TITLE: RNA and Reclassification: Assessing RNA Data in Rare Disease

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OBJECTIVE RNA analysis holds potential for improving the diagnostic yield of clinical exome sequencing (ES). We examine two cases where RNA analysis revealed substantial splicing defects but led to contrasting classification decisions.

METHODS ES identified variants with predicted splice impacts for two patients with rare syndromic neurodevelopmental disorders: homozygous *ATP6V0A2* c.2055+4A>C (case 1) and heterozygous *CNOT3* c.387+5G>A (case 2). Both were initially classified as variants of unknown significance (VUS) due to lack of direct evidence. Targeted RT-PCRseq on RNA from whole blood assessed the splicing impact. The magnitude, specificity, reproducibility, and protein functional impact were evaluated based on ClinGen SVI splicing subgroup recommendations.

RESULTS Both variants demonstrated aberrant splicing not seen in controls. Case 1 showed *ATP6V0A2* exon 16 skipping in 99.03% of transcripts, while case 2 showed *CNOT3* exon 5 skipping in 49.82% of transcripts. Both produced in-frame transcripts expected to escape nonsense-mediated decay. Despite similar magnitude (after accounting for zygosity), specificity, and reproducibility, the predicted protein functional impact interpretations differed. The *ATP6V0A2* variant was reclassified as likely pathogenic because germline deletion of exon 16 is a recurrent pathogenic variant. The *CNOT3* variant remained a VUS due to uncertainty about the effect of exon 5 skipping on protein function.

CONCLUSIONS Careful consideration of splicing impact on protein function is essential for variant classification. While confirming aberrant splicing often leads to upgraded classification, it represents only one line-of-evidence and may not always be sufficient for reclassification. Nevertheless, RNA analysis