

A novel DMD intronic alteration: a potentially disease-causing variant of an intermediate muscular dystrophy phenotype

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Pathogenic germline variants in *DMD* gene, which encodes the well-known cytoskeletal protein named dystrophin, are associated with a wide range of dystrophinopathies disorders, such as Duchenne muscular dystrophy (DMD, severe form), Becker muscular dystrophy (BMD, mild form) and intermediate muscular dystrophy (IMD). Muscle biopsy, immunohistochemistry, molecular (multiplex ligation-dependent probe amplification (MLPA)/next-generation sequencing (NGS) and Sanger methods) and in silico analyses were performed in order to identify alterations in *DMD* gene and protein in a patient with a clinical manifestation and with high creatine kinase levels. Herein, we described a previously unreported intronic variant in *DMD* and reduced dystrophin staining in the muscle biopsy. This novel *DMD* variant allele, c.9649+4A>T that was located in a splice donor site within intron 66. Sanger sequencing analysis from maternal DNA showed the presence of both variant c.9649+4A>T and wild-type (WT) *DMD* alleles. Different computational tools suggested that this nucleotide change might affect splicing through a WT donor site disruption, occurring in an evolutionarily conserved region. Indeed, we observed that this novel variant, could explain the reduced dystrophin protein levels and discontinuous sarcolemmal staining in muscle biopsy, which suggests that c.9649+4A>T allele may be re-classified as pathogenic in the future. Our data show that the c.9649+4A>T intronic sequence variant in the *DMD* gene may be associated with an IMD phenotype and our findings reinforce the importance of a more precise diagnosis combining muscle biopsy, molecular techniques and comprehensive *in silico* approaches in the clinical cases with negative results for conventional genetic analysis.

Key words: *DMD* gene, muscular dystrophy, dystrophinopathies, intronic sequence variant

Introduction

Dystrophinopathies are X-linked recessive disorders associated with pathogenic variants in the *DMD* gene (OMIM # 300377) which result in abnormal synthesis of dystrophin protein, a cytoskeletal protein with a major structural role in muscle¹. These disorders lead to muscle weakness and progressive degeneration of muscle function^{1,2}. Although most of the pathogenic variants in *DMD* gene are large rearrangements, it is estimated that 25-35% of affected patients have small-scale sequence variants affecting the dystrophin structure and/or function³. These disorders are progressive neuromuscular commonly diagnosed between the ages of 2 and 6 years due to delay in walking, unsteady gait, repeated stumbling, frequent falls, and difficulty at climbing stairs as well as increased levels of serum creatine kinase (CK)⁴.

The functional impact of genetic variants is believed to be the main driver of variability in clinical manifestations. Based on that, these disorders can be classified as Duchenne muscular dystrophy (DMD; OMIM #310200), when a patient presents the severe form or Becker muscular dystrophy (BMD; OMIM #300376) and intermediate muscular dystrophy (IMD), both characterized by early-onset, and by milder forms caused by a partially functional dystrophin⁵.

Several clinical cases with an IMD phenotype have recently been reported in the literature mainly due to widely spread, availability and cost reduction of genetic analysis such as multiplex ligation-dependent probe amplification (MLPA) and next-generation sequencing (NGS) techniques⁶. These molecular methods provide precise and early diagnosis combined with the microscopic study of invasive muscle biopsy⁷. Also, advances in NGS technologies have allowed improvements in molecular diagnosis and identification of new sequence variants in gene regions not previously evaluated, such as intronic alterations which constitute less than 0.5% of the currently reported causative variants but their value is presumably underestimated in dystrophinopathies⁸.

In this study, we described a novel point variant in intron 66 of the *DMD* gene in a patient with intermediate manifestation of dystrophinopathy.

Case presentation

A 9-year-old boy patient from Rio Grande do Sul state (Southern region) of Brazil was born by cesarean delivery at 36 weeks, weighing 2495 g, head circumference 34 cm and discharged from hospital 48 hours later. His parents were not consanguineous. He sat independently at 9 months, never crawled, and walked at 22 months. He had no other comorbidities, never suffered any surgical

procedure. His parents noticed that since he was three years old, he often fell on the ground, and had difficulties to go up the stairs, stand up or do physical activities.

On physical examination, the patient exhibited head circumference of 53cm, medium and photoreactive pupils, eye movements preserved in all directions, posture with hyperlordosis and global hypotonia. Moreover, he had proximal muscle weakness in upper limbs and deep hyporeflexia, bilateral flexion-cutaneous-plantar reflex, calf pseudohypertrophy, and Gowers sign. Wechsler Intelligence Scale for Children IV (WISC-IV) intelligence tests revealed a low intelligence quotient (IQ) of 59, characterizing mild cognitive deficit. An elevated determination of creatine phosphokinase (CPK; 11.150 U/I) and aldolase (10.9 IU/L) enzymes were also observed. Other laboratorial tests showed: aspartate transaminase (AST) 282 mg/dL; thyroid stimulating hormone (TSH) 4.06 nI-U/L; B12 vitamin 494 pg/mL; lactic acid 6.5 mg/dL. Skull magnetic resonance imaging (MRI) and transthoracic Doppler echocardiogram indicated no evidence of significant abnormalities. Considering these clinical features, specially impaired muscle function and high levels of CPK, this patient was referred to a molecular analysis of the *DMD* gene and muscle biopsy. There was no family history of muscle weakness or cardiac abnormalities. Indeed, the patient's mother showed normal CPK levels (62 U/I) and normal echocardiogram.

Methods and results

Muscle biopsy was collected at the quadriceps muscle, frozen in isopentane cooled in liquid nitrogen and fresh-frozen cryostat sections were used for histochemistry, enzyme histochemistry and immunohistochemistry (IHC) analysis. Transverse serial frozen sections were stained with hematoxylin-eosin (HE) (Fig. 1A) and modified trichrome gomori (Fig. 1B) revealed great variation in muscle fiber sizes, round hypotrophic fibers and myonuclei internalization. Necrotic muscle fibers surrounded by myophagocytosis, increased endomysial connective tissue (masson trichrome stain) and interstitial adipose tissue was observed in several muscle areas. No intracytoplasmic vacuoles and no rods or ragged red fibers (RRF) were observed. The normal distribution of glycogen in muscle biopsy was evaluated by Periodic Acid Schiff (PAS) staining. Enzyme histochemistry did not show cytochrome c oxidase (COX) -negative/SDH-positive muscle fibers (Fig. 1C). Additionally, no conspicuous intra-vacuolar or perimysial amyloid deposits were revealed by Congo Red staining. Core- and target-defects in succinate dehydrogenase (SDH) (Fig. 1D) and nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) were not observed in this sample.

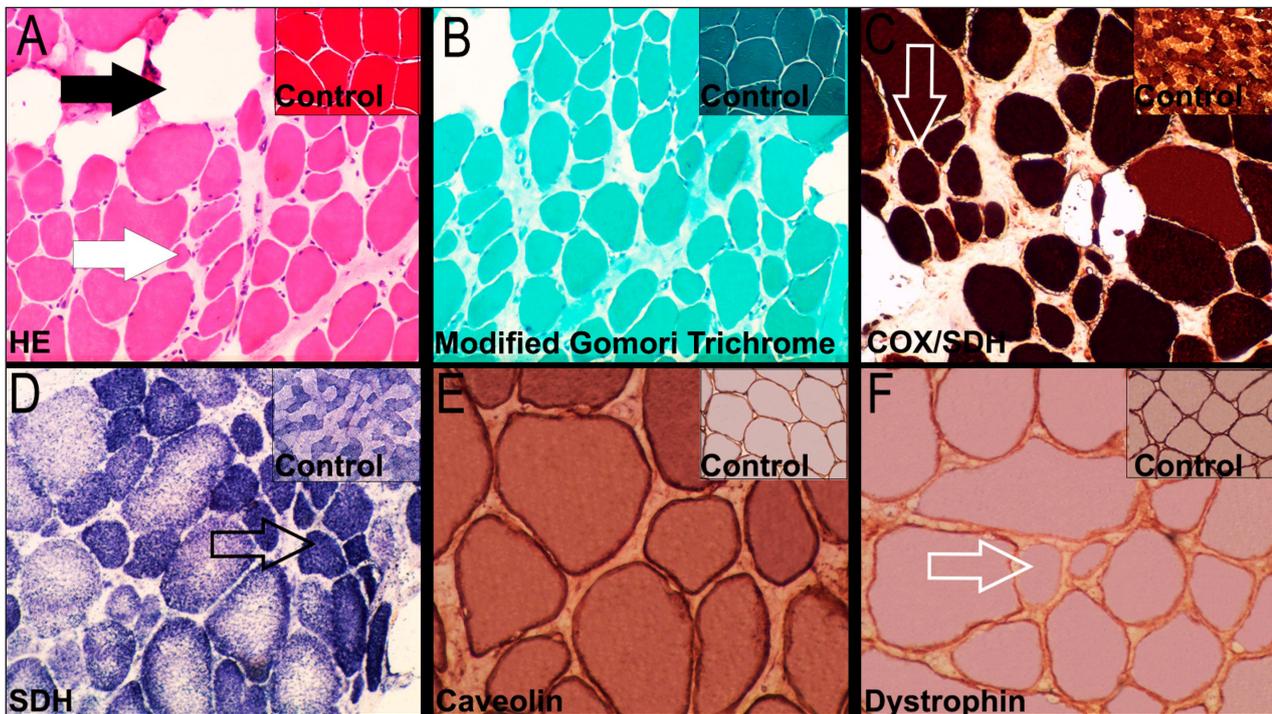


Figure 1. Quadriceps muscle biopsy with features of dystrophy. A) Muscle fibres showing variation in size and atrophic fibres (white arrow) surrounded by endomysial connective tissue/adipose tissue (dark arrow) (Hematoxylin and Eosin, 10x); B) Muscle section with variation in fibre size, internal nuclei, increased endomysial connective tissue and adipose tissue (modified Gomori trichrome, 10x); C) and D) Oxidative enzymes showing variation in fibre size with slight predominance of type 1 fibres (open arrow) [cytochrome c oxidase/succinate dehydrogenase (COX/SDH) and succinate dehydrogenase (SDH), respectively; 10x]. (E) Immunolabelling of caveolin-3 using peroxidase label in the same muscle (20x). (F) Immunolabelling of dystrophin revealing some fibres with weak and uneven (open arrow; 20x) labelling compared with controls.

Muscle fibers exhibited diffuse sarcolemmal immunoreactivity for dysferlin (DYSF), neuronal nitric oxide synthase (nNOS) and laminin alpha 2. Strong diffuse immunoreactivity with alpha-sarcoglycan (data not shown) and caveolin-3 (Fig. 1E) in the membranes of muscle fibers were accompanied by diffuse emerin staining in myonuclei. Reduced and discontinuous sarcolemmal staining on hypertrophic fibers were evidenced in the immunoreactivity using a human -dystrophin monoclonal antibody that reacts with the N-terminal domain of this protein (Fig. 1F). Sparse satellite and regenerated muscle fibers were highlighted by immunoreaction with CD56 and scattered macrophages (CD68 positivity) and lymphocytes (CD45 positivity) were evidenced in the endomysial area (data not shown). A significant hypotrophy of type 1 fibers was verified in the slow myosin heavy chain (MHC) class I immunoreaction (data not shown). The source, clone and dilution of antibodies used for each staining are as follows: anti-dystrophin (Accurate Chemical & Scientific Corporation; Dy10/12B2;1:20), anti-nNOS (Santa Cruz Biotechnology; R-20; 1:70), anti-caveolin-3

(Abcam; ab2912; 1:100), anti-laminin-2 (Enzo; 4H8-2; 1:100), anti-SGCA (Sigma-Aldrich; polyclonal; 1:50), anti-DYSF (Abnova; Ham1/7B6; 1:20), anti-MHC class I (Abcam; W6/32; 1:130), anti-CD56 (Bio-Rad; Eric-1; 1:50), anti-CD68 (Dako; KP1; RTU) and anti-CD45 (Dako; 2B11 + PD7/26; RTU).

Molecular and in silico analysis

Afterwards, genomic DNA of the proband was isolated from peripheral blood leukocytes and the screening for exon deletions or duplications in the *DMD* gene was performed by MLPA technique using the SALSA® MLPA® P034 and P035 (*DMD*/Becker) kits (MRC Holland, Amsterdam, Netherlands), according to the manufacturer's instructions. A reference DNA (no deletions and/or duplications in the *DMD* gene) was used as a normal copy number control. MLPA amplified fragments were separated by capillary gel electrophoresis in an ABI 3500xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the results were analyzed using the Coffalyser. Net Software (<https://coffalyser.wordpress.com/>) (MRC®

Holland, Amsterdam, Netherlands). MLPA analysis identified no *DMD* deletions and/or duplications in the proband. In parallel, we performed target sequencing of the *DMD* gene using a NGS approach. The *DMD* gene regions were amplified using standard multiplex polymerase chain reaction (PCR) reactions through an Ion AmpliSeq customized panel (Thermo Fisher Scientific), which covers the 79 exons and at least 5 bp of exon-intron boundaries of *DMD*. PCR products were then sequenced by Ion Torrent Personal Genome machine (Ion Torrent Systems Inc, Gilford, NH), according to manufacturer's instructions. Human *DMD* sequence corresponding to the NM_004006.2 was used as a wild-type (WT) reference. A novel hemizygous *DMD* variant, described as c.9649+4A>T, was detected in intron 66 (mean coverage of this genic region = 2080x).

To confirm the origin of this *DMD* intronic sequence variant, genomic DNA was obtained from his mother (an obligatory carrier) and, after amplification by PCR using primers that flank the variant region previously described by Lenk and colleagues⁹, the purified PCR product was analyzed by Sanger sequencing. The sequencing analysis showed the presence in heterozygosis of the c.9649+4A>T variant (Fig. 2).

Considering the lack of information regarding *DMD* c.9649+4A>T variant, an *in silico* approach was employed. First, the search for this intronic alteration in several population databases, including 1000 genomes Project, Exome Sequencing Project (ESP), Exome Aggregation Consortium database (ExAC), Genome Aggregation Database (gnomAD), and Online Archive of Brazilian Mutations (AbraOM), indicated that c.9649+4A>T was not previously reported in healthy individuals. Additionally, the variant was not described neither in Leiden Open Variation Database (LOVD)¹⁰, ClinVar nor in a specific *DMD*/*BMD* mutation database (TREAT-NMD *DMD* Global database)¹¹. Based on this, it was considered a novel *DMD* sequence variant. Next, *in silico* analysis was performed in order to investigate the biological effect on splicing motifs (including exonic enhancers and silencers). Mutation Taster¹², Human Splicing Finder¹³ and Berkeley Drosophila Genome Project (BDGP)¹⁴ algorithms suggested that the c.9649+4A>T intronic variant might affect splicing through a WT donor site disruption. Supplementary Figure 1 depicts the output provided by Human Splicing Finder algorithm, showing the reduction in the splicing prediction raw scores comparing WT *DMD* sequence vs. variant sequence. Moreover, PhyloP score derived from alignment of 46 vertebrate species genomic sequences¹⁵ indicated that this nucleotide change occurs in an evolutionarily conserved region. The SpliceAI, a deep learning-based tool¹⁶, predicts the identified variant to affect most probably the splicing (Delta

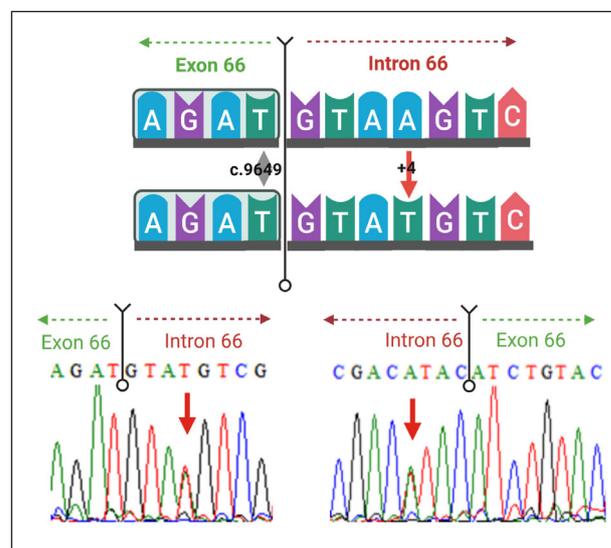


Figure 2. Schematic representation of the *DMD* gene region encompassing the novel sequence variant c.9649+4A>T (indicated by the red arrow) in the splice donor site within intron 66 (upper panel; Created with BioRender.com), and bidirectional Sanger sequencing analysis from maternal DNA showing the presence of both variant and wild-type alleles (lower panel). Sense and antisense *DMD* sequences are shown by indicating the orientation of the DNA strands in the panels, as well as a range of 4-8 base pairs are underlined in both directions at the exon-intron junction in order to highlight this boundary. WT, wild-type sequence.

score = 0.58). Finally, the c.9649+4A>T was classified according to the guidelines proposed by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP)¹² and by Sherlock classification system¹⁷ for the interpretation of sequence variants. After careful analysis of all available evidence about this novel *DMD* variant, it was classified as a variant of uncertain significance (VUS) by both classification approaches. Briefly, PM2 (pathogenic moderate evidence: absent from controls), PP3 and PP4 (supporting pathogenic evidence: multiple lines of computational evidence support a deleterious effect and patient's phenotype is highly specific for a disease with a single genetic etiology, respectively) were the applied criteria using the ACMG guidelines. In contrast, EV0135, EV0184 and EV0024 (pathogenic evidence: absent from general population; variant involving a donor +3A/G, +4A or +5G; and weak functional evidence for protein function disrupted, respectively), as well as EV0211 (neutral evidence: first case report with the variant) were the selected criteria based on Sherlock framework.

Discussion

More than 890 *DMD* pathogenic variants have been described so far (detailed information in: <https://clinvarminer.genetics.utah.edu/variants-by-gene/DMD/condition/Duchenne%20muscular%20dystrophy/pathogenic>), covering the different functional domains of dystrophin protein. The majority (~65%) of these causative variants are intragenic deletions/duplications that often lead to frameshift errors. Among the remaining ones, we find intronic alterations that usually create cryptic exons by activating potential splice sites^{8,18}. Of note, the pre-mRNA of *DMD* gene is composed by 99% of introns¹⁹ that exhibit a very complex pattern of expression and different alternative splicing²⁰, contributing to high rates of point variants, insertions and deletions^{21,22}. This study presents a novel intronic sequence variant in the *DMD* gene, c.9649+4A>T in a patient at 9 years of age with an intermediate manifestation of muscular dystrophy. Remarkably, the variant was absent from all queried databases (1000 Genomes Project, ExAC, ESP, GnomAD, LOVD).

The novel *DMD* sequence variant described here is at the 5' donor splice site of intron 66 which is essential during the post-transcriptional modifications, specifically the pre-mRNA splicing process²³. Disruptions on donor site, defined by the three terminal nucleotides of each exon and the first seven bases of the downstream intron, tended to generate an alternative 5' splice site, resulting in different protein isoforms²³ that leads to a wide variety of clinical Duchenne phenotypes²⁴.

Importantly, at the same splice donor site of intron 66, two different germline *DMD* variants at the nucleotide position +5 were previously reported in the LOVD database¹⁰, namely c.9649+5 G>T²⁵ and c.9649+5 G>A²⁶, being classified as pathogenic and likely pathogenic alterations, respectively. In another sequence variant, also in the same position, c.9649+5G>C, was classified as pathogenic in the ClinVar database¹¹. This finding represents an indirect evidence that nucleotide changes at c.9649+4 position might have functional impact associated with the splicing efficiency alteration in the *DMD* intron 66. In accordance with it, previous studies showed that the point intronic variants c.9649+1G>A²⁴, c.9649+2T>C²⁴ and c.9649+2insT²⁷ also at the 5' donor splice site of intron 66, led to severe phenotype (Duchenne dystrophy). Other point variants within or in the vicinity of the intron 66 region also leads to a range of variety clinical Duchene phenotype as variants c.9649+15T>C^{9,28}, c.9807+5G>A²⁸ and c.9857+15C>T²⁹ but its pathogenic effects are unknown.

Indeed, the novel *DMD* intronic variant is located within in the cysteine-rich domain of the protein, consisting in a region required for a β -dystroglycan interaction

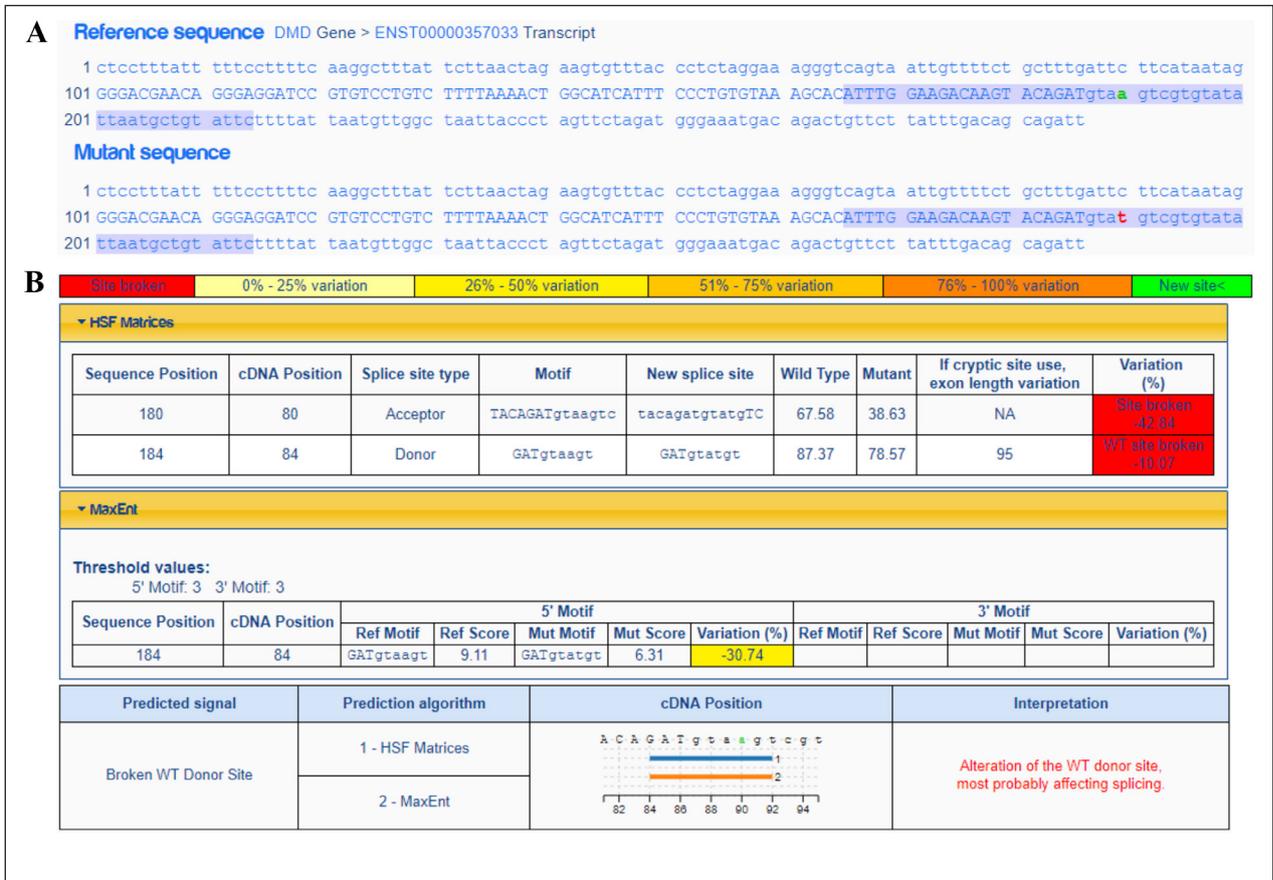
with dystrophin complex³⁰. Therefore, we can speculate that this genetic alteration might destabilize this complex, a functional consequence which could explain the discontinuous sarcolemmal staining observed in our muscle biopsy analysis, leading to the clinical manifestation of muscle weakness observed in the proband. As note, it is well known that germline *DMD* pathogenic variants in the cysteine-rich domain are among the possible underlying genetic defects associated with DMD phenotype^{31,32}.

As recently well reviewed^{33,34}, the global cognition functions are often affected in DMD patients, even in severe or mild phenotypes and, likewise, our patient also shows a mild cognitive deficit. At the same time, our patient has a healthy heart function, suggesting that the intronic variant reported here produces enough amounts of dystrophin protein in the cardiac muscle as observed in normal echocardiogram. Based on the muscle biopsy analysis and *in silico* results obtained in the current study, we may suggest that, even considering its uncertain clinical significance using both ACMG-AMP and Sherloc criteria, the c.9649+4A>T variant leads to a decrease in the dystrophin protein production levels and hypotrophic fibers as showed in Figure 1. Moreover, the reduced levels of dystrophin on patient's muscle might explain his high levels of serum CK which can be released from dystrophic fibers³⁵. Further functional and molecular approaches such as patient-derived induced pluripotent stem (iPS) cells³⁶ and analyses based on dystrophin reporter minigene³⁷ are needed in order to characterize the tissue-specific effects of this novel variant in the dystrophin protein in detail. Indeed, segregation of clinical phenotype within the family may clarify the impact of this variant on its classification.

As limitations of this study, the RNA isolation from the biopsied muscle tissue of proband could not be performed due to the small amount of material collected. It would be important in order to evaluate the *DMD* transcript expression levels and abundance of specific isoforms. Furthermore, the amount of dystrophin protein in the brain or cardiac muscle in our patient was not evaluated. Finally, although this is the first report of the intronic *DMD* variant c.9649+4A>T and the clinical suspicion of molecular alterations in this gene was strong, our molecular approach was based on the single-gene analysis of one patient, not involving additional whole exome or genome sequencing tests to screen for potential causative variants in other genes.

Conclusions

Our data show that the c.9649+4A>T intronic sequence variant in the *DMD* gene may be associated with an IDM phenotype and further studies are needed



Supplementary Figure 1. Analysis of the novel intronic *DMD* mutation identified in this case report (c.9649+4A>T, intron 66) using the Human Splicing Finder (HSF) algorithm. A) Comparison between the reference (wild-type, WT) and mutated *DMD* sequences, encompassing exon 66 (upper case nucleotides letters, 86 base pairs) and 100 intronic nucleotides at exon ends (lower case letters). The highlighted blue sequences were analyzed by HSF and MaxEntScan predictors. Green and red letters indicate the WT and mutated nucleotide change, respectively. B) Raw and interpreted tables show relevant results related to the mutation position and context. Variations in the tables were depicted in colored boxes, according to the scale showed in the upper panel. c.9649+4A>T was predicted to affect splicing by disrupting a WT donor site (note the reduction in the raw scores when compared WT *DMD* vs mutant sequence).

to clarify the complete functional effects of this genetic alteration, specially its functional impact in the mRNA processing. Overall, our findings reinforce that the variant described here initially classified as VUS, may be re-classified in the future as likely pathogenic or pathogenic. Indeed, our study reinforces the importance of a more precise diagnosis combining muscle biopsy, new generation molecular techniques and comprehensive *in silico* approaches in the clinical cases with negative results for conventional genetic analysis.

Ethical consideration

Written informed consent was obtained from the parents of the index patient/proband for publication of this case report and accompanying laboratory tests results and images.

Acknowledgement

We appreciate the cooperation of the patient and his parents for this study. Schematic representations were created with BioRender.com.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

APBS, IAV, GB and RS designed the study, coordi-

nated the project, performed sequence analysis, analyzed the data and wrote the paper; RS, FQ, provided patient samples and patient data. IVA designed and performed bioinformatics analysis. JCB, MLB performed immunohistochemical experiments and assisted in drafting and critical reading. ACBF, GB performed molecular experiments, assisted in drafting and critical reading. All authors read and approved the final manuscript.

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