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Reanalysis of Undiagnosed Neurodevelopmental Disorder Cases: From *RNU4-2* Variants to Clinical Phenotypes

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Abstract

Background and Objectives

Neurodevelopmental disorders (NDDs) are often the results of genetic factors, whose identification and key role in their etiology may be elusive. Despite advancements in genetic testing, many cases remain unsolved. Single-nucleotide variants in a critical 18-bp region of the noncoding gene *RNU4-2*, a crucial component of the spliceosome complex, have been recently recognized as being responsible for ReNU syndrome, also known as NDD with hypotonia, brain anomalies, distinctive facies, and absent language (NEDHAFA). The aim of this study was to investigate the prevalence of *RNU4-2* variants within a cohort of unsolved patients exhibiting NDDs from the Telethon Undiagnosed Disease Program (TUDP).

Methods

We conducted a reanalysis of genomic data using bioinformatic tools, followed by direct sequencing to identify variants in the *RNU4-2* critical region.

Results

Causative variants were detected in 11 of 375 tested individuals, allowing us to diagnose 2.93% of our unsolved cohort. All heterozygous variants in *RNU4-2* occurred de novo, including 10 with the recurrent n.64_65insT insertion and 1 with n.77_78insT. Structural modeling suggested that these variants disrupt the U4/U6 snRNA interaction, potentially impairing spliceosome function.

Discussion

Our findings reinforce the critical role of *RNU4-2* variants in syndromic NDDs and underscore the importance of noncoding regions in genetic diagnoses. These findings show that *RNU4-2* variants account for approximately 2.9% of the patients with TUDP in this study and highlight the need for integrating advanced molecular techniques and data sharing to refine diagnoses and enhance our understanding of rare genetic disorders.

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Supplementary Material

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Glossary

ASD = autism spectrum disorder; **ES** = exome sequencing; **GDD** = global developmental delay; **HPO** = Human Phenotype Ontology; **ID** = intellectual disability; **NDD** = neurodevelopmental disorder; **TUDP** = Telethon Undiagnosed Disease Program.

Introduction

Neurodevelopmental disorders (NDDs) are a heterogeneous group of conditions, including attention-deficit/hyperactivity disorder, autism spectrum disorder (ASD), intellectual disability (ID), specific learning disorders (SLDs), and movement disorders.[1] Syndromic forms of NDDs are characterized by the co-occurrence of multiple neurodevelopmental conditions along with systemic involvement, with an overall prevalence lower than 1%.[2] Genetic factors play a crucial role in NDDs, with more than 1,500 genes associated with these conditions.[3] However, achieving a definitive diagnosis is not always possible. This challenge arises from the complexity of disease onset, which ranges from single disease-gene associations to polygenic influences and environmental factors.[4] Moreover, most individuals with NDDs remain without a genetic diagnosis because of the limitations of current testing methods, such as exome sequencing (ES), which focuses only on protein-coding regions.

Recent studies[5,6] have identified pathogenic variants in the noncoding gene *RNU4-2*, a component of the major spliceosome complex essential for RNA maturation and highly expressed in the brain,[7] through whole-genome sequencing. This recently recognized NDD has been named "ReNU syndrome" and has been estimated to account for approximately 0.4% of individuals with NDDs.[6] In addition to NDDs, individuals with *RNU4-2* variants exhibited hypotonia, seizures, microcephaly, short stature,[8] and a recognizable pattern of facial anomalies. The genetic defect of ReNU syndrome highlights the role of neglected noncoding regions in NDDs. A recurrent de novo variant, n.64_65insT, in a highly conserved region of *RNU4-2* has been found in most individuals with ReNU syndrome.[5] This variant disrupts a critical 18-bp region (hg38:chr12:120291825–120291842) essential for the activity of the spliceosome.[9] Additional *RNU4-2* pathogenic variants, including other insertions and single-nucleotide variants within the same critical region, suggest an important role of these nucleotides in *RNU4-2* function.[5]

In this study, following the identification of *RNU4-2* as the genetic cause of a syndromic form of NDD, we screened our unsolved cases from the Telethon Undiagnosed Disease Program (TUDP) for variations in the *RNU4-2* critical region. We identified a significant percentage of individuals harboring de novo pathogenic variants in RNU4-2, including 10 with the recurrent n.64_65insT insertion and 1 with n.77_78insT. Structural modeling supported that these variants may disrupt the U4/U6 snRNA interaction, potentially impairing spliceosome function. Our findings highlight the critical role of *RNU4-2* variants in syndromic NDD diagnoses and emphasize the importance of noncoding regions in advancing genetic diagnostics.

Methods

Cohort Selection

Our cohort consists of 1,019 patients enrolled in the TUDP, an Italian multicentric project, which aims to solve rare complex genetic diseases. The program inclusion criteria were pediatric age at the time of initial recruitment, severe neurocognitive involvement, dysmorphic features and/or severe multisystem manifestations (Figure 1), and negative results from at least 180K array comparative genomic hybridization (array CGH). Clinical characterization was standardized using Human Phenotype Ontology (HPO) terms,[10] collected by the physicians directly involved in patient care, who also provided detailed clinical records and additional relevant information to support the genetic investigation. Patients are generally followed longitudinally by their center of origin, ensuring that clinical data are regularly updated. From the TUDP unsolved cohort, we screened 375 cases that remained undiagnosed after ES and custom high-resolution array CGH.

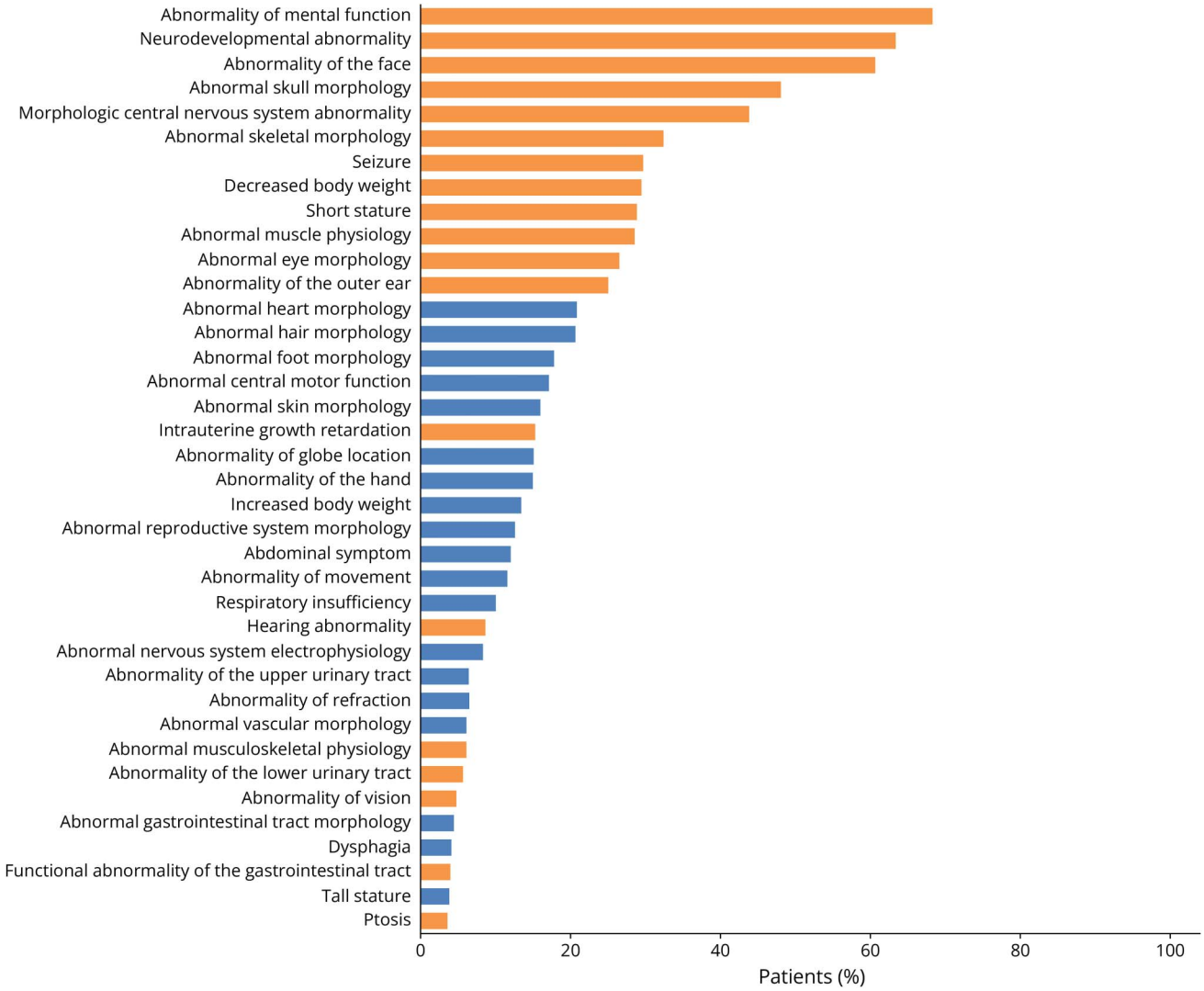
Standard Protocol Approvals, Registrations, and Participant Consents

The study was approved by local ethics committees of the participating institutions (protocol number: 0018726 of 04/07/2024). Patients provided written informed consent for the participation in the study according to the Italian National Health System guidelines and the Declaration of Helsinki. Written informed consent for the publication of images was also obtained from all patients included in this study. The families of patients 4 and 6 have declined the publication of their images.

ES, Data Analysis, and 3D Modeling

Genomic DNA from multiple runs over the years was enriched using 2 distinct capture kits, SureSelect Human All Exon v7 and v8 (Agilent Technologies, Santa Clara, CA), and sequenced using the NovaSeq6000 system with paired-end runs covering at least 2 × 150 nt (Illumina Inc.). The raw output files produced from the sequencer, in the form of BCL (Base Call) files, were demultiplexed using Illumina bcl2fastq software (v2.19.0.316, Illumina Inc.) to generate individual FASTQ for each sample. Bwa-mem2 (v 2.2.1) was then used to align the paired reads to the human reference genome (hg38), producing sequence alignment map (SAM) files.

Figure 1 Prevalence of HPO Terms



Bar plots illustrate the prevalence of HPO terms observed in the TUDP unsolved cohort. HPO terms are listed on the y-axis in the order of decreasing prevalence while the x-axis represents the percentage of patients exhibiting each term. Orange bars highlight the HPO features that are observed in our ReNU cohort. HPO = Human Phenotype Ontology; TUDP = Telethon Undiagnosed Disease Program.

Using SAMtools, they were subsequently converted to the binary alignment map (BAM) format. Retrospectively, we used SAMtools to interrogate BAM files, seeking for putative variants in the *RNU4-2* critical region.

Alphafold3[11] and ChimeraX 1.9[12] were used to generate a prediction of wild-type and mutated snRNA U4 in its interaction with U6.

Sanger Sequencing

The *RNU4-2* genomic region was amplified by PCR using a proper primer pair (forward primer: 5′-GTTCCAACAACA AGAAACCTCC-3′, reverse primer: 5′-TCACGGAATACT CCTGAACAA-3′). Sanger sequencing of the amplified fragment was performed using the BigDye version 3.1 sequencing kit (Applied Biosystems, Waltham, MA) and the 3,500 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. All variants were annotated on the non–protein-coding transcript NR_003137.2.

Data Availability

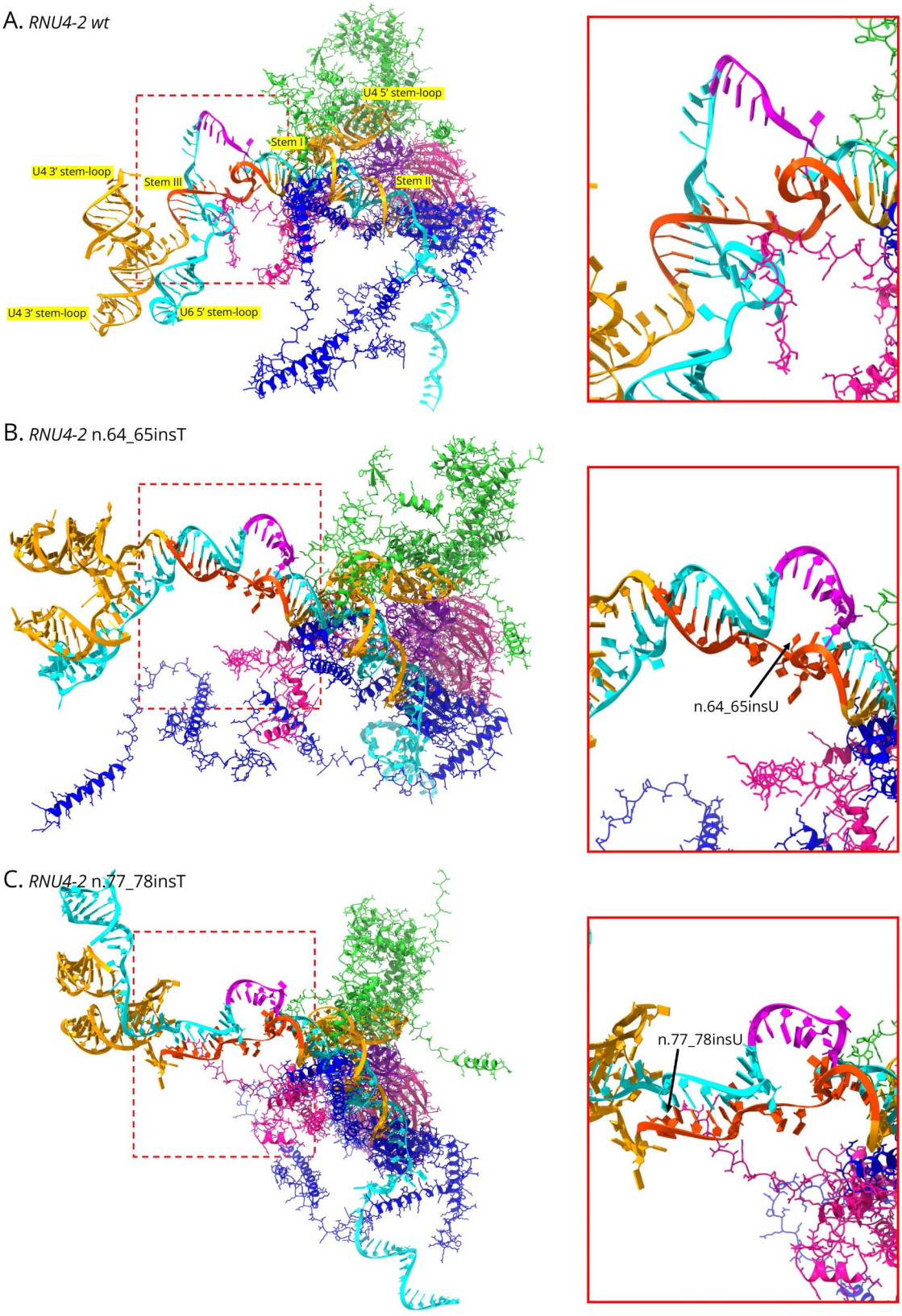
Additional data are available from the corresponding author on reasonable request.

Results

RNU4-2 Variants

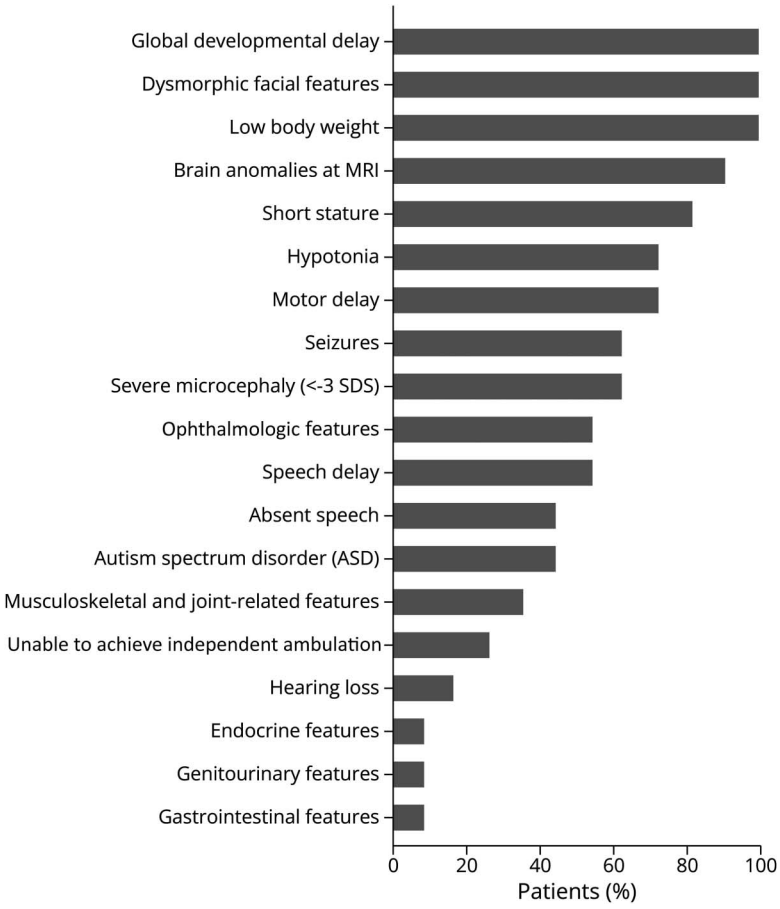
Among the 375 selected individuals, we identified 11 patients (8 male patients and 3 female patients) carrying de novo variants in *RNU4-2*; 3 of them were found through reanalysis of ES raw data, driven by the SolveRD consortium,[13] while the remaining 8 were identified through direct gene sequencing.

Figure 2 Modeling of *RNU4-2* Wild-Type and Pathogenic Variants



In each panel, predicted 3D structures of the U4/U6 di-snRNP complex are shown. *RNU4-2* is shown in orange, with the critical region highlighted in red; *RNU6-1* is shown in cyan with the 5′-ACAGAGA sequence in magenta, Prp31 in green, Prp3 in blue, Prp4 in pink, and Snu13 in violet. The critical region is highlighted with a red dotted square. The left panels display the full complex while the right ones zoom in on the critical region. (A) The U4/U6 complex with wild-type *RNU4-2* shows the physiologic intramolecular and intermolecular interactions. (B) n.64_65insT shows mispairing in the critical region and stem-loop mis-orientation. (C) n.77_78insT shows misfolding in the critical region, bringing aberrant structure bending.

Figure 3 Clinical Features of Individuals With Pathogenic Variants in RNU4-2



The bar graphs illustrate the percentage of patients with pathogenic *RNU4-2* variants exhibiting each of the clinical conditions represented, arranged by prevalence (data presented as percentages). ASD = autism spectrum disorder; SDS = SD score.

Ten patients harbored the recurrent variant n.64_65insT (hg38:chr12:120291839T>TA) and 1 had the n.77_78insT (hg38:chr12:120291826T>TA) variant, both previously reported[5,6] and located within the 18-bp critical region.

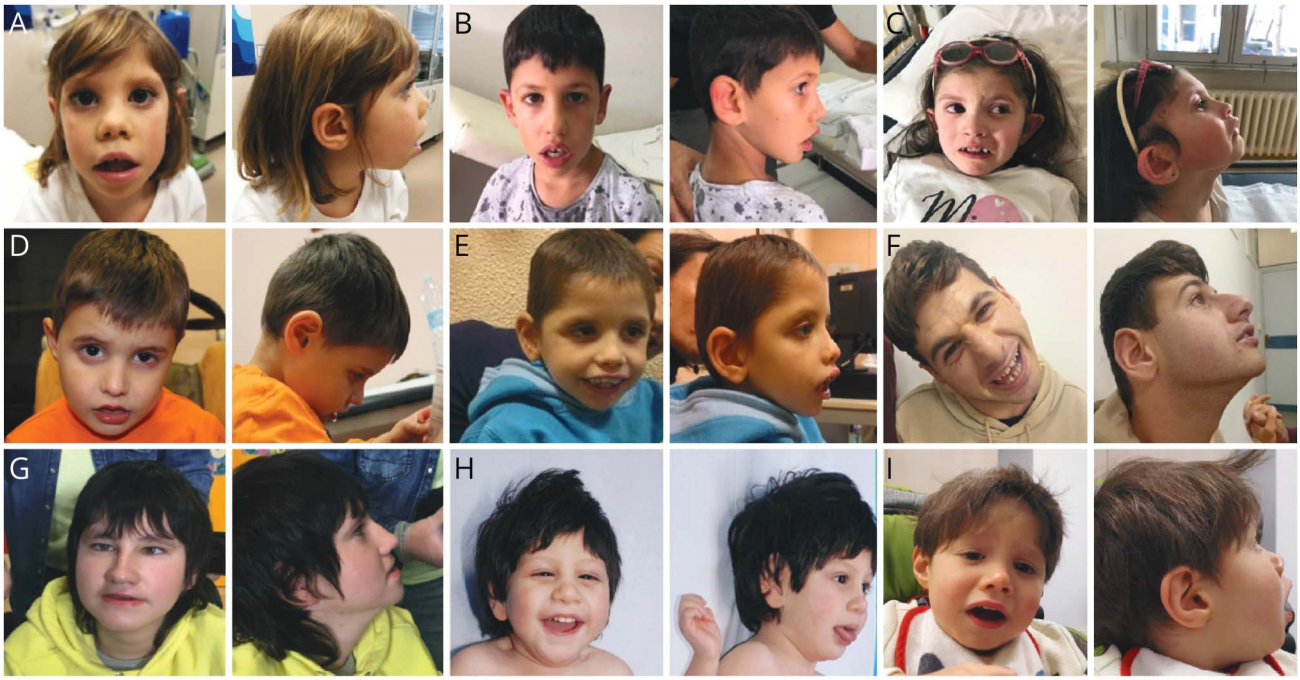
To investigate how the U4/U6 is altered by the *RNU4-2* variants, we generated the 3D model through Alphafold3 and visualized it with ChimeraX 1.9.1. In silico prediction confirmed the previously characterized intramolecular structures of wild-type U4 and U6 snRNAs, including the 5′ and 3′ stem-loops; the 3 intermolecular stems; and the U4/U6 di-snRNP complex conformation involving Prp31, Prp3, Prp4, and Snu13 proteins.[5,14,15] By contrast, prediction analysis of the 2 variants found in affected individuals revealed disruptions of RNA secondary structure and defective intermolecular pairing: (1) n.64_65insT induces mispairing in a critical region, altering the orientation of U6 and resulting in the loss of orientation of the U4 3′ stem-loops and (2) n.77_78insT disrupts pairing in the critical region as well as in stem III, resulting in the loss of complex spatial conformation, which prevents functional loop arrangement for correct Brr2 interaction. Although the structural effects are different, both variants ultimately misplace and constrain the U6 5′-ACAGAGA sequence, a key element for the 5′ splice-site recognition within the major spliceosome complex (Figure 2).

Clinical Presentations

The 11 patients were all born to nonconsanguineous parents with unremarkable family histories and uncomplicated pregnancies, except for intrauterine growth restriction detected in 45% of patients (5/11). The age of the affected individuals ranged from 2.9 to 20.5 years at the time of the most recent evaluation, and they all exhibited severe-to-moderate global developmental delay (GDD), ID, growth retardation, microcephaly, and facial dysmorphisms (Figure 3 and eTable 1).

Approximately half of the individuals (55%, 6/11) were able to speak a few words while the other half exhibited no speech. Although delayed (average age: 4.2 years) and presenting with ataxic gait and severe motor clumsiness, most individuals (73%, 8/11) achieved independent ambulation and had hypotonia, whereas 27% (3/11) remained nonambulatory. ASD was diagnosed in 45% of patients (5/11). Most patients (63%, 7/11) had epilepsy with variable age at onset and clinically heterogeneous manifestations, comprising focal, generalized tonic-clonic, and febrile seizures. Ophthalmologic anomalies were observed in 55% of patients (6/11), and they included vision

Figure 4 Clinical Photographs Illustrating the Facial Features of Individuals With Pathogenic Variants in *RNU4-2*



Panels A–I show 9 children with de novo pathogenic variants in *RNU4-2*, highlighting the distinct facial characteristics.

impairment, optic nerve hypoplasia, strabismus, nystagmus, ptosis, and megalocornea. Hearing impairment, including bilateral sensorineural hearing loss, was observed in 2 of 11 individuals (17%).

Most individuals (82%, 9/11) presented with short stature, and all of them exhibited low weight. Despite the presence of short stature, none of the patients received growth hormone treatment during the evaluation period. Microcephaly, present either at or shortly after birth, was identified in 9 of 11 patients (82%). Among these, 2 patients (17%, 2/11) had standard microcephaly, defined as a head circumference more than 2 SDs below the mean, while 7 patients (63%, 7/11) exhibited severe microcephaly, with a head circumference more than 3 SDs below the mean. In addition, 2 patients (17%) exhibited progressive microcephaly (range: from −2.0 SDs to −3.5 SDs).

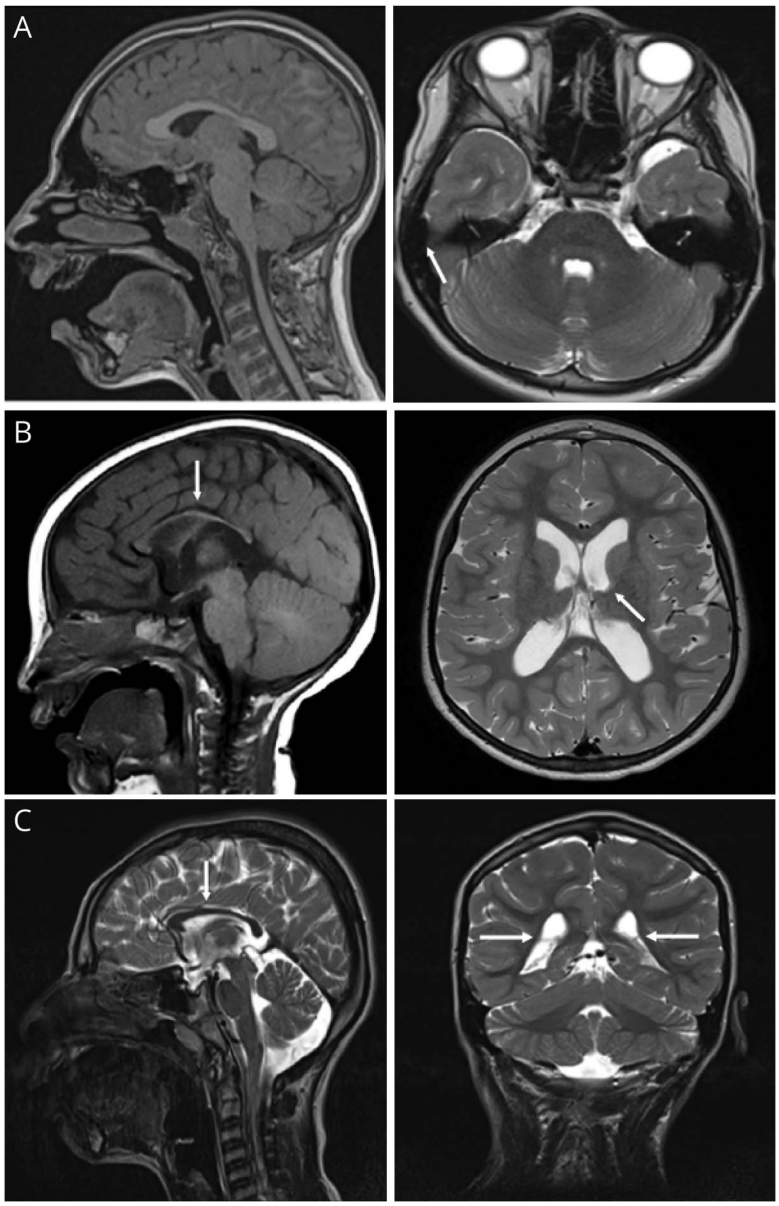
Facial dysmorphic features included deep-set eyes, sparse eyebrows, epicanthal folds and short palpebral fissures, prominent philtrum, a tented open mouth with thick upper and lower lips and downturned mouth corners, a wide nasal bridge, anteverted nares or underdeveloped ala nasi, and dysplastic ears (large, cupped ears; low-set ears; short/attached earlobes; and periauricular pits).[8,16,17] Additional frequent features comprised micrognathia, retrognathia, and a sloping forehead (Figure 4). Broad first toes and clinodactyly were also observed.

Several systemic features were observed, including hypothyroidism, cryptorchidism, gastroesophageal reflux disease, pes planus, joint hypermobility, severe osteoporosis, and scoliosis. Brain MRI identified a range of brain abnormalities in 91% of patients (10/11), such as thin corpus callosum, reduced white matter volume, ventriculomegaly with ventricular dysmorphism or asymmetry, periventricular gray matter heterotopia, olfactory bulb hypoplasia, hypoplastic pons, and arachnoid cysts (Figure 5).

Discussion

The groundbreaking discovery on *RNU4-2* involvement in a syndromic form of NDD (5,6) prompted us to perform a reanalysis of unsolved patients from our TUDP cohort, selecting 375 patients who exhibited NDD but had previously remained undiagnosed. By both bioinformatic inspection and direct sequencing of the critical region, we identified 11 individuals with de novo variants in *RNU4-2*, including the recurrent n.64_65insT and n.77_78insT. Phenotypes overlapped with the core features of the ReNU syndrome, defining a consistent clinical profile characterized by GDD, ID, growth retardation, microcephaly, and distinctive facial dysmorphisms.[5,6,8,16-21] Motor and speech impairments were common, with most achieving independent ambulation despite ataxia. Epilepsy and brain MRI abnormalities were also prevalent, primarily involving structural anomalies across multiple regions, including the white matter, corpus callosum, and ventricles, as previously described.[5,8] A high prevalence of ASD was observed, along with frequent ophthalmologic and hearing impairments. Beyond neurologic and cognitive features, a significant proportion of patients exhibited a variety of additional systemic anomalies, including musculoskeletal

Figure 5 Brain MRI Findings of Individuals With Pathogenic Variants in *RNU4-2*



Panel (A) shows sagittal (left) and axial (right) images of patient 4 from eTable 1. The axial image demonstrates mild enlargement of the subarachnoid space in the left middle fossa (white arrow), attributed to a small arachnoid cyst, along with a slightly hypoplastic appearance of the ipsilateral temporal pole. Panel (B) presents sagittal (left) and axial (right) images of patient 2 from eTable 1. The sagittal image reveals hypoplasia of the corpus callosum (white arrow) while the axial image highlights periventricular gray matter heterotopia (white arrow). Additional features include thinning of the white matter, hypoplasia of the olfactory bulbs, hypoplasia of the optic nerves, and an arachnoid cyst (not shown). Panel (C) displays sagittal (left) and coronal (right) images of patient 1 from eTable 1. The sagittal image illustrates hypoplasia of the corpus callosum (white arrow) while the coronal image shows reduced periventricular white matter volume and ventricular asymmetry (white arrows).

features such as pes planus, joint hypermobility, osteoporosis, and scoliosis,[5,22] as previously described. Endocrine (hypothyroidism) and genitourinary (cryptorchidism) abnormalities were also present, further highlighting the multifaceted clinical presentation of the disorder. These findings offer crucial insights into the diverse manifestations of the disorder and enhance diagnostic accuracy, while also laying a solid foundation for future genetic and therapeutic research.

Structural analysis by 3D modeling gave us hints about the possible damaging effect of these variants. Specifically, we found that core secondary structures and the 3 intermolecular interactions remained intact, but variants in the 18-bp critical region caused stem mispairing and altered loop orientation. Complex unwinding, essential for splicing, is catalyzed by the Brr2 helicase, which properly positions U6 5′-ACAGAGA to intercept 5′ splice sites on pre-mRNA. These structural changes may affect the U4/U6 complex by either disrupting Brr2 docking due to altered spatial arrangement or by impairing U6 positioning, potentially reducing or inhibiting 5′ splice-site recognition. Given the well-established and finely regulated U4/U6 positioning on the spliceosome, it has been hypothesized that disease-causing variants may ultimately disrupt 5′ splice-site recognition by altering U6 spatial conformation.[5] This hypothesis is also supported by our predictive model because these variants seem to highly destabilize this functional region.

This screening allowed us to diagnose 2.93% of our unsolved cases, emphasizing the importance of noncoding regions in

advancing genetic diagnostics. Although the frequency of *RNU4-2*–associated NDD has been estimated to be 0.4%,[5] our cohort showed a higher prevalence of *RNU4-2* pathogenic variants. This finding reflects the phenotypic overlap—based on HPO terms—between the TUDP unsolved cohort and our ReNU syndrome subcohort, in which the most prevalent HPO terms also closely align with those most frequently associated with ReNU syndrome (Figure 1), supporting the specific enrichment in this widely genetically investigated syndromic NDD cohort.

Our findings highlight the importance of continued data reanalysis in undiagnosed patients, as 3 cases were identified through re-examination by the Solve-RD consortium. This underscores the evolving nature of genomic research and the necessity of maintaining comprehensive genetic records for periodic reassessment.[13]

Overall, this study deepens our understanding of ReNU syndrome, by expanding the global cohort and providing further clinical characterization. By including a broader range of cases, we gain more profound insights into the phenotypic variability and clinical spectrum of the disorder. However, to fully unravel the complexity of ReNU syndrome, functional studies and long-term follow-up assessments will be crucial for a more comprehensive understanding of the underlying molecular mechanisms and disease progression.

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Author Contributions

P. Di Letto: major role in the acquisition of data; analysis or interpretation of data. C. De Leonibus: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. F.P. Palmieri: analysis or interpretation of data. M. Zanobio: analysis or interpretation of data. M. Scarpato: analysis or interpretation of data. V. Cetrangolo: analysis or interpretation of data. S.I. Rahman: major role in the acquisition of data. A. Selicorni: drafting/revision of the manuscript for content, including medical writing for content. M. Mariani: drafting/revision of the manuscript for content, including medical writing for content. S. D'Arrigo: drafting/revision of the manuscript for content, including medical writing for content. C. Ciaccio: drafting/revision of the manuscript for content, including medical writing for content. D. Milani: drafting/revision of the manuscript for content, including medical writing for content. P.F. Ajmone: major role in the acquisition of data. M. Morleo: analysis or interpretation of data. C. Spampanato: study concept or design. G. Piluso: drafting/revision of the manuscript for content, including medical writing for content. M. Zollino: major role in the acquisition of data. F.F. L'Erario: major role in the acquisition of data. D. Greco: major role in the acquisition of data. V. Capra: major role in the acquisition of data. M. Scala: major role in the acquisition of data. F. Romano: major role in the acquisition of data. G. Terrone: major role in the acquisition of data. A. De Falco: major role in the acquisition of data. C. Paolella: major role in the acquisition of data. M. Mastrangelo: major role in the acquisition of data. G. Ricciardi: major role in the acquisition of data. N. Brunetti-Pierri: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. V. Nigro: drafting/revision of the manuscript for content, including medical writing for content; study concept or design. A. Torella: drafting/revision of the manuscript for content, including medical writing for content; study concept or design.

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Disclosure

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Appendix Coinvestigators

Coinvestigators are listed at Neurology.org/N.

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