	deletion exons 03-16	c.94-?_1992+?del p. (Phe31_Ile664del) [IF, ABD]	DMD [11]	DMD	inherited	1	WES
2 11 / 3 / ambulant	Clinical: motor delay, muscle weakness, muscle atrophy, Gower sign, calf muscle pseudohypertrophy, unstable gait, scapular winging. Rx: hyperlordosis, scoliosis. Lab: elevated serum CK.	deletion exons 03-16	c.94-?_1992+?del p.(Phe31_Ile664del) [IF, ABD]	DMD [11]	DMD	inherited	1	WES
3 11 / 2 / 10	Clinical: motor delay, muscle weakness, muscle atrophy, Gower sign, calf muscle pseudohypertrophy, unstable gait, flexion contracture. Lab: elevated serum CK. Rx: hyperlordosis, scoliosis. IHC: dystrophin and γ-sarcoglycan deficiency	deletion exons 03-41	c.94-?_5922+?del p.(Phe31_His1974del) [IF, ABD]	DMD [12-14]	DMD	de novo	1	MPLA
4 26 / 2 / 12	Clinical: motor delay, proximal hypotonia, Gower sign, abnormal gait. Lab: elevated serum CK. Rx: hyperlordosis, scoliosis. ECG: arrhythmia	deletion exon 45*	c.6439-?_6614+?del p.(Glu2147Alafs*17) [OF, CRD]	DMD [16]	DMD	de novo	1	MPLA
5 12 / 3 /ambulant	Clinical: motor delay, muscle weakness and atrophy, Gower sign, calf muscle, unstable gait, pseudohypertrophy, flexion contracture. Lab: elevated serum CK. Rx: hyperlordosis, scoliosis. IHC: dystrophin and γ-sarcoglycan deficiency	deletion exons 45-49*	c.6439-?_7200+?del p.(Glu2146_Arg2400del) [IF, CRD]	BMD [16-18]	DMD	inherited	1	MPLA
6 16 / 3 / 11	Clinical: motor delay, proximal and progressive weakness, Gower sign, muscle atrophy, calf muscle pseudohypertrophy, gait disturbances, flexion contracture. Lab: elevated serum CK. Rx: hyperlordosis.	deletion exons 45-50*	c.6439-?_7309+?del p.(Glu2147Leufs*9) [OF, CRD]	DMD [11,19,20]	DMD	-	1	MPLA
7 9 / 2 / ambulant	Clinical: motor delay, muscle weakness, progressive proximal muscle involvement, calf muscle pseudohypertrophy, hyporeflexia, joint contractures, Gower sign, gain disturbances. Lab: elevated serum CK. Rx: hyperlordosis & scoliosis.	deletion exons 46-47*	c.6615-?_6912+?del p.(Leu2206Phefs*16) [OF, CRD]	DMD [11, 21, 22]	DMD	-	1	Amplicon-based NGS
8	Clinical: motor delay, proximal and	deletion	c.6615-?_7542+?del	DMD/BMD	DMD	-	1	MPLA

11 / 2 / 10	progressive weakness, Gower sign, muscle atrophy, calf muscle pseudohypertrophy, gait disturbances, flexion contracture. Lab: elevated serum CK. Rx: Hyperlordosis	exons 46-51*	p.(Leu2206Glnfs*23) [OF, CRD]	[21,23-25]				
9 11 / 3 / ambulant	Clinical: motor delay, muscle weakness and atrophy, calf muscle pseudohypertrophy, Gower sign gait disturbances, restrictive ventilatory defect. Lab: elevated serum CK Rx: hyperlordosis & scoliosis.	deletion exons 46-51*	c.6615-?_7542+?del p.(Leu2206Glnfs*23) [OF, CRD]	DMD/BMD [21,23-25]	DMD	-	1	MPLA
10 20 / 2 / 12	Clinical: motor delay, muscle weakness and atrophy, calf muscle pseudohypertrophy, unstable gait, Gower sign, hyporeflexia. Lab: elevated serum CK. Rx: hyperlordosis & scoliosis.	deletion exons 51-53*	c.7310-?_7872+?del p.(Pro2438Valfs*17) [OF, CRD]	DMD [26]	DMD	-	1	MPLA
11 11 / 2 / 11	Clinical: motor delay, muscle weakness and atrophy, progressive proximal muscle involvement, Gower sign, calf muscle pseudohypertrophy, unstable gait, joint contractures. Lab: elevated serum CK. US: right ventricular hypertrophy. Rx: hyperlordosis, scoliosis.	duplication exons 12-13	c.1332-?_1602+?dup p.(Val535Phefs*3) [OF, CRD]	DMD [27]	DMD	-	1	MPLA
12 17 / 3/ 11	Clinical: motor delay, muscle weakness, muscle atrophy, Gower sign, calf muscle pseudohypertrophy, gait disturbance. Lab: elevated serum CK Rx: hyperlordosis, scoliosis.	point nonsense deletion exon 12	c.1382_1383del p.(Asn461Argfs*21) [OF, CRD]	no supportive evidence	DMD	inherited	2	Amplicon-based NGS
13 11 / 2 / 10	Clinical: motor delay, muscle weakness, muscle atrophy, calf muscle pseudohypertrophy. Unbalanced gait, hyporeflexia, Gower sign. Lab: elevated serum CK. Rx: hyperlordosis, scoliosis.	point nonsense deletion exon 12	c.1382_1383del p.(Asn461Argfs*21) [OF, CRD]	no supportive evidence	DMD	inherited	2	Targeted sequencing
14 11 / 2 / ambulant	Clinical: motor delay, muscle weakness, progressive proximal muscle involvement, calf muscle pseudohypertrophy, unstable gait, Gower sign. Lab: elevated serum CK. Rx: hyperlordosis, scoliosis.	point nonsense deletion exon 37	c.5175del p.(Asn1725Lysfs*17) [OF, CRD]	no supportive evidence	DMD	-	2	Amplicon-based NGS
15 11 / 2 / ambulant	Clinical: motor delay, progressive proximal muscle weakness, muscle atrophy, calf muscle pseudohypertrophy, unsteady gait, flexion contracture, Gower sign. Lab: elevated serum CK. Rx: hyperlordosis , scoliosis.	stop gain point nonsense substitution exon 22	c.2869C>T p.(Gln957*) [CRD]	DMD [28]	DMD	de novo	1	Targeted sequencing
16 15 / 14 / ambulant	Clinical: muscle weakness, proximal muscle involvement, myalgia, exercise intolerance, difficulty in climbing stairs. Lab: elevated serum CK.	splice site point nonsense substitution intron 20	c.2623-2A>C	BMD [29]	BMD	-	2	Amplicon-based NGS
17 19 / 3 / 14	Clinical: motor delay, muscle weakness and atrophy, calf muscle pseudohypertrophy, gait disturbance, Gower sign, flexion contracture. Lab: elevated serum CK. Rx: hyperlordosis & scoliosis.	splice site insertion intron 50	c.7309+2_7309+3insTT	no supportive evidence	DMD	-	2	Targeted sequencing
18 9 / 9 / ambulant	Clinical: proximal muscle weakness, myalgia, exercise intolerance, difficulty in climbing stairs. Lab: elevated serum CK.	splice site substitution intron 62	c.9225-647A>G	BMD [30, 31]	BMD	-	1	Amplicon-based NGS

ABD, actin-binding domain; ACMG, American College of Medical Genetics and Genomics; BMD, Becker muscular dystrophy; CK, creatine kinase; CRD, central rod domain; DMD, Duchenne muscular dystrophy; ECG, electrocardiography; IF, in frame; IHC, immunohistochemistry; Lab, laboratory test; MPLA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; No., number; OF, out-of-frame; Rx, radiology; US, ultrasonography; WES, whole exome sequencing; y, years; *, variant in hotspot; -, unknown

Conclusions

At the end of this investigation, we can state unequivocally that genetic analysis cannot predict whether a patient will follow a rapidly progressive or indolent course. It is also difficult to anticipate the possibility of cardiomyopathy or respiratory tract involvement. Genetic test results must always be evaluated in the context of clinical findings, family history, and other laboratory data as in some cases identical variants have been demonstrated to result in different phenotypes. Knowing the patient's genotype is essential for therapy customization.

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Conflict of interests
The authors declare no conflict of interest.

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