Short report

Genetic diagnosis of Duchenne and Becker muscular dystrophy through mRNA analysis: new splicing events

Alba Segarra-Casas ,[1,2] Cristina Domínguez-González ,[3,4] Aurelio Hernández-Laín,[5] Maria Teresa Sanchez-Calvin,[6,7] Ana Camacho,[8] Eloy Rivas,[9] Andrea Campo-Barasoain,[10] Marcos Madruga,[11,12] Carlos Ortez,[13,14] Daniel Natera-de Benito,[13,14] Andrés Nascimento,[4,13,14] Anna Codina,[14] Maria Jose Rodriguez,[1] Pia Gallano,[1,4] Lidia Gonzalez-Quereda[1,4]

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jmg-2022-108828).

For numbered affiliations see end of article.

Correspondence to
Dr Pia Gallano, Genetics Department, Hospital de Sant Pau, Universitat Autònoma de Barcelona, Institut d'Investigació Biomèdica Sant Pau IIB Sant Pau, Barcelona, Catalunya, Spain; pgallano@santpau.cat

Received 18 July 2022
Accepted 27 October 2022
Published Online First 19 December 2022

ABSTRACT

Background Up to 7% of patients with Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD) remain genetically undiagnosed after routine genetic testing. These patients are thought to carry deep intronic variants, structural variants or splicing alterations not detected through multiplex ligation-dependent probe amplification or exome sequencing.

Methods RNA was extracted from seven muscle biopsy samples of patients with genetically undiagnosed DMD/BMD after routine genetic diagnosis. RT-PCR of the *DMD* gene was performed to detect the presence of alternative transcripts. Droplet digital PCR and whole-genome sequencing were also performed in some patients.

Results We identified an alteration in the mRNA level in all the patients. We detected three pseudoexons in *DMD* caused by deep intronic variants, two of them not previously reported. We also identified a chromosomal rearrangement between Xp21.2 and 8p22. Furthermore, we detected three exon skipping events with unclear pathogenicity.

Conclusion These findings indicate that mRNA analysis of the *DMD* gene is a valuable tool to reach a precise genetic diagnosis in patients with a clinical and anatomopathological suspicion of dystrophinopathy that remain genetically undiagnosed after routine genetic testing.

INTRODUCTION

Duchenne muscular dystrophy (DMD, OMIM #310200) and Becker muscular dystrophy (BMD, OMIM #300376) are X-linked recessive disorders caused by pathogenic variants in the *DMD* gene (OMIM *300377), which encodes the dystrophin protein.[1] DMD is the most severe form of the disease and the most common neuromuscular disorder in childhood, affecting 1 in 3500 live male births.[2] Patients with BMD present a less severe phenotype with a more progressive and variable disease course. Disease severity can be explained by the reading frame rule in 90% of patients: frameshift variants in the *DMD* gene result in a premature termination codon (PTC) and a non-functional dystrophin leading to DMD. In contrast, in-frame variants usually give place to a partially functional protein and a BMD phenotype.[3]

Currently, the diagnostic algorithm consists of multiplex ligation-dependent probe amplification (MLPA) followed by exome sequencing (ES) in genomic DNA (gDNA). Exonic deletions and duplications are the most prevalent pathogenic variants (65%–75% of patients), and subsequently, small disease-causing variants are detected in 20%–25% patients.[4–6] With this diagnostic approach, up to 7% of patients with dystrophinopathy remain without a precise genetic diagnosis.[7 8] As more than one-third of disease-causing variants in the Human Genome Mutation Database are estimated to perturb pre-mRNA splicing,[9] patients with undiagnosed DMD/BMD are thought to carry complex structural variants (SVs) or splicing alterations in the *DMD* gene. Nonetheless, pathogenic variants in non-coding regions potentially disrupting canonical splicing of *DMD* are missed in routine diagnosis. Identifying and characterising splicing variants in the *DMD* gene are essential to obtain a precise genetic diagnosis of these patients, to be eligible to mutation-specific therapies, and to offer families appropriate genetic and reproductive counselling.

The aim of this study was to evaluate the utility of mRNA analysis of the *DMD* gene in patients with dystrophinopathy who remained genetically undiagnosed after MLPA and ES. Through *DMD* mRNA analysis in seven patients, we detected three pseudoexons (PEs) in the *DMD* gene, one chromosomal rearrangement between Xp21.2 and 8p22, and three exon skipping events with unclear pathogenicity.

METHODOLOGY

Patients

Patients were recruited from several hospitals in Spain, and they were included when (1) DMD and BMD were suspected in the clinical examination, and it was supported by alterations in dystrophin immunohistochemistry and/or western blot; and (2) MLPA of the *DMD* gene (P034 and P035 Sauce Kit; MRC-Holland, Amsterdam, Netherlands) and ES had negative results. In total, cDNA analysis was performed on seven patients (table 1).

© Author(s) (or their employer(s)) 2023. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

To cite: Segarra-Casas A, Domínguez-González C, Hernández-Laín A, *et al*. *J Med Genet* 2023;**60**:615–619.

J Med Genet: first published as 10.1136/jmg-2022-108828 on 19 December 2022. Downloaded from http://jmg.bmj.com/ on April 20, 2024 by guest. Protected by copyright.

Table 1 Relevant clinical and genetic findings of patients

Patient	Phenotype	Onset age*	Relevant clinical findings	Loss of ambulation*	Muscle biopsy findings†	Genomic variant‡	mRNA alteration‡
P1	BMD	Early childhood	Clumsy walking and muscle weakness	No (midle childhood)	Myopathic changes with partial deficiency of DYS-3 staining	g.32584731A>G	PE15
P2	BMD	Early adolescence	Muscle weakness	Yes (adult)	Numerous atrophic and hypertrophic fibres with size variability and internal nuclei Overexpression of utrophin and almost absent expression of DYS3	g.32371864A>C	PE37
P3	BMD	Middle childhood	HyperCKemia	No (early adolescence)	Conserved skeletal muscle architecture, with occasional internalised nuclei Reduction of DYS-2 staining in WB	–	Exon nine skipping
P4	DMD	Early childhood	Difficulties in climbing stairs, difficulties with fine and gross motor skills, mild pseudohypertrophy of gastrocnemius, biceps, triceps and paraspinal muscles	No (early childhood)	Reduction of DYS-1 expression and absence of DYS-2 and DYS-3 expression Reduction of sarcoglycan expression	g.31279418T>C	PE62
P5	BMD	Adult	HyperCKemia, calf hypertrophy, muscle cramps and myalgia after exercise	No (adult)	Mild dystrophic pattern with alterations in dystrophin staining	–	Exon 71 and 78 skipping
P6	BMD/DMD	Early childhood	Down syndrome, with frequent falls and proximal muscle weakness	Yes (early adolescence)	Dystrophic pattern with reduced DYS-2 and DYS-3 staining Partial alteration of dystrophin WB	g.32873008_32873015delins (CCA;chr8:g.16346710_16422443)	Cryptic exon (chr8)
P7	BMD	Midle childhood	HyperCKemia and rhabdomyolysis	No (midle childhood)	Mild dystrophic pattern with reduced DYS-2 and DYS-3 staining Almost absence of DYS-2 in WB	–	Exon 71 skipping

* Early childhood, 2–5 years; middle childhood, 6–11 years; early adolescence, 12–18 years; late adolescence, 19–21 years.
†Monoclonal antibodies detecting the rod domain (DYS1), C-terminal domain (DYS2) and N-terminal (DYS3) domain of dystrophin.
‡Variant description based on NC_000023.11, NC_000008.10 and NM_004006.2.
BMD, Becker muscular dystrophy; CK, creatine kinase; DMD, Duchenne muscular dystrophy; PE, Pseudoexon; WB, western blot.

Amplification of *DMD* cDNA sequence

RNA was extracted from muscle biopsies using the Animal Tissue RNA Purification Kit (Norgen Biotek, Ontario, Canada). RNA was retrotranscribed using Oligo(dT)20 Primer (Invitrogen, California, USA). The whole mRNA *DMD* was sequenced with overlapping primer pairs (online supplemental table 1). PCR products were sequenced with BigDye Terminator V.1.1 Cycle Sequencing Kit (ThermoFisher) and analysed in an Applied Biosystems 3500 Dx Series Genetic Analyzer (ThermoFisher). Splicing in silico analyses were performed with Alamut Visual software V.2.6.1. Variants have been submitted to the 'Global Variome shared LOVD' (https://databases.lovd.nl/shared/references/DOI:10.1136/jmg-2022-108828).

gDNA analysis and whole-genome sequencing (WGS)

gDNA was extracted from total peripheral blood using QIAsymphony SP (Qiagen). The presence of deep intronic variants (in patient 1 (P1), patient 2 (P2) and patient 4 (P4)) and intronic variants near exon boundaries (in patient 3 (P3), patient 5 (P5) and patient 7 (P7)) was evaluated through Sanger sequencing.

WGS was performed in P6. One microgram of fragmented gDNA was used for library preparation with Truseq DNA PCR-Free (Illumina, California, USA). Sequencing was performed in a NovaSeq 6000 (Illumina). Manta V.1.6.0 was used to detect SVs and junction breakpoints.[10] SVs passing the default filtering were inspected on IGV (Integrative Genomics Viewer) . Breakpoints were confirmed by Sanger sequencing.

Droplet digital PCR (ddPCR)

Mini Affinity Plus qPCR probes (Integrated DNA Technologies, Iowa, USA) were designed to quantify exon 9 and 71 skipping (online supplemental table 3). ddPCR reactions were performed with 10 ng of cDNA and ddPCR Supermix for Probes (no dUTP (deoxyuridine Triphosphate)) (Bio-Rad Laboratories, California, USA) following manufacturer protocol. Droplets were generated with Droplet Generation Oil for Probes (Bio-Rad) in the QX200 Droplet Generator (Bio-Rad). Thermocycling conditions were as follows (ramping rate 2°C/s): 95°C 10 min, 40 cycles of 94°C for 30 s and 58°C for 1 min, and 98°C for 10 min. Droplets were loaded in the QX200 Droplet Reader (Bio-Rad). For each sample, four replicates were performed and a non-template control was included in each assay. The data were analysed with the QX Manager Software Standard Edition V.1.2 (Bio-Rad). Mann-Whitney test was performed. Only wells with more than 10 000 droplet reads were considered.

RESULTS

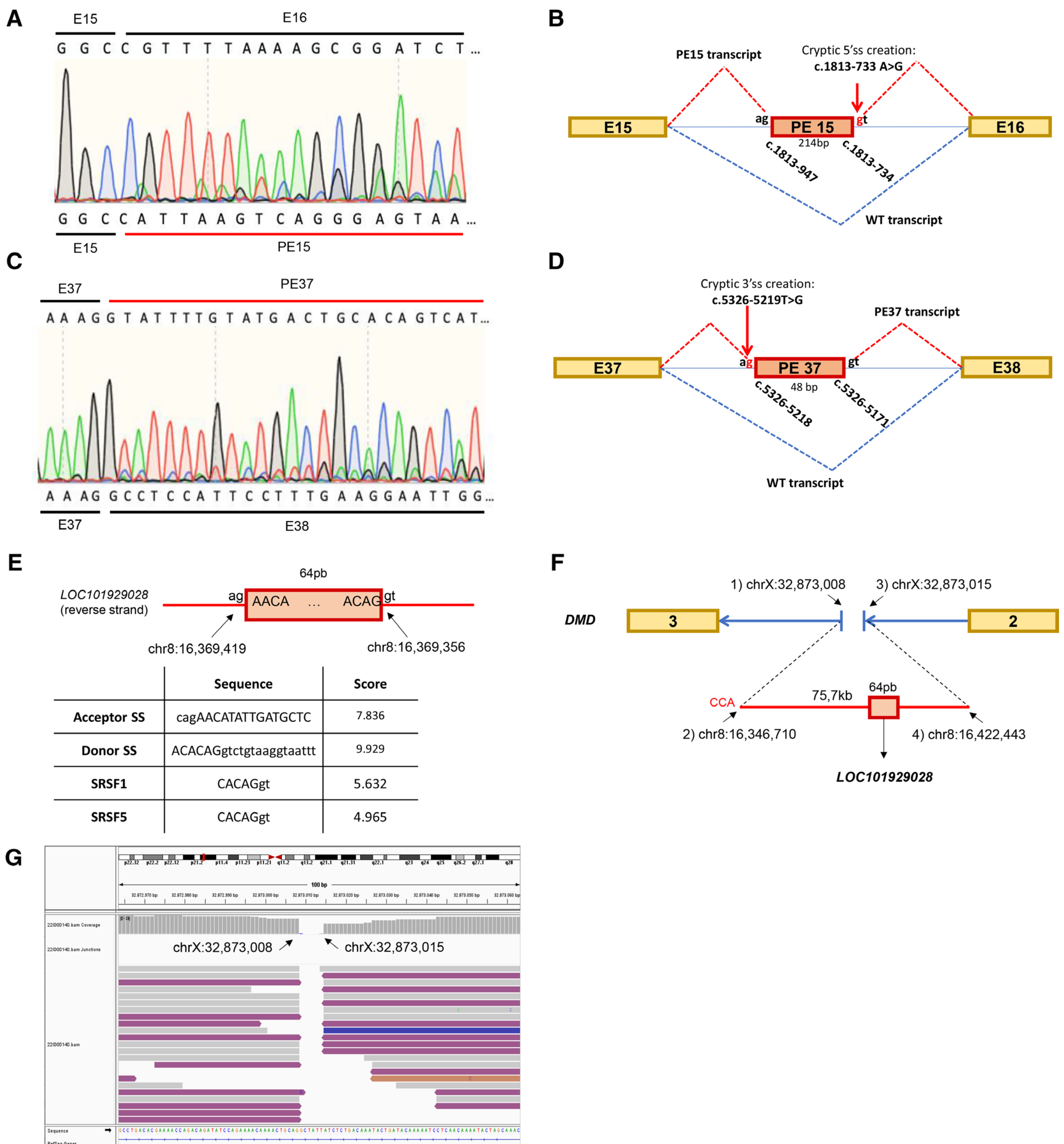
Deep intronic variants and PE inclusion

We identified the inclusion of PEs in dystrophin mRNA in three patients (P1, P2 and P4) due to deep intronic variants (table 1).

In P1, we detected an inclusion of 214 bp from intron 15 (from c.1813–947 to c.1813–734) into the *DMD* transcript (PE15) (figure 1A). We detected the deep intronic variant g.32584731A>G (c.1813–733A>G) in intron 15, classified as a 'variant of uncertain significance' (VUS) following the American College of Medical Genetics guidelines.[11] This variant, not present in human genetic variation databases, creates a new donor splice site (SS) in intron 15 near a cryptic acceptor SS (figure 1B and online supplemental table 2). The deep intronic variant c.1813–733A>G results in the disruption of the reading frame of *DMD* transcripts (p.Val605Ilefs*33), probably targeted by nonsense-mediated decay.

In P2, we observed the presence of an in-frame PE between exons 37 and 38 (PE37) that incorporated a PTC (p.Ala1776Valfs*9) (figure 1C). gDNA analysis revealed the intronic variant g.32371864A>C (c.5326–5219T>G), not previously reported in databases, that results in the retention of 48 bp from intron 37. This variant creates a new cryptic acceptor SS and activates a pre-existing donor SS (figure 1D, (online supplemental table 2)).

J Med Genet: first published as 10.1136/jmg-2022-108828 on 19 December 2022. Downloaded from http://jmg.bmj.com/ on April 20, 2024 by guest. Protected by copyright.



	Sequence	Score
Acceptor SS	cagAACATATTGATGCTC	7.836
Donor SS	ACACAGgtctgtaaggtaattt	9.929
SRSF1	CACAGgt	5.632
SRSF5	CACAGgt	4.965

Figure 1 Characterisation of two novel PEs and one chromosomal rearrangement between *DMD* and 8p22. (A) Sanger sequencing in P1 showed two different transcripts: WT transcript and aberrant transcript with PE15 between exons 15 and 16, from c.1813–947 to c.1813–734. (B) Schematic representation of canonical splicing (in blue) and alternative splicing (in red) taking place in P1. At the gDNA level, it was detected that the variant g.32584731A>G (c.1813–733A>G) creates a novel donor SS. (C) mRNA analysis in P2 revealed residual levels of WT transcript and an alternative transcript with an inclusion of 48 bp of intron 37 (PE37). (D) Schematic view of canonical (in blue) and alternative (in red) splicing due to the presence of the hemizygous variant g.32371864A>C (c.5326–5219T>G), which creates a new acceptor SS and results in the inclusion of the in-frame PE37. (E) Splicing predictor scores of the cryptic exon from the *LOC101929028* locus (8p22) detected in the mature *DMD* transcript in p6. Acceptor, donor SSs and binding sites of SR proteins were predicted by ESEfinder V.3.0. (F) Schematic view of the translocation of 75.7 kb from chromosome 8 (chr8: 16 346 710–16 422 443) to intron 2 of the *DMD* gene involving a 6 bp deletion in the breakpoint junctions, and an insertion of three nucleotides 'CCA'. (G) IGV screenshot of P6 WGS in the junction breakpoint. The upper panel displays the depth coverage, showing the deletion of 6 bp in *DMD* intron 2 (chrX:32,873,008–32,873,015). The bottom panel shows the aligned reads on chromosome X (in grey) and the chimeric reads aligning both chromosome X and chromosome 8 (in purple). PE, pseudoexon; SR, serine-rich; SS, splice site; WT, wild type.

J Med Genet: first published as 10.1136/jmg-2022-108828 on 19 December 2022. Downloaded from http://jmg.bmj.com/ on April 20, 2024 by guest. Protected by copyright.

In P4, besides residual levels of full-length dystrophin, we detected a PE of 58 bp between exons 62 and 63 (PE62). The deep intronic variant g.31279418T>C (c.9225–285A>G) was identified, inherited from the patient's mother. PE62 had already been described in patients with BMD.[12] The pathogenic deep intronic variant c.9225–285A>G, located in position+5 from the cryptic donor SS, increases complementarity between the pre-mRNA and U1snRNA, and thus increases the splicing efficiency of the cryptic donor SS.[12]

Chromosomal rearrangement

P6 cDNA sequencing revealed a 64 bp cryptic exon (p.Phe32Asnfs*5) between exons 2 and 5 apart from the wild-type dystrophin transcript. BLAT (BLAST-like alignment tool) analysis of the cryptic exon resulted in 100% identity with *LOC101929028* located in 8p22 (chr8:16 369 356–16 369 419). The flanking region of 64 bp inserted in the mRNA of dystrophin probably confers an exon-like profile with an acceptor, donor SS and exonic splicing enhancers that can be recognised by the spliceosome complex (figure 1E), suggesting the presence of a chromosomal rearrangement between chromosome 8 and *DMD*. WGS was performed in P6 to determine the exact breakpoints of the rearrangement. We detected an insertion of chromosome 8 (chr8: 16 346 710–16 422 443) in intron 2 of the *DMD*, and a 6 bp deletion in *DMD* intron 2 (chrX:32 873 009–32 873 014) (figure 1F). IGV showed the junction breakpoints surrounded by chimeric reads aligning both *DMD* and chromosome 8 (figure 1G). The insertion of 75.7 kb encompassed the *LOC101929028* detected in the mRNA analysis and did not contain any protein-coding gene. In-depth analysis of the flanking sequences of the breakpoint junctions revealed the presence of LINE elements in 5′ and 3′ junctions.

Alternative exon skipping events with unclear pathogenicity

In three patients with BMD (patient 3 (P3), P5 and P7) (table 1), no other alteration was detected in dystrophin mRNA apart from transcripts with exon skipping events, together with full-length transcript. In P3, a patient with BMD manifesting only hyperCKemia, we detected exon 9 skipping. In P5, who had mild BMD, exon 71 and 78 skipping was revealed. Finally, in P7, we detected exon 71 skipping. No intronic variants were found near canonical SS that could explain the induction of these exon skipping events. Exon 9 and 71 *DMD* skipping isoforms were quantified by ddPCR in P3 and P7. Both exon skipping events were present in slightly higher proportion in patients than in control samples (exon 9 skipping: 0.97%±0.2 in controls and 11.15%±0.3 in P3; exon 71 skipping: 1.34%±0.16 in controls and 16.45%±0.9 in P7) (online supplemental figure 1).

DISCUSSION

Here we show the relevance of performing an mRNA-based analysis of the *DMD* gene to establish a definite genetic diagnosis in patients with undiagnosed dystrophinopathy after MLPA and ES. Up to 7% of the patients remain genetically undiagnosed after routine genetic testing.[7,8] Nonetheless, having a precise genetic diagnosis is essential for these patients to be eligible for mutation-specific therapies and for their relatives to receive an accurate genetic and reproductive counselling. Here we studied seven patients with clinical suspicion of DMD/BMD but no precise genetic diagnosis after clinical genetic testing (table 1). We detected a transcriptional alteration in the *DMD* in all patients and established a final genetic diagnosis in four of the seven patients. Our findings indicate that variants that alter *DMD* splicing, such as deep intronic variants and SVs, are an important cause of DMD/BMD in patients with undiagnosed dystrophinopathy.

The implementation of high-throughput technologies in clinical testing has enabled a faster and a more efficient exploration of the human genome. As a result, invasive procedures such as muscle biopsy tend to be avoided. However, this procedure remains an essential tool in the differential diagnosis of several neuromuscular disorders. If in the first approach we had performed WGS instead of mRNA sequencing of *DMD*, we would not have been able to interpret and validate some of the findings responsible for the pathology in our cohort. For instance, through WGS, we would have detected two novel deep intronic variants in P1 and P2 (figure 1A–D), but we would not have been able to assess their pathogenicity. Computational algorithms for predicting alternative splicing are usually less reliable for deep intronic variants than for variants in canonical SSs.[13] In these cases, performing an RNA-based analysis is essential to assess the impact of deep intronic variants in the canonical splicing of the *DMD* gene and to reclassify intronic variants previously classified as VUS.

The *DMD* is a complex gene for the spliceosome machinery due to its large protein-coding sequence and intronic regions. In muscle tissue, few alternative splicing events are observed in comparison with other tissues, as a tight splicing regulation is needed in the muscle tissue to maintain dystrophin functionality.[14,15] In our cohort, we detected three exon skipping events with unclear pathogenicity that commonly occur in non-muscle tissues (exon 9 skipping in P3, exon 71 skipping in P5 and P7, and exon 78 skipping in P5).[16,17] P3, P5 and P7 present mild forms of BMD with hyperCKemia, muscle cramps or rhabdomyolisis (table 1). Similar works have also reported exon skipping events in patients with BMD with no other genomic alteration.[18,19] However, further analyses in a larger patient cohort will be needed to clarify the role of these exon skipping events.

In summary, after evaluating splicing alterations in the *DMD* gene through cDNA sequencing, we found a transcriptional alteration in all patients who remained undiagnosed after clinical genetic testing and established a precise diagnosis in four of the patients. Recently, RNA sequencing (RNA-seq) is being considered as a promising diagnostic approach in undiagnosed patients after ES. It is estimated that RNA-seq increases the genetic diagnostic rate in Mendelian disorders by up to 35%.[20] One of the main advantages of this approach is the ability to evaluate not only alternative splicing events but also transcript isoform abundance, monoallelic expression and differential gene expression in the targeted tissue. However, to detect the presence of SVs, RNA-seq can only detect those that alter canonical splicing, such as the insertion identified in P6 (figure 1E–G). SVs that do not have a direct effect in RNA processing would have been missed through an RNA-based analysis. Hence, an integrated approach with transcriptomics and WGS can help to achieve a precise genetic diagnosis in patients with dystrophinopathy and may allow them the accessibility to mutation-specific therapies and genetic and reproductive counselling.

Author affiliations
[1]Genetics Department, Institut d'Investigació Biomèdica Sant Pau (IIB SANT PAU), Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
[2]Genetics and Microbiology Department, Universitat Autonoma de Barcelona, Bellaterra, Spain

J Med Genet: first published as 10.1136/jmg-2022-108828 on 19 December 2022. Downloaded from http://jmg.bmj.com/ on April 20, 2024 by guest. Protected by copyright.

[3]Neuromuscular Unit, Neurology Department, imas12 Research Institute, Hospital Universitario 12 de Octubre, Madrid, Spain
[4]Centre for Biomedical Network Research on Rare Diseases (CIBERER), Instituto de Salud Carlos III, Madrid, Spain
[5]Neuropathology Unit, imas12 Research Institute, Hospital Universitario 12 de Octubre, Madrid, Spain
[6]Clinical Genetics Unit, Genetics department, Hospital Universitario 12 de Octubre, Madrid, Spain
[7]UDISGEN (Unidad de Dismorfología y Genética), Hospital Universitario 12 de Octubre, Madrid, Spain
[8]Paediatric Neurology Unit, Universidad Complutense de Madrid, Hospital Universitario 12 de Octubre, Madrid, Spain
[9]Pathology Department, Instituto de Biomedicina de Sevilla, CSIC, Universidad de Sevilla, Hospital Universitario Virgen del Rocio, Sevilla, Spain
[10]UGC Pediatrics Department, Hospital Universitario Virgen Macarena, Sevilla, Spain
[11]Neurology Department, Neurolinkia, Sevilla, Spain
[12]Hospital Viamed Santa Ángela de la Cruz, Sevilla, Spain
[13]Neuromuscular Unit, Department of Neurology, Hospital Sant Joan de Déu, Barcelona, Spain
[14]Applied Research in Neuromuscular Diseases, Institut de Recerca Sant Joan de Déu, Barcelona, Spain

Twitter Alba Segarra-Casas @AlbaSegarraC and Lidia Gonzalez-Quereda @lidiagquer

Acknowledgements We thank the patients and their relatives for their participation in this research study and the European Reference Network for Neuromuscular Diseases. We also thank Carolyn Newey for English language editing.

Contributors PG, LG-Q and AS-C: study design, acquisition and interpretation of data, preparation of the draft of the manuscript, and reviewing and editing of the final manuscript. PG and LG-Q: funding acquisition. PG, AH-L, ACa, MTS-C, CD-G, AC-B, MM, ER, ACo, CO, DN-dB, AN and MJR: clinical data collection, data analysis, and reviewing and editing the final manuscript. All authors approved the final manuscript.

Funding This study was supported by Instituto de Salud Carlos III and FEDER, 'Una manera de hacer Europa' (grant number FIS PI18/01585, LG-Q and PG) and the Ministerio de Universidades (grant number FPU20/06692, AS-C).

Competing interests None declared.

Patient consent for publication Consent obtained from parent(s)/guardian(s).

Ethics approval This study involves human participants and was approved by the ethics committee of Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) (REF: IIBSP-CAN-2021-110). Written informed consent was obtained from all patients and signed by parents if the patient was a minor.

Provenance and peer review Not commissioned; externally peer reviewed.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/.

ORCID iDs
Alba Segarra-Casas http://orcid.org/0000-0001-5185-5031
Cristina Domínguez-González http://orcid.org/0000-0001-5151-988X

REFERENCES

1 Mercuri E, Bönnemann CG, Muntoni F. Muscular dystrophies. *Lancet* 2019;394:2025–38.
2 Emery AE. Population frequencies of inherited neuromuscular diseases--a world survey. *Neuromuscul Disord* 1991;1:19–29.
3 Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* 1988;2:90–5.
4 Tuffery-Giraud S, Béroud C, Leturcq F, Yaou RB, Hamroun D, Michel-Calemard L, Moizard M-P, Bernard R, Cossée M, Boisseau P, Blayau M, Creveaux I, Guiochon-Mantel A, de Martinville B, Philippe C, Monnier N, Bieth E, Khau Van Kien P, Desmet F-O, Humbertclaude V, Kaplan J-C, Chelly J, Claustres M. Genotype-phenotype analysis in 2,405 patients with a dystrophinopathy using the UMD-DMD database: a model of nationwide knowledgebase. *Hum Mutat* 2009;30:934–45.
5 Aartsma-Rus A, Van Deutekom JCT, Fokkema IF, Van Ommen G-JB, Den Dunnen JT. Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle Nerve* 2006;34:135–44.
6 Juan-Mateu J, Gonzalez-Quereda L, Rodriguez MJ, Baena M, Verdura E, Nascimento A, Ortez C, Baiget M, Gallano P. Dmd mutations in 576 Dystrophinopathy families: a step forward in genotype-phenotype correlations. *PLoS One* 2015;10:e0135189.
7 Dent KM, Dunn DM, von Niederhausern AC, Aoyagi AT, Kerr L, Bromberg MB, Hart KJ, Tuohy T, White S, den Dunnen JT, Weiss RB, Flanigan KM. Improved molecular diagnosis of dystrophinopathies in an unselected clinical cohort. *Am J Med Genet A* 2005;134:295–8.
8 Okubo M, Minami N, Goto K, Goto Y, Noguchi S, Mitsuhashi S, Nishino I. Genetic diagnosis of Duchenne/Becker muscular dystrophy using next-generation sequencing: validation analysis of DMD mutations. *J Hum Genet* 2016;61:483–9.
9 Lim KH, Ferraris L, Filloux ME, Raphael BJ, Fairbrother WG. Using positional distribution to identify splicing elements and predict pre-mRNA processing defects in human genes. *Proc Natl Acad Sci U S A* 2011;108:11093–8.
10 Chen X, Schulz-Trieglaff O, Shaw R, Barnes B, Schlesinger F, Källberg M, Cox AJ, Kruglyak S, Saunders CT. Manta: rapid detection of structural variants and indels for germline and cancer sequencing applications. *Bioinformatics* 2016;32:1220–2.
11 Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and genomics and the association for molecular pathology. *Genet Med* 2015;17:405–24.
12 Tuffery-Giraud S, Saquet C, Chambert S, Claustres M. Pseudoexon activation in the DMD gene as a novel mechanism for Becker muscular dystrophy. *Hum Mutat* 2003;21:608–14.
13 Riepe TV, Khan M, Roosing S, Cremers FPM, 't Hoen PAC. Benchmarking deep learning splice prediction tools using functional splice assays. *Hum Mutat* 2021;42:799–810.
14 Miro J, Bougé A-L, Murauer E, Beyne E, Da Cunha D, Claustres M, Koenig M, Tuffery-Giraud S. First identification of RNA-binding proteins that regulate alternative exons in the dystrophin gene. *Int J Mol Sci* 2020;21:7803.
15 Tuffery-Giraud S, Miro J, Koenig M, Claustres M. Normal and altered pre-mRNA processing in the DMD gene. *Hum Genet* 2017;136:1155–72.
16 Reiss J, Rininsland F. An explanation for the constitutive exon 9 cassette splicing of the DMD gene. *Hum Mol Genet* 1994;3:295–8.
17 Bougé A-L, Murauer E, Beyne E, Miro J, Varilh J, Taulan M, Koenig M, Claustres M, Tuffery-Giraud S. Targeted RNA-seq profiling of splicing pattern in the DMD gene: exons are mostly constitutively spliced in human skeletal muscle. *Sci Rep* 2017;7:39094.
18 Xie Z, Sun C, Zhang S, Liu Y, Yu M, Zheng Y, Meng L, Acharya A, Cornejo-Sanchez DM, Wang G, Zhang W, Schrauwen I, Leal SM, Wang Z, Yuan Y. Long-Read whole-genome sequencing for the genetic diagnosis of dystrophinopathies. *Ann Clin Transl Neurol* 2020;7:2041–6.
19 Okubo M, Noguchi S, Awaya T, Hosokawa M, Tsukui N, Ogawa M, Hayashi S, Komaki H, Mori-Yoshimura M, Oya Y, Takahashi Y, Fukuyama T, Funato M, Hosokawa Y, Kinoshita S, Matsumura T, Nakamura S, Oshiro A, Terashima H, Nagasawa T, Sato T, Shimada Y, Tokita Y, Hagiwara M, Ogata K, Nishino I. Rna-Seq analysis, targeted long-read sequencing and in silico prediction to unravel pathogenic intronic events and complicated splicing abnormalities in dystrophinopathy. *Hum Genet*;27.
20 Cummings BB, Marshall JL, Tukiainen T, Lek M, Donkervoort S, Foley AR, Bolduc V, Waddell LB, Sandaradura SA, O'Grady GL, Estrella E, Reddy HM, Zhao F, Weisburd B, Karczewski KJ, O'Donnell-Luria AH, Birnbaum D, Sarkozy A, Hu Y, Gonorazky H, Claeys K, Joshi H, Bournazos A, Oates EC, Ghaoui R, Davis MR, Laing NG, Topf A, Kang PB, Beggs AH, North KN, Straub V, Dowling JJ, Muntoni F, Clarke NF, Cooper ST, Bönnemann CG, MacArthur DG, Genotype-Tissue Expression Consortium. Improving genetic diagnosis in Mendelian disease with transcriptome sequencing. *Sci Transl Med* 2017;9:eaal5209.

J Med Genet: first published as 10.1136/jmg-2022-108828 on 19 December 2022. Downloaded from http://jmg.bmj.com/ on April 20, 2024 by guest. Protected by copyright.