Clinical Genetics

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Effectiveness and Impact of Transcript Analysis in Clinical Genetics Daily Practice

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ABSTRACT

Broad-spectrum genetic tests often lead to the identification of variants of uncertain significance (VUS), a major issue in modern clinical genetics. A fair proportion of VUS may alter the splicing processes, but their interpretation is challenging. This study aimed at providing a classification approach for VUS potentially-affecting splicing by integrating transcript analysis from peripheral blood mRNA into routine diagnostics. VUS in *DICER1*, *MSH2*, *MLH1*, *DYNC1H1*, *RPS6KA3*, and *SCN9A*, found in patients with phenotypes compatible with the related syndromes, altered splicing, leading to their re-classification as Pathogenic/Likely Pathogenic. This had a significant clinical impact for different diseases, from hereditary tumor predisposition to neurological and congenital syndromic disorders. Transcript analysis is valuable in VUS clinical evaluation, and its incorporation into routine diagnostic workflows facilitates timely and accurate clinical decision-making.

1 | Short Report

The ever-growing use of broad-spectrum genetic tests for rare disease diagnosis increase the detection of variants of uncertain significance (VUS), with consequent challenges for counseling and patient management [1]. Although some authors hypothesized that the majority of genomic variants will be classified in the near future [2], to date VUS remain one of the major issues in clinical genetics. Among pathogenic genomic variants, up to 60% alters gene splicing, that is, the process of intron removal from pre-mRNA transcripts [3]. The identification of such variants is still challenging, because bioinformatic predictors do not yet reach absolute sensitivity and specificity [4], and the evaluation of their actual effect through functional assays remains time-consuming and demanding for many diagnostic laboratories, therefore it is not systematically carried out in clinical practice.

In this work, we show our pipeline used to clarify the role of VUS predicted to alter splicing in patients with suspected genetic diseases.

Patients underwent genetic counseling sessions following standard care protocol. The variants herein reported were detected through diagnostic next generation sequencing (NGS) analyses, including single-gene analysis, target gene panels or whole exome sequencing, as detailed in Supporting Information. Variants were classified using the ACMG/AMP guidelines [5] and ClinGen variant curation expert panels with gene-specific

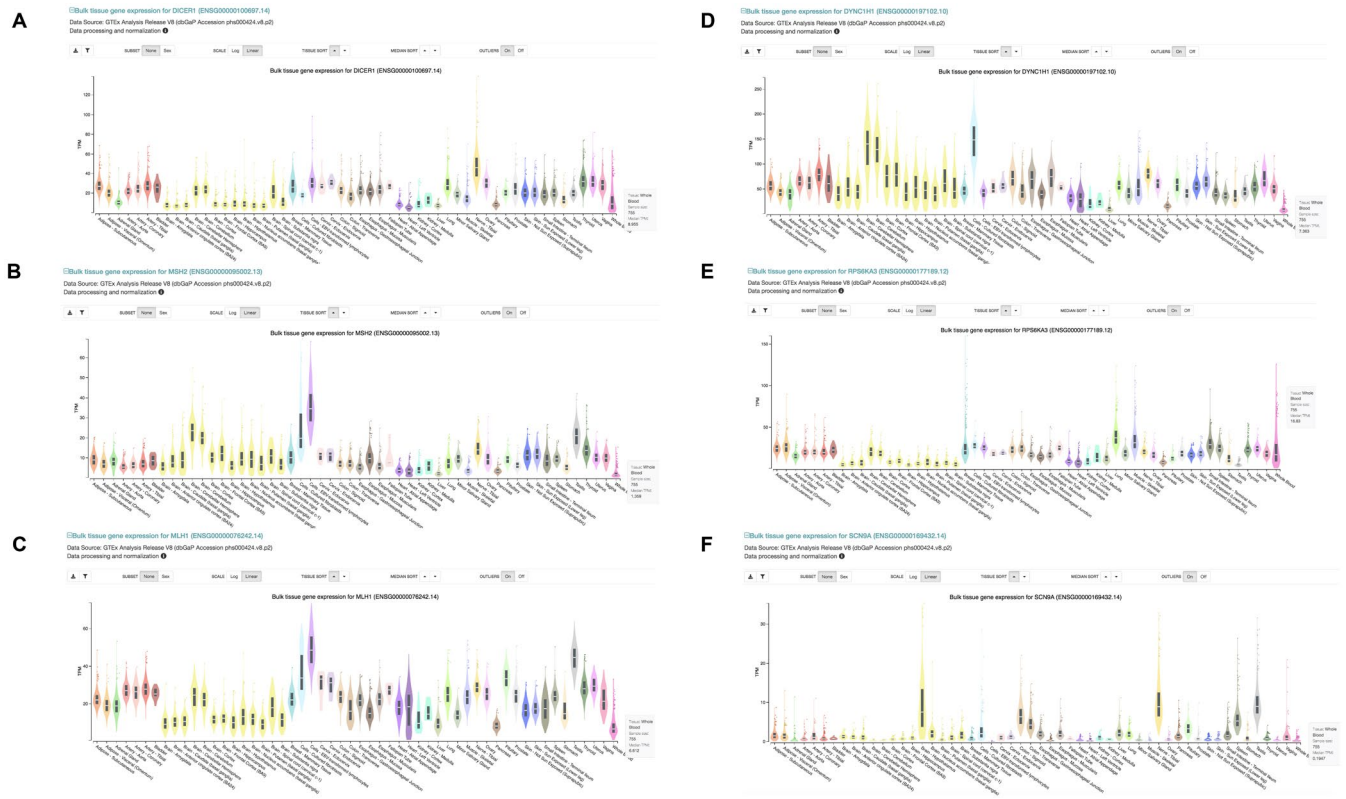

FIGURE 1 | GTEx portal expression data for the analyzed genes. (A–F) Bulk RNAseq data (TPM, transcript per million) in human normal tissues. Accession June 2024. [Colour figure can be viewed at wileyonlinelibrary.com]

criteria. VUS emerging from diagnostic NGS tests were selected for transcript analysis if they met the following criteria:

- Variant in a gene where alterations are associated with phenotypes compatible with the ones of patients;
- Variant predicted to alter splicing according to MaxEntScan (Figures S1 and S2), Human Splice Finder v3.0, SpliceAI and/or SPiP [6];
- Absence of Pathogenic/Likely Pathogenic variants in other regions analyzed for diagnostic purposes that could explain patients' phenotype;
- Variant mapping to the canonical gene transcripts and expressed in peripheral blood (according to Genotype-Tissue Expression portal, GTEx, Figure 1A–E). We performed this analysis also for a particularly credible variant in a gene despite the very low expression in peripheral blood, that is, *SCN9A* (Figure 1F).

According to these criteria, a new blood draw for transcript analysis was proposed to the patients.

The analyzed variants are reported in Table 1; clinical descriptions and transcript analyses are detailed in Supporting Information.

1.1 | Case 1—*DICER1*

A male proband with clinical suspicion of *DICER1* syndrome [7] carried the heterozygous variant c.2468G>T (p.Gly823Val) in exon 16, absent in gnomAD, subsequently found in the mother and older brother, who both shared the patient's phenotype. This variant, classified VUS according to ACMG/AMP (PM2, PP3, PP2), maps close to an acceptor splice site and *in silico* splicing predictions (Table 1, Figure S1A, *panels i, ii*) indicated that it might unmask an exonic internal splice site. RT-PCR amplification from patient's peripheral blood-derived cDNA (exons 14–18) resulted in a wild-type fragment (365 bp) and a smaller fragment, with a 34 base pair deletion in exon 16, as detected by Sanger sequencing (Figure 2A, *panels i–ii*, respectively), due to the use of the cryptic acceptor splice site inside exon 16, unmasked by the variant. The deletion induces a frameshift, with a premature stop codon at aminoacid 818. The PS3 criterion could be added to classify the variant Likely Pathogenic, according to *DICER1*-specified ACMG/AMP criteria. The proband and other family members carrying the germline variant were therefore included in *DICER1* syndrome surveillance program [8].

1.2 | Case 2—*MSH2*

A male proband with clinical suspicion of Lynch syndrome [9], likely derived from the paternal branch of the family, carried the heterozygous synonymous *MSH2* c.1275A>G (p.Glu425=) variant, classified VUS according to ACMG/AMP guidelines (PM2, PP3, BP6), VUS in InSiGHT, the reference database for variant classification in Lynch syndrome, and Likely Benign/ VUS in ClinVar (Variation ID: 90589, Accession: VCV000090589.67). *In silico* predictions (Table 1, Figure S1B, panels *i-ii*) and previous studies [10] suggested an

TABLE 1 | Characteristics of the identified variants.

Patient ID	Chromosome position (hg38)	Gene	RefSeq transcripts[a]	cDNA position	Type of change	Effect on protein translation	gnomAD (v.4.1.0)	dbSNP ID
Case 1	Chr14:95108062C>A	*DICER1*	NM_177438.3	c.2468G>T	p.(Gly823Val)	p.Gln812fs8Ter	Absent	rs1889808547
Case 2	Chr2:47429940A>G	*MSH2*	NM_000251.3	c.1275A>G	p.(Glu425=)	p.Gly410_Glu425del	Absent	rs63751650
Case 3	Chr3:37007064G>A	*MLH1*	NM_000249.4	c.453+1G>A	intronic	p.Arg128fs8Ter	Absent	rs267607750
Case 4	Chr14:102018621G>A	*DYNC1H1*	NM_001376.5	c.8343+5G>A	intronic	p.Arg2726fs18Ter	0.0000217	rs369653555
Case 5	ChrX:20169503A>C	RPS6KA3	NM_004586.3	c.1354-12T>G	intronic	p.Lys451fs261Ter	Absent	Absent
Case 6	Chr2:166284475T>C	SCN9A	NM_001365536.1	c.1952A>G	p.(Asp651Gly)	p.Asp651fs13Ter	8.48E-07	Absent

Patient ID	Varsome/ClinVar/ Franklin	*In Silico* prediction	MaxEntScan lVariationl (%)	HSF v3.1		SpliceAI	SPiP
				Variation	Signal and interpretation of effect on splicing		
Case 1	VUS/Absent/VUS	**CADD:** Pathogenic Strong (33)	No significant MaxEnt scores found	−10.64 > 4.25 (139.64%)	New acceptor splice site. Activation of a cryptic Acceptor site. Potential alteration of splicing	0.66 (AG)	Alteration of an exonic splicing regulatory element (30.67%)
Case 2	Likely benign/ VUS/VUS	**CADD:** Uncertain (23.3)	[45.07]	8.92 > 4.90 (−45.07%)	Broken WT donor site. Alteration of the WT Donor site, most probably affecting splicing	0.62 (DG)	Alteration of the consensus splice site + Alteration of an exonic splicing regulatory element (98.41%)
Case 3	Pathogenic/ Pathogenic/ Pathogenic	**CADD:** Pathogenic Strong (35)	[76.81]	91.02 > 63.88 (−29.82%)	Broken WT donor site. Alteration of the WT Donor site, most probably affecting splicing	1.00 (DL)	Alteration of the consensus splice site (98,41%)
Case 4	Benign/VUS/VUS	**CADD:** Benign Strong (0.2579)	[27.96]	89.35 > 79.78 (−10.71%)	Broken WT donor site. Alteration of the WT Donor site, most probably affecting splicing	0.00 (DL)	Alteration of the consensus splice site (98,41%)
Case 5	VUS/Absent/VUS	**CADD:** Benign Supporting (22.5)	[168.96]	49.08 > 76.95 (56.78%)	New Acceptor splice site. Activation of a cryptic Acceptor site. Potential alteration of splicing (cryptic exon activation)	0.89 (AG)	Alteration of the consensus splice site (98,41%)
Case 6	VUS/Absent/VUS	**CADD:** Uncertain (24.6)	[723.89]	68.76 > 95.9 (39.47%)	New Donor splice site. Activation of a cryptic Donor site. Potential alteration of splicing.	0.99 (DG)	Creation of a new splice site (85.91%)

Note: Genomic coordinates (hg38), cDNA position, amino acid change, Minor Allele Frequency (MAF) in Genome Aggregation Consortium (gnomAD v4.01, European Non Finnish population), rsID in dbSNP(when available) and *in silico* prediction of pathogenicity using different programs are reported.
Abbreviations: DG = donor gain; AG = Acceptor gain; DL = donor loss.
[a]MANE transcript.

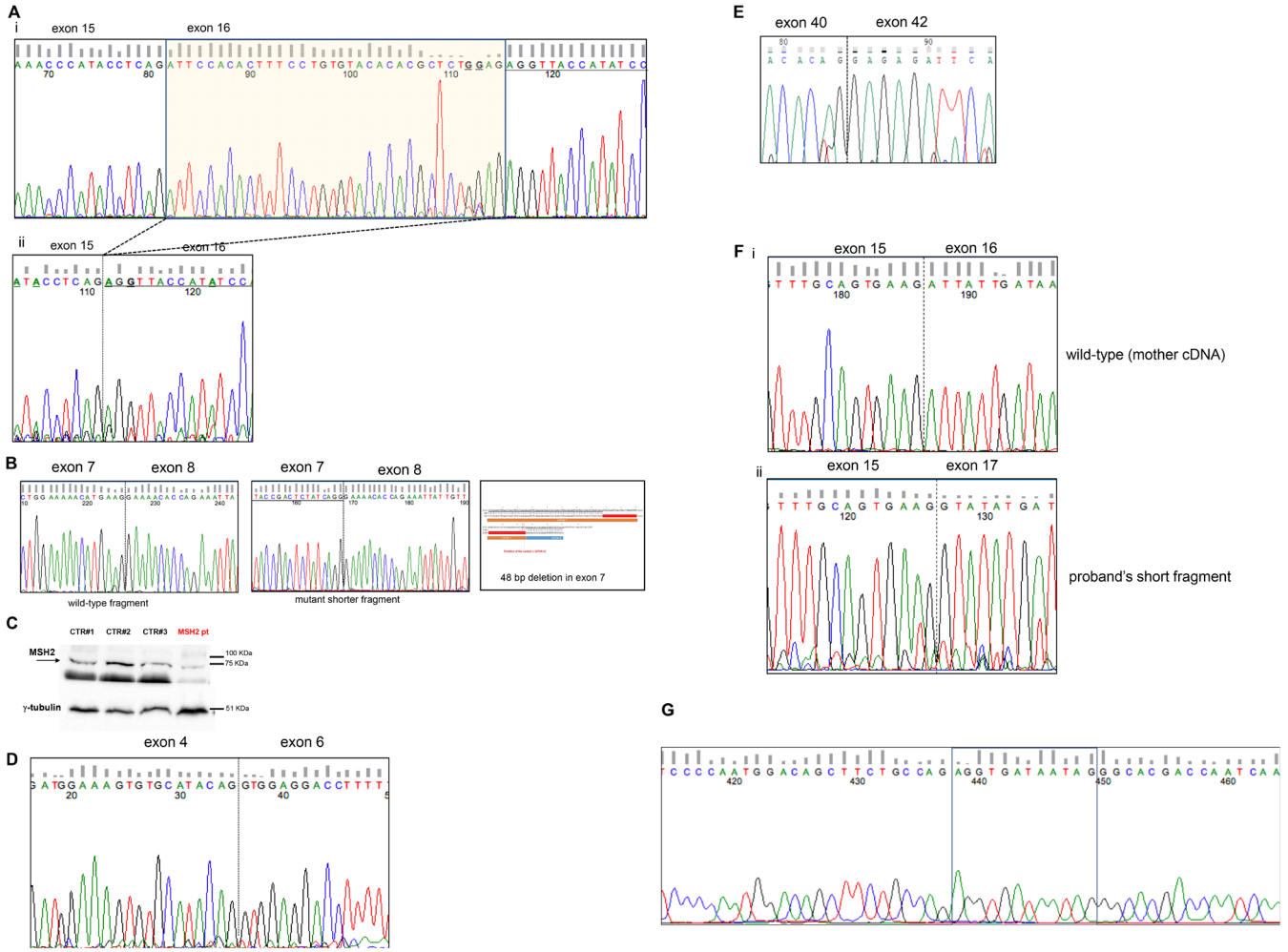

FIGURE 2 | Transcript analyses from blood RNA. (A) cDNA of patient carrying the constitutive *DICER1* variant in exon 16 was amplified by RT-PCR. Representative electropherograms of (i) wild-type fragment, and (ii) smaller band with a 34-bp deletion in exon 16 (yellow boxed area in wild-type fragment (i)). (B) cDNA of patient carrying the heterozygous *MSH2* silent variant was amplified by RT-PCR. Representative elecropherograms of wild-type and mutant fragments are reported, indicating the 48-bp deletion in exon 7. Right panel: The deleted sequence in highlighted in red. (C) Representative western blot analysis for MSH2 in lysates derived from WBC of controls (CNT#1–3) and *MSH2*-mutant patient. γ-tubulin was used as endogenous control. MSH2 quantity is severely reduced in patient versus controls. (D) cDNA of patient carrying the heterozygous *MLH1* variant was amplified by RT-PCR. Representative elecropherograms of wild-type and mutant fragments are shown, indicating exon 5 skipping in the mutant fragment. (E) The cDNA of patient carrying the heterozygous variant in *DYNC1H1* was amplified by RT-PCR, resulting in a wild-type fragment and a smaller one with exon 41 skipping, as shown by direct sequencing. (F) cDNA of patient with the hemizygous variant in *RPS6KA3* and of his mother was amplified by RT-PCR. Sanger sequencing of the maternal wild-type fragment showed the correct splicing pattern (upper electropherogram). Representative electropherogram of the small fragment (the only one in the patient, and present also in the heterozygous carrier mother) showed the aberrant exon 16 skipping. (G) cDNA of patient with the homozygous *SCN9A* variant was amplified by RT-PCR. Sanger sequencing of the RT-PCR product revealed a shortened exon 12 included in the final transcript (dashed line). [Colour figure can be viewed at wileyonlinelibrary.com]

impact on splicing. RT-PCR amplification from patient's peripheral blood-derived cDNA (spanning exons 6–8) resulted in a wild-type fragment (288 bp) and a smaller fragment with a 48-bp deletion in exon 7, confirmed by Sanger sequencing, due to the use of a cryptic splice site as reported [10] (Figure 2B). AlphaFold prediction suggested that the 16-aminoacids loss would severely affect the folding of an alpha-helix portion. Western blot showed lower MSH2 protein levels in the lysate of white blood cells (WBC) from the patient versus three healthy controls (Figure 2C). Variant segregation in the mother resulted negative, suggesting paternal inheritance. These results, concordant with immunohistochemical data on the patient's tumor tissue, strongly support variant pathogenicity. Although it was not possible to extend targeted genetic testing to first-degree relatives, it was suggested to them to undergo an initial colonoscopy and annual gynecological check-ups (for females).

1.3 | Case 3—*MLH1*

A male proband with clinical suspicion of Lynch syndrome [9] carried the heterozygous *MLH1* c.453+1G>A variant, classified Pathogenic/Likely pathogenic according to ACMG/AMP (PS4, PVS1, PM2) and ClinVar (single submission), but reported as VUS in InSiGHT. Its location in the first base of a canonical

donor splice site is predicted to impair splicing (Table 1). RT-PCR of the cDNA derived from patient's peripheral blood showed a wild-type band (222 bp) and a smaller band with the expected exon 5 skipping, as confirmed by Sanger sequencing (Figure 2D), inducing a frameshift and a premature stop codon at aminoacid 134. This allowed to extend targeted genetic test to the four daughters. After the clinical management of this case, the variant was formally classified Pathogenic also in InSiGHT.

1.4 | Case 4—*DYNC1H1*

A male proband with peripheral neuropathy carried the heterozygous *DYNC1H1* c.8343 + 5G>A variant, classified VUS according to ACMG/AMP (PM2) and ClinVar. Since its location within the consensus splice site of intron 41, and considering *in silico* predictions results (Table 1, Figure S2A, *panels i-ii*), transcript analysis was performed. RT-PCR amplification from patient-derived peripheral blood cDNA (exons 40–43) resulted in a wild-type fragment (299 bp) and a smaller fragment, confirming the exon 41 skipping, as shown via Sanger sequencing (Figure 2E). This result led to add the PS3 criterion and classify the variant Likely Pathogenic, confirming the diagnosis of *DYNC1H1*-related disorder [11].

1.5 | Case 5—*RPS6KA3*

A male proband with a syndromic clinical picture carried the hemizygous *RPS6KA3* c.1354-12T>G variant, absent in gnomAD and dbSNP and classified VUS according to ACMG/AMP (PM2, PP3). The variant was found to be inherited from the mother, in whom it was present in heterozygosis in blood-derived DNA, with no indication of mosaicism. Given the *in silico* predictions results (Table 1, Figure S2B, *panels i-ii*), transcript analysis was performed on both proband and his mother. RT-PCR amplification from peripheral blood-derived cDNA of the region spanning exons 14–18 revealed in the mother both the band corresponding to the wild-type allele (Figure 2F, *panel i*) and a shorter isoform, which was the only one present in the patient, as expected for a X-linked inheritance. Sanger sequencing of the short band confirmed the altered spliced form with exon 16 skipping (Figure 2F, *panel ii*). This result led to add the PS3 criterion and classify the *RPS6KA3* c.1354-12T>G variant as Likely Pathogenic, confirming the diagnosis of Coffin-Lowry syndrome [12]. Segregation analysis in the maternal grandparents proved the *de novo* origin of the variant in the mother. Notably, the mother presented a very mild phenotype, with the hand conformation as the only clinical sign present, in accordance with the nuanced phenotypes sometimes observed in female carriers. The splicing data led to integrate patient's follow-up with evaluations and exams based on the genetic diagnosis, and offered the possibility of carrying out a prenatal diagnosis for the ongoing pregnancy in the mother.

1.6 | Case 6—*SCN9A*

A male proband, born of consanguineous parents and presenting with neurologic issues, carried the homozygous *SCN9A* variant c.1952A>G in NM_001365536.1 (p.Asp651Gly) (intronic in transcript NM_002977.3:c.1941 + 11A>G), located within a run of homozygosity of 9.5 Mb (total patient homozygosity of 65.8 Mb) and classified VUS according to ACMG/AMP (PM2, PP3). Since its location near the canonical splice site, and considering *in silico* prediction results (Table 1, Figure S2C, panels *i, ii*), transcript analysis was performed.

RT-PCR amplification (region spanning exons 11–13) of the cDNA derived from patient's peripheral blood showed a single fragment, and Sanger sequencing revealed that the homozygous variant generated a premature alternative donor splice site in exon 12, resulting in a shortened exon 12 (−23 bp), leading to a frameshift and a premature stop codon in exon 13 (Figure 2G). The PS3 and PP4 criteria could therefore be added to classify the variant Likely Pathogenic, confirming the diagnosis of Hereditary Sensory and Autonomic Neuropathy type IID (HSAN2D) [13] and reassuring the patient for the recurrence risk of the ongoing pregnancy of the spouse.

2 | Discussion

In the precision medicine era, identification and proper interpretation of disease variants play a pivotal role in clinical diagnosis and patient management. In this perspective, our work confirmed how transcript analysis, whenever variants potentially affecting splicing are identified, can represent an efficient support for standard diagnostic procedures [14, 15]. Transcript analysis offers a rapid and cost-effective approach to validate variant pathogenicity, providing evidence of their impact for clinical decision-making and patient management. Moreover, it can uncover splicing events missed by *in silico* predictions alone, particularly relevant for variants located in non-canonical splice sites or regulatory regions where computational algorithms may lack accuracy, providing a more comprehensive understanding of variant effect [4]. Several challenges remain, including protocol standardization, interpretation of complex splicing patterns, and differential tissue expression. Future efforts are needed to address these issues. However, this approach has had a significant clinical impact in patients with different types of disease, ranging from hereditary tumor predisposition syndromes to congenital syndromic disorders [6, 16–18], allowing to resolve a diagnostic process that for several affected individuals lasted years (the longest period in this case-study was 4 years for Case 3). In the present study, in Cases 1–3 confirmation of the variant effects on splicing and the consistency with other features made it possible to support a clinical diagnosis, set up adequate surveillance and offer predictive tests to relatives. In Case 5, the quick result of transcript study, leading to a specific diagnosis in the proband, made it possible to promptly offer a prenatal diagnosis in the parents' new pregnancy. This case is also notable, because it is the third report of restrictive cardiomyopathy to date associated with a *RPS6KA3*-related disorder [12], highlighting the importance of cardiological surveillance in these patients.

Even when transcript analysis did not support a splicing effect for a variant in patient-derived fibroblasts (*CACNA1G* gene, transcript *ENST00000359106.5* c.2454-4G>A, data not shown), there was an impact for the patient, since the functional results

allowed to rule out those gene variants and therefore evaluate differential diagnoses for the patient's condition.

In conclusion, transcript analysis represents a valuable tool in the clinical evaluation of VUS potentially affecting splicing, and by incorporating this approach into routine diagnostic workflows, clinical geneticists can improve variant interpretation, advancing patient care and outcomes.

Author Contributions

Conceptualization: G.I., A.V., E.B., D.T.; methodology: G.I., C.D., A.V., E.B.; investigation and data curation: all authors; writing – original draft preparation: G.I., E.C., E.Z., I.B., S.C.; writing – review and editing: G.I., A.V., E.B., D.T.; All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/cge.14684.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.