
This is the **accepted version** of the journal article:

Valenzuela, Irene [et al.]. «Deep phenotyping of 11 individuals with pathogenic variants in RNU4-2 reveals a clinically recognizable syndrome». *Genetics in medicine*, Vol. 26, Num. 12 (December 2024), art. 101288 DOI 10.1016/j.gim.2024.101288

This version is available at <https://ddd.uab.cat/record/336071>

under the terms of the  IN COPYRIGHT license.

This is the **accepted version** of the journal article:

Valenzuela, Irene [et al.]. «Deep phenotyping of 11 individuals with pathogenic variants in RNU4-2 reveals a clinically recognizable syndrome». *Genetics in medicine*, Vol. 26, Num. 12 (December 2024), art. 101288 DOI 10.1016/j.gim.2024.101288

This version is available at <https://ddd.uab.cat/record/336071>

under the terms of the  IN COPYRIGHT license.

Deep phenotyping of 11 individuals with pathogenic variants in *RNU4-2* reveals a clinically recognizable syndrome

Irene Valenzuela^{1*}, Marta Codina-Solà^{1*}, Elida Vazquez², Anna Cueto-González¹, Jordi Leno-Colorado¹, Amaia Lasa-Aranzasti¹, Laura Trujillano¹, Bárbara Masotto¹, Miriam Masas¹, Mar Escobar¹, Elena García-Arumí¹, Eduardo F. Tizzano¹

1. Clinical and Molecular Genetics Area, Vall d'Hebron Hospital; Medicine Genetics Group, Vall d'Hebron Research Institute (VHIR), Barcelona, Spain.
2. Department of Pediatric Radiology, Hospital Vall d'Hebron, Barcelona, Spain

*Both authors contributed equally to this work

^aCorresponding Author, Department of Clinical and Molecular Genetics, Hospital Vall d'Hebron, Passeig Vall d'Hebrón 119-129, 08035 Barcelona Hospital Vall d'Hebron Barcelona, Spain. [irene.valenzuela@vallhebron.cat], +34934893141.

ORCID

Valenzuela, I 0000-0003-2350-6058

Codina-Solà, M 0000-0002-5382-413X

Vázquez Méndez, Elida 0000-0003-0930-154X

Cueto-Gonzalez, A 0000-0001-7694-6124

Jordi Leno-Colorado 0000-0001-6049-256X

Lasa-Aranzasti, A 0000-0002-9136-5106

Trujillano, L 0000-0002-3028-6156

Masotto, B 0009-0009-8041-1507

Miriam Masas 0009-0009-7735-074X

Garcia Arumi, E 000-0003-1848-005X

Tizzano, EF 0000-0002-7116-6310

Abstract

Purpose: Despite ever-increasing knowledge of the genetic etiologies of neurodevelopmental disorders, approximately half remain undiagnosed following exome or genome sequencing.

Here, we provide a deep clinical characterization of 11 previously unreported patients with a recently described neurodevelopmental disorder (NDD) due to pathogenic variants in *RNU4-2*.

Methods: The eleven patients were identified in a pool of 70 patients selected for targeted *RNU4-2* sequencing on the basis of their clinical phenotypes from a cohort of 1032 individuals with a NDD and without a prior genetic diagnosis.

Results: The eleven patients were aged between 13 months and 36 years. All patients showed moderate to severe developmental delay and/or intellectual disability. Height and weight were below 10th percentile and most showed microcephaly. In almost 50% of the patients, intrauterine growth retardation was detected. All patients showed a distinctive pattern of dysmorphic features, including hooded upper eyelid and epicanthus, full cheeks, tented philtrum, mouth constantly slightly open with an everted lower lip vermillion, high palate and profuse drooling. Of 11 patients, 64% also presented with ophthalmological problems (mainly strabismus, nystagmus and refraction errors) and 64% had musculoskeletal features (joint hypermobility, mild scoliosis, easy fractures).

Conclusion: This work provides an improved characterization of the phenotypic spectrum of *RNU4-2* syndrome across different age groups, and demonstrates that thorough clinical assessment of patients with an NDD can be enhanced significantly for this novel syndrome.

KEYWORDS

Neurodevelopmental disorder, *RNU4-2*, deep phenotyping, non-coding spliceosomal snRNA, diagnostic criteria

Abbreviations

Neurodevelopmental disorders (NDD), exome sequencing (ES), genome sequencing (GS), non-coding RNAs (ncRNAs), single nucleotide variant (SNV), intrauterine growth retardation (IUGR), global developmental delay (GDD), intellectual disability (ID), small nuclear ribonucleoprotein (snRNP), alternative splicing (AS)

Introduction

The clinical features of individuals with neurodevelopmental disorders (NDDs) are often nonspecific, and diagnosis relies on genetic testing using broad genetic panel sequencing, exome sequencing (ES) or genome sequencing (GS). Over the past decade, phenotype-driven ES and GS, has demonstrated utility for clinical diagnostic testing in individuals with suspected Mendelian conditions (1–5). Nevertheless, a large fraction of patients (~30%–80%, depending on prior testing, categorical phenotype, analytical approach, and other factors) tested by ES/GS remain without a precise genetic diagnosis (5–11) and a substantial proportion of these individuals may harbor variants in novel candidate genes that explain their clinical findings. An increasing proportion of cases with a suspected genetic disorder, including NDDs, are receiving genetic diagnoses involving variants in non-coding RNA (ncRNA) genes. The term ncRNA refers to a heterogeneous group of transcripts that, by definition, are not translated into proteins, but may nonetheless have important biological functions(12,13). Since their discovery, ncRNAs have emerged as important regulators of multiple cellular processes across a range of cell types and tissues, and their dysregulation has been implicated in disease (14). Given the number of ncRNAs (around 40000 according to Ensembl) and their role in biological processes such as splicing or regulation of gene expression, it is to be expected that some of them will be associated with human disorders (12,13). Some already known examples include disorders caused by pathogenic biallelic variants in *RNU4ATAC* (HGNC:34016) (15), or cartilage-hair hypoplasia, associated to pathogenic variants in *RMRP* (HGNC:10031) (16). Given that most commercially available exome capture kits target only a small fraction of non-coding genes, patients with these conditions may remain undiagnosed due to a lack of sequencing coverage on etiological non-coding genes. Therefore, a comprehensive delineation of their clinical presentation is essential to recognize them, and guide targeted molecular studies to confirm the clinical diagnosis.

The recent identification of the non-coding RNA *RNU4-2* (HGNC:10193) as a novel syndromic NDD gene demonstrated the advantage of considering large genome-sequenced cohorts to discover new candidate genes in the non-coding genome. *RNU4-2* encodes the U4 small nuclear RNA (snRNA), which is a critical component of the U4/U6.U5 tri-snRNP complex of the major

spliceosome. Greene et al and Chen et al., reported a constrained 18 bp region of *RNU4-2* with de novo heterozygous variants in 119 individuals with NDD (17,18). The vast majority of individuals (77.3%) have the same highly recurrent single base-pair insertion (n.64_65insT). The authors estimate that variants in this region explain around 0.5% of undiagnosed individuals with NDD with previous non-conclusive genome study. Furthermore, a recent paper by Schot et al., reported a patient detected by GS reanalysis with clinical characteristics overlapping with published patients (19)

This study aims to report a deep clinical characterization of 11 patients at different ages with NDD previously undiagnosed and followed in our center. All individuals were studied by targeted DNA sequencing, demonstrating that *RNU4-2* is likely a clinically recognizable disorder. The description of our cohort further details the clinical characteristics of *RNU4-2* as a novel syndromic entity that should be recognized within the wide spectrum of NDD.

MATERIALS AND METHODS

Participants and procedure

This study was conducted in the Genetics Department of the Vall d'Hebron University Hospital, Barcelona (Spain). Clinical geneticists made the selection starting from a cohort of 1032 undiagnosed individuals with NDD considering the clinical characteristics described by Greene et al. and Chen et al. (17,18). Specifically, the main criteria for selection included: presence of microcephaly, moderate to severe intellectual disability, short stature, and presence of epicanthus, tented philtrum and drooling. We also considered those patients with a differential diagnosis within the Angelman-like and Rett-like spectrum. Finally, based on these criteria, 70 patients were prioritized for a targeted study. All clinical and molecular data were collected including prenatal, morphologic, and neurodevelopmental characteristics as well as previous genetic studies. Informed consent approved by the local IRB was signed by their legal guardians.

Molecular studies

Targeted study of *RNU4-2* was performed by Sanger sequencing using the following oligonucleotides (sequence 5' to 3'): TCTCCATTCAGATTTTGTCACAG and GGGAATCTGTGACCTTCAGTG. PCR reaction was performed at a 25uL following standard conditions and standard PCR conditions.

RESULTS

Clinical features

We identified and characterized a total of eleven individuals (Cases 1 to 11) with variants in *RNU4-2*, including 5 males and 6 females. The mean age of our case series was 12 years and 6 months, with a range of 13 months to 36 years. Parental consanguinity was not reported, and all cases were sporadic.

The main clinical features are presented in Table 1 and supplementary table 1. All numbers and percentages are related to the total number of patients that could be evaluated for a given feature. Figure 1 shows photographs of patients at various ages.

Prenatal and perinatal observations

Most children were born at term (mean gestational age 37.3 weeks, range 32–40 weeks). Intrauterine growth retardation (IUGR) was detected in five of the children during the third trimester of pregnancy without detecting associated placental abnormalities in any of them. In one of the children, thickened nuchal skin fold and cardiomegaly was detected at 21 weeks of gestation. The birth was by cesarean section in five of the individuals, eutocic in 4, and dystocic with the need for forceps in 2 individuals. Regarding perinatal complications, one of the children presented neonatal hypoglycemia and respiratory distress that required monitoring during the first 24 hours of life in a neonatal intensive care unit. One of the infants, born at 34+6 weeks of gestation, presented neonatal jaundice as a complication that required phototherapy.

Dysmorphic facial features

All patients presented a distinctive facial appearance that renders the condition recognizable (Figure 1). The eye shape is very characteristic, with the majority of patients presenting hooded upper eyelid and epicanthus. Additionally, 6 out of 11 patients showed hypotelorism (54.5%). Full cheeks are also observed in a high proportion of patients and mostly evident at pediatric age. The mouth shape is also very characteristic, with a tented philtrum, a tendency to keep the mouth constantly slightly open with an everted lower lip vermilion, and significant drooling. A high palate was observed in 8 out of 11 patients (72.7%) and one of the patients presented bifid uvula. Tooth abnormalities are also evident in the majority of the patients, mainly tooth malposition.

Some of the dysmorphic features become more evident with age, such as the triangular facies, the increased tendency for lower lip eversion, and the prominent nasal bridge with a broad base (Figure 1). Hand and foot abnormalities were also detected (Figure 2). Prominent fingertip pads were evident in infancy in five of the patients (45%) but not detected at older ages. Small and slender hands and feet were observed in four patients. One patient presented shortening of the third and fourth metacarpal in both feet.

Growth and head circumference

Microcephaly, i.e., occipital frontal circumference at least 2 SDs below the mean, was sometimes observed at birth (3/11), but more frequently tended to develop gradually throughout infancy but not all children were microcephalic. Microcephaly at birth was particularly evident in individuals who also exhibit IUGR (P2 OFC of -2.62SD and P10 of -2.23SD). Postnatally, microcephaly was noted in 10 of 11 patients (90%) in the age range from 0 to 2 years. Occipital frontal circumference, already mainly under the 25th centile at birth, tended to fall progressively below normal values (median value -3.55SD, ranging from -6.29 to -0.44 SD).

The majority of patients had a height below the 10th centile (8/11; 72%). Short stature (at least 2 SDs below the mean) was observed in five patients (5/11; 45%). One of the patients with short stature was treated with growth hormone during childhood without clear improvement.

Most patients were of slender build, with normal body proportions. Body weight was below the 10th centile in the majority of patients (9/11, 82%). Overall, height (length) and weight were globally normal at birth, but often showed marked delay over time.

Development, behavior and neurology

All *RNU4-2* patients have moderate to severe global developmental delay (GDD)/intellectual disability (ID). Hypotonia was often found in the first years of life in all patients. Developmental delay was present in all individuals, with motor delay being one of the key features.

Developmental milestones such as sitting and walking were considerably delayed: the mean age of sitting unsupported was 12 months, and the mean age of walking was 34 months (range: 20 months to 6 years). A number of patients had a wide-based or ataxic-like gait, typical of individuals with Angelman syndrome. All milestones for fine motor skills were delayed. Most of the older individuals (aged over 19 years) require help with dressing and other everyday

activities. Speech rarely extends to more than a few words. Many patients have absence of speech (7/11 (64%), 2 of them still under 4 years of age) but show receptive language skills, a strong communicative intent and communicate using alternative methods. No regression of developmental milestones was observed in any of the patients so far. Eighty-one percent of the children had a considerable impaired toileting ability, and many were still not toilet trained when approaching puberty.

All the individuals presented with autistic features. Repetitive use of objects, hand and finger mannerisms, and stereotyped movements such as rocking back and forth, or hand flapping were common. They had a strong sensory interest, illustrated by putting fingers or objects in their mouth or being attracted to water. Most parents also report a strong love for music.

Although parents reported that their children were happy and friendly, behavioral problems were reported in 55% of them. Sleep problems were not described.

Profuse drooling is a consistent trait in all patients. Although the majority of patients were treated with botulinum toxin, they demonstrated only a slight response to the treatment. Two of the patients have shown a better response to treatment with glycopyrrolate.

Fifty five percent had seizures, including absence seizures, focal seizures, fever-triggered seizures or generalized seizures.

Regarding the neuroradiological findings, we can observe some relevant signs. In two of the individuals, within the context of IUGR, a fetal MRI was performed. The main findings included: pathologic biometrics in the brain, -2SD respect to gestational age, consistent with microcephaly, dysgenesis of the corpus callosum, with short and thin corpus callosum, even absent rostrum in one case, abnormal cerebellar and brain stem biometrics, delayed gyral development, particularly in frontal lobes and suspected white matter anomaly (Figure 3).

Concerning postnatal brain MRI, 10 of the 11 individuals had postnatal follow-up. The main findings detected were: craniofacial dysmorphia, with dolichocephaly, microcephaly with sunken frontal area, dysgenesis of the corpus callosum, very thin, with absent rostrum in one case, ventriculomegaly with colpocephaly, simplified cortical gyral pattern, more striking at the frontal lobes, pathological white matter, with reduced volume, delayed myelination, non-specific abnormalities of the white matter, with areas of gliosis, small periventricular gray matter heterotopias, prominence of cerebral cortical sulci, and intracranial arterial tortuosity. Other less specific findings were presence of cavum vergae, small temporal arachnoid cysts, and

hippocampal malrotation. MRI spectroscopy did not demonstrate abnormal lactate or other findings of interest. Cerebral arterial tortuosity was detected in two of the patients (Figure 4).

Gastrointestinal manifestations

Forty-five percent of the individuals have feeding difficulties in infancy. Failure to thrive was also reported in a high proportion of patients (7/11, 64%) some of them requiring nutritional supplements. Several of the patients have experienced challenges in transitioning from liquid to solid food. Two of the patients required a gastrostomy, and the oldest patient in the series, although able to eat by mouth, requires pureed food. Other gastrointestinal problems detected were mainly gastro-esophageal reflux and constipation.

Ophthalmologic manifestations

Visual problems were present in 64% of the individuals. The most frequent anomaly by far was strabismus (7/11, 64%). Quite common conditions were astigmatism, hypermetropia and myopia. Nystagmus was also present in a relevant proportion of patients (7/11, 63.6%) and it is a finding that appears to be more evident in early childhood. One of the patients presented retrolental fibroplasia that was attributed to prematurity. Many of them are prescribed glasses.

Musculoskeletal features

Musculoskeletal anomalies were frequent and varied. In addition to mild joint hypermobility, scoliosis was present in five individuals (5/11; 45.5%). Since scoliosis can be a progressive manifestation, it may not have been detected yet in some patients due to their age. Besides scoliosis two of the patients presented also pectus carinatum (18%) (Figure 5).

Three of the patients experienced fractures at different ages with minimal trauma. In fact, one of the 19-year-old individuals has been diagnosed with osteoporosis.

Other manifestations

Although major malformations are not common in our cohort, congenital heart disease was detected in two individuals (2/11; 18%): one with a ventricular septal defect and the other with coarctation of the aorta. Inguinal hernias were detected in two patients and needed surgery. Umbilical hernia was present in two individuals.

Recurrent bronchitis was present in four individuals (4/11, 36.4%) and two of them also had recurrent pneumonia. Recurrent otitis media was reported in two individuals one of them required the placement of transtympanic drains (TTD). This feature must be taken into account whenever children present with fever because affected children tend to have a high pain threshold. It can contribute to conductive hearing loss.

Molecular data

In relation to the genetic studies conducted prior to the sequencing of *RNU4-2*, all patients except one had undergone microarray (11/11) and ES analysis (10/11), and in most of them (8/10), re-analysis of ES data had already been performed at least once. In one of the patients, after microarray, a targeted study of *RNU4-2* was directly conducted. All patients except one presented with the recurrent NR_003137.2:n.64_65insT variant, whereas the remaining one (patient 4) presented with the single nucleotide variant (SNV) NR_003137.2:n.69C>T. Segregation studies in their parents showed that all variants were de novo. Variant NR_003137.2:n.64_65insT was classified as pathogenic (PM1_supporting, PM2_supporting, PS2_VeryStrong) and variant NR_003137.2:n.69C>T as likely pathogenic (PM1_supporting, PM2_supporting, PS2_strong, PP3_supporting) according to the ACMG guidelines and subsequent adaptations (20,21).

DISCUSSION

NDD has a prevalence of ~2-3% in the general population and is a common cause of referral to pediatricians, geneticists, and pediatric neurologists (22). The underlying cause of syndromic NDD is largely of genetic origin. A thorough clinical evaluation and stepwise genetic testing can help in arriving at a causative diagnosis. Improvements in diagnostic technologies have led to these causes being more and more detectable and narrowing diagnostic odyssey. Despite a growing number of associated genes that have been identified as causative of NDD, most cases remain undiagnosed after extensive genomic analysis (22).

Recently, Greene et al. and Chen et al. described a new genetic entity caused by pathogenic variants in *RNU4-2* (17,18) one of the genes encoding the U4 snRNA, component of the small nuclear ribonucleoprotein (snRNP) U4, which in turn is one of the five snRNPs of the major spliceosome. Schot et al. reported a single patient detected by GS re-analysis (19). Here we

describe the natural history of 11 individuals of different ages with a variant in *RNU4-2*, contributing to delineate the clinical phenotype as a clearly recognizable entity. All patients showed moderate to severe developmental delay or intellectual disability. Interestingly, in four of the individuals (4/11; 36.4%), the diagnosis of Angelman syndrome was considered previously due to the degree of disability, behavior, microcephaly and broad-based gait. Two other individuals (2/11; 18.2%); were clinically diagnosed with Rett-like syndrome. Therefore, the NDD caused by pathogenic variants in *RNU4-2* could be included in the differential diagnosis of Angelman and Rett syndromes. Beyond Angelman and Rett syndrome a broader range of potential diagnosis should be considered including adenylosuccinate lyase deficiency, alpha-thalassemia X-linked ID syndrome (ATR-X), Kleefstra syndrome (KS), *HERC2*-ID, *MBD5* haploinsufficiency, Christianson syndrome (CS), severe MTHFR deficiency, Pitt-Hopkins syndrome (PTHS), Mowat-Wilson syndrome (MWS), *CDKL5* Deficiency Disorder, *FOXG1* syndrome. Indeed, in individuals with these characteristics, targeted sequencing of *RNU4-2* may be relevant for economic and practical reasons when it is not feasible to proceed to GS.

Although there is a wide variety of manifestations in individuals with alterations in *RNU4-2*, some of the phenotype characteristics can help distinguish within the spectrum of NDD entities. Postnatal microcephaly (median value -3.55SD) was present in approximately 90% (10/11) of the individuals. Although some of them already presented congenital microcephaly (2/10, 20%), primarily those who also presented with IUGR, most exhibited progressive postnatal microcephaly. Chen et al (18) reported that the onset of microcephaly was congenital in the majority of cases. In our cohort, except for two cases (with one patient lacking head circumference data at birth), microcephaly was progressive during the first or second year of life. Height and weight below 10th percentile were noticed for most patients (8/11; 72%) with five of them with height under the third centile. In individuals whose height was already low at birth, there was no catch-up growth postnatally. About half of the participants (5/11; 45.5%) showed this small size already in utero, during the third trimester, without findings of placental abnormalities. In comparison to what was reported by Chen et al.(around 20%) (18), the proportion of IUGR is higher in our cohort. Intrauterine manifestations in most entities related to intellectual disability, in the absence of major malformations, are scarce and make it difficult to diagnose in a prenatal context. Indeed, the observation of IUGR can be an important clue at the

prenatal level to include *RNU4-2* in the differential diagnosis, especially considering that in most centers, GS is not currently included among routine prenatal genetic tests.

Feeding difficulties are relevant in infancy in *RNU4-2* individuals (45%), likely secondary to oromotor hypotonia, but only two of them in our case series have required gastrostomy. Follow-up by gastroenterology and nutritional support is crucial for these patients, not only to assess feeding difficulties but also to monitor growth and development in order to determine the best approach to managing feeding challenges.

Postnatally, patients show a distinguishable pattern of dysmorphic features (summarized in Table 1 and more detailed in Supplementary Table 1) being the eye, cheek, and mouth regions the most distinctive overall. Some dysmorphic features are age dependent, as everted lower lip is more pronounced with age, nasal base seemed to be wider based, cheeks are less full, and the facial shape becomes triangular.

Concerning brain MRI findings, in the two individuals who have undergone fetal study, microcephaly and corpus callosum dysgenesis were observed. Postnatal MRI, besides microcephaly, showed some notable findings such as dysgenesis of the corpus callosum with specifically absent rostrum, ventriculomegaly and simplified cortical gyral pattern, more striking at the frontal lobes. Furthermore, intracranial arterial tortuosity was observed in two of the patients, which is a rare finding related to NDD. This finding is more commonly described in syndromes associated with extracellular matrix alterations such as Loeys-Dietz syndrome, arterial tortuosity syndrome, or cutis laxa. A review of cerebral MRIs from more patients is required to determine if there are specific patterns of neuroimaging that, together with the other clinical findings, may better guide the indication of the genetic study.

Regarding musculoskeletal features, scoliosis and pectus anomalies, are relevant clinical manifestations to note to establish proper follow up. Small and slender feet and shortening of both third and fourth metacarpal were also detected in these patients. In this line, metatarsal and metacarpal abnormalities have been reported in individuals with Rett syndrome together with small hands and feet (23,24). Thus, some of the manifestations observed in hands and feet bring the diagnosis of *RNU4-2* closer to Rett syndrome. Follow-up for a person with the *RNU4-2* variant should include regular monitoring of bone health due to the increased risk of fractures with minimal trauma. Although it appears that the risk of fractures from minimal impact is overrepresented in individuals with *RNU4-2* variants, it is important to consider the vulnerability

of people with disabilities and include in the differential diagnosis the possibility of non-accidental trauma. Notably, two of them showed osteoporosis with previous fractures at 19 years old. The natural history of these two individuals is relevant for considering the establishment of specific measures to monitor bone health in patients with the *RNU4-2* variant. This should include periodic bone density scans and assessments for osteoporosis starting in adolescence together with preventive measures, such as calcium and vitamin D supplementation.

Multidisciplinary care should also include regular neurological evaluations to monitor developmental progress. Given that epilepsy is a common feature, in cases of suspected seizures or other neurological complications, additional diagnostic tests, such as EEGs and brain MRI, should be conducted promptly. Physical and occupational therapy are essential to manage motor skills and enhance mobility. Speech therapy should be included to address communication difficulties. Gastrointestinal follow up is also recommended to monitor for feeding difficulties and other common gastrointestinal problems such as constipation or gastroesophageal reflux. Regular ophthalmological evaluations are also recommended due to the frequent occurrence of eye-related manifestations. While major malformations in other organs are rare, it is relevant to perform echocardiography and abdominal ultrasound to rule out associated malformations that may require specific management. Psychological support for both the patient and their family is also vital to address the emotional and behavioral challenges associated with this condition. By adjusting to this comprehensive follow-up plan, individuals with *RNU4-2* can receive an optimized multidisciplinary care necessary to manage their condition and improve their overall quality of life.

It is relevant to note, that the study in the eleven individuals was conducted in a targeted manner, according to a recognizable phenotype based on the combination of clinical manifestations and dysmorphisms. In fact, using this approach, one of the patients, directly underwent a targeted study of *RNU4-2* after microarray analysis without ES. Given the expected high prevalence of the disorder (17,18)(17,18) as well as the simple diagnostic approach (it involves 141 nucleotides and 18 bp considering only the highly constrained region), it is entirely feasible to screen selected cohorts of undiagnosed individuals with intellectual disability to identify affected individuals.

Based on our initial selection criteria (70/1032), a pathogenic variant in *RNU4-2* was identified in 11/70 cases (16%). Therefore, in our experience, comprehensive clinical examination and

targeted study in selected individuals can identify a significant percentage among undiagnosed patients with NDD. However, this approach may miss some affected individuals with atypical features.

In 10 individuals of our cohort, the recurrent variant n.64_65insT was identified, and in one individual, the variant n.69C>T (17,18). Although it has been suggested that perhaps SNV could be associated with a milder phenotype (18) our patient has a severe phenotype (P4, Supplementary Table1). Larger cohorts of individuals need to be assessed to determine whether there is a genotype- phenotype correlation.

RNU4-2 encodes the U4 snRNA component, which is a part of the U4/U6 snRNP complex essential for assembling the major spliceosome (18). While alternative pre-mRNA splicing typically accomplishes specific cellular requirements, aberrant splicing events can occur, potentially leading to various diseases (25–28). Recently, the pivotal role of alternative splicing (AS) in neural development and the formation and function of neuronal networks has become increasingly evident (25,29,30). Disruptions in neural AS patterns have been linked to neurological disorders and diseases (31). Research has demonstrated that misregulation of microexons in the brain is associated with autism, where disruptions in the normal splicing of these small exons lead to altered protein function (32,33). Variations in U4 snRNA can impact the efficiency and accuracy of pre-mRNA splicing. Given the crucial role of AS in neurogenesis (29,30) and the phenotype of individuals with pathogenic variants in *RNU4-2*, which predominantly affects neurodevelopment, it is reasonable to hypothesize that this phenotype could be connected to splicing disturbances. Such disruptions can have downstream effects on gene expression and the production of protein isoforms. Further RNA-seq studies would be crucial for identifying and analyzing AS events in patients with *RNU4-2* variants. Understanding these processes is essential for unraveling the complexities of gene expression regulation, gaining better insight into the pathophysiology of the condition, and potentially developing targeted therapies.

Conclusion

Our study delineates the clinical presentation of eleven individuals with *RNU4-2* pathogenic variants, through a careful and systematic comparison of the clinical manifestations. This is a deep phenotyping description of this new syndrome diagnosed through a targeted approach,

demonstrating that it has a highly recognizable phenotype among patients with NDD. At present, ES is the gold standard for the diagnosis of NDDs and routine GS is still not available in most centers. Therefore, targeted studies of *RNU4-2* may be a useful tool to identify affected individuals.

By providing insights into the clinical characterization and molecular spectrum of *RNU4-2* syndrome, we contribute to a broader understanding of this entity potentially aiding to improve diagnosis, prognosis, and targeted management strategies. Long-term follow-up studies are necessary to define the developmental path as well as the natural history of this disorder.

DATA AVAILABILITY STATEMENT: The data that support the molecular findings of these participants are available from the corresponding author upon request.

ACKNOWLEDGMENTS

We are sincerely thankful to all the individuals and their parents who participated in the study.

FUNDING STATEMENT

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

AUTHOR CONTRIBUTIONS

Conceptualization: I.V, MCS, EFT; Data curation: I.V, MCS, AMC, EV, ALA, LT, BM, EGA, E.F.T; Formal analysis: IV, MCS, EV; Writing-original draft: IV, MCS, EV, EFT; Writing-review & editing: all authors; Guarantor: IV.

ETHICS DECLARATION

Written informed consent was obtained for use of medical history, genetic testing report, and photographs, as approved by the Institutional Review Board of Hospital Vall d'Hebron.

CONFLICT OF INTEREST

The enclosed manuscript has been revised and approved by all the authors and they have taken care to ensure the integrity of the work. The authors have no conflicts of interest or financial disclosures to report.

SUPPLEMENTARY FILE LISTING

The supplementary material section includes a table with detailed clinical and molecular information of all individuals with the RNU4-2 variant included in the publication (Supplementary Table 1).

References

1. Srivastava S, Love-Nichols JA, Dies KA, Ledbetter DH, Martin CL, Chung WK, et al. Meta-analysis and multidisciplinary consensus statement: exome sequencing is a first-tier clinical diagnostic test for individuals with neurodevelopmental disorders. *Genet Med* [Internet]. 2019 Nov;21(11):2413–21. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/31182824>
2. Brockman DG, Austin-Tse CA, Pelletier RC, Harley C, Patterson C, Head H, et al. Randomized prospective evaluation of genome sequencing versus standard-of-care as a first molecular diagnostic test. *Genet Med* [Internet]. 2021 Sep;23(9):1689–96. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/33976420>
3. Stark Z, Schofield D, Alam K, Wilson W, Mupfeki N, Macciocca I, et al. Prospective comparison of the cost-effectiveness of clinical whole-exome sequencing with that of usual care overwhelmingly supports early use and reimbursement. *Genet Med* [Internet]. 2017 Aug;19(8):867–74. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28125081>
4. Manickam K, McClain MR, Demmer LA, Biswas S, Kearney HM, Malinowski J, et al. Exome and genome sequencing for pediatric patients with congenital anomalies or intellectual disability: an evidence-based clinical guideline of the American College of Medical Genetics and Genomics (ACMG). *Genet Med* [Internet]. 2021 Nov;23(11):2029–37. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/34211152>
5. Chung CCY, Hue SPY, Ng NYT, Doong PHL, Hong Kong Genome Project, Chu ATW, et al. Meta-analysis of the diagnostic and clinical utility of exome and genome sequencing in pediatric and adult patients with rare diseases across diverse populations. *Genet Med* [Internet]. 2023 Sep;25(9):100896. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/37191093>
6. Shashi V, McConkie-Rosell A, Rosell B, Schoch K, Vellore K, McDonald M, et al. The utility of the traditional medical genetics diagnostic evaluation in the context of next-generation sequencing for undiagnosed genetic disorders. *Genet Med* [Internet]. 2014 Feb;16(2):176–82. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23928913>
7. Splinter K, Adams DR, Bacino CA, Bellen HJ, Bernstein JA, Cheatle-Jarvela AM, et al. Effect of Genetic Diagnosis on Patients with Previously Undiagnosed Disease. *N Engl J Med* [Internet]. 2018 Nov 29;379(22):2131–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/30304647>
8. Fung JLF, Yu MHC, Huang S, Chung CCY, Chan MCY, Pajusalu S, et al. A three-year follow-up study evaluating clinical utility of exome sequencing and diagnostic potential of reanalysis. *NPJ Genom Med* [Internet]. 2020;5(1):37. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/32963807>

9. Yang Y, Muzny DM, Xia F, Niu Z, Person R, Ding Y, et al. Molecular findings among patients referred for clinical whole-exome sequencing. *JAMA* [Internet]. 2014 Nov 12;312(18):1870–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25326635>
10. Posey JE, Rosenfeld JA, James RA, Bainbridge M, Niu Z, Wang X, et al. Molecular diagnostic experience of whole-exome sequencing in adult patients. *Genet Med* [Internet]. 2016 Jul;18(7):678–85. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26633545>
11. Retterer K, Juusola J, Cho MT, Vitazka P, Millan F, Gibellini F, et al. Clinical application of whole-exome sequencing across clinical indications. *Genet Med* [Internet]. 2016 Jul;18(7):696–704. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26633542>
12. Mattick JS, Makunin I V. Non-coding RNA. *Hum Mol Genet* [Internet]. 2006 Apr 15;15 Spec No:R17-29. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16651366>
13. Nemeth K, Bayraktar R, Ferracin M, Calin GA. Non-coding RNAs in disease: from mechanisms to therapeutics. *Nat Rev Genet* [Internet]. 2024 Mar;25(3):211–32. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/37968332>
14. Nemeth K, Bayraktar R, Ferracin M, Calin GA. Non-coding RNAs in disease: from mechanisms to therapeutics. *Nat Rev Genet*. 2024 Mar;25(3):211–32.
15. Merico D, Roifman M, Braunschweig U, Yuen RKC, Alexandrova R, Bates A, et al. Compound heterozygous mutations in the noncoding RNU4ATAC cause Roifman Syndrome by disrupting minor intron splicing. *Nat Commun* [Internet]. 2015 Nov 2;6:8718. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26522830>
16. Hermanns P, Bertuch AA, Bertin TK, Dawson B, Schmitt ME, Shaw C, et al. Consequences of mutations in the non-coding RMRP RNA in cartilage-hair hypoplasia. *Hum Mol Genet* [Internet]. 2005 Dec 1;14(23):3723–40. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16254002>
17. Greene D, Thys C, Berry IR, Jarvis J, Ortibus E, Mumford AD, et al. Mutations in the U4 snRNA gene RNU4-2 cause one of the most prevalent monogenic neurodevelopmental disorders. *Nat Med* [Internet]. 2024 May 31; Available from: <http://www.ncbi.nlm.nih.gov/pubmed/38821540>
18. Chen Y, Dawes R, Kim HC, Ljungdahl A, Stenton SL, Walker S, et al. De novo variants in the RNU4-2 snRNA cause a frequent neurodevelopmental syndrome. *Nature* [Internet]. 2024 Jul 11; Available from: <http://www.ncbi.nlm.nih.gov/pubmed/38991538>
19. Schot R, Ferraro F, Geeven G, Diderich KEM, Barakat TS. Re-analysis of whole genome sequencing ends a diagnostic odyssey: Case report of an RNU4-2 related neurodevelopmental disorder. *Clin Genet* [Internet]. 2024 Jun 11; Available from: <http://www.ncbi.nlm.nih.gov/pubmed/38859706>

20. Ellingford JM, Ahn JW, Bagnall RD, Baralle D, Barton S, Campbell C, et al. Recommendations for clinical interpretation of variants found in non-coding regions of the genome. *Genome Med*. 2022 Jul 19;14(1):73.
21. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. 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 May;17(5):405–24.
22. Wright CF, Campbell P, Eberhardt RY, Aitken S, Perrett D, Brent S, et al. Genomic Diagnosis of Rare Pediatric Disease in the United Kingdom and Ireland. *N Engl J Med* [Internet]. 2023 Apr 27;388(17):1559–71. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/37043637>
23. Leonard H, Thomson M, Bower C, Fyfe S, Constantinou J. Skeletal abnormalities in Rett syndrome: increasing evidence for dysmorphogenetic defects. *Am J Med Genet* [Internet]. 1995 Sep 11;58(3):282–5. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8533832>
24. Schultz R, Glaze D, Motil K, Hebert D, Percy A. Hand and foot growth failure in Rett syndrome. *J Child Neurol* [Internet]. 1998 Feb;13(2):71–4. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9512306>
25. Chabot B, Shkreta L. Defective control of pre-messenger RNA splicing in human disease. *J Cell Biol*. 2016 Jan 4;212(1):13–27.
26. Griffin C, Saint-Jeannet JP. Spliceosomopathies: Diseases and mechanisms. *Dev Dyn*. 2020 Sep;249(9):1038–46.
27. Carroll R, Kumar R, Shaw M, Slee J, Kalscheuer VM, Corbett MA, et al. Variant in the X-chromosome spliceosomal gene GPKOW causes male-lethal microcephaly with intrauterine growth restriction. *Eur J Hum Genet*. 2017 Sep;25(9):1078–82.
28. Engal E, Oja KT, Maroofian R, Geminder O, Le TL, Marzin P, et al. Bi-allelic loss-of-function variants in WBP4, encoding a spliceosome protein, result in a variable neurodevelopmental syndrome. *The American Journal of Human Genetics*. 2023 Dec;110(12):2112–9.
29. Barbosa-Morais NL, Irimia M, Pan Q, Xiong HY, Gueroussov S, Lee LJ, et al. The Evolutionary Landscape of Alternative Splicing in Vertebrate Species. *Science* (1979). 2012 Dec 21;338(6114):1587–93.
30. Merkin J, Russell C, Chen P, Burge CB. Evolutionary Dynamics of Gene and Isoform Regulation in Mammalian Tissues. *Science* (1979). 2012 Dec 21;338(6114):1593–9.
31. Licatalosi DD, Darnell RB. Splicing Regulation in Neurologic Disease. *Neuron*. 2006 Oct;52(1):93–101.

32. Gonatopoulos-Pournatzis T, Blencowe BJ. Microexons: at the nexus of nervous system development, behaviour and autism spectrum disorder. *Curr Opin Genet Dev.* 2020 Dec;65:22–33.
33. Irimia M, Weatheritt RJ, Ellis JD, Parikshak NN, Gonatopoulos-Pournatzis T, Babor M, et al. A Highly Conserved Program of Neuronal Microexons Is Misregulated in Autistic Brains. *Cell.* 2014 Dec;159(7):1511–23.

Figures and tables legends

Figure 1. Photographs of patients with pathogenic variants *RNU4-2* at various ages illustrating the dysmorphisms described in the text. **P1:** 1A-1C at 2 years old. **P2:** 2A-2C at 7 months old, 2D with 1 year old, 2E and 2F with 24 months old and 2G, 4 years old. **P3:** 3A with 8 months old and 3B with 4 years old. **P11:** 4A with 3 years old, 4B and 4C with 7 years old, 4D and 4E with 15 years. **P4:** 5A-5C aged 2 years and 5D aged six. **P6:** 6A and 6B 4 years old and 6C-6E with 6

years old. **P7:** 7A 23 months, 7B-7C 15 years old and 7D 19 year old. **P8:** 8A-8C 29 years old. Note the hooded upper eyelid and epicanthus, full cheeks, tented philtrum, mouth constantly slightly open with an everted lower lip vermilion and high palate. Informed consent had been obtained for publication of all images present in this paper.

Figure 2. Photographs of hands and feet of individuals with pathogenic variants *RNU4-2*. A-C Prominent fingertip pads in infancy P1 (7 months), P3 (3 years old) and P4 (2 years old). D Mild edema dorsum feet of P1 at 7 months old. E shortening of third and fourth metacarpal in P7 at 19 years old. F. small and slender feet of P8 at 29 years old.

Figure 3. Prenatal brain MRI of individuals with pathogenic variants *RNU4-2*. **Patient 2 (A-C)** (36.5 weeks of gestation). **Patient 9 (D-F)** (36.1 weeks of gestation). (A) Axial HASTE image at 36 weeks of gestation demonstrates small biometrics, slight colpocephaly and delayed gyral pattern. (B) Sagittal HASTE image shows an abnormal corpus callosum, short and thin, respect to the gestational age. (C) Axial HASTE image at a lower level remarks the delayed gyral development predominantly seen in frontal lobes. (D, E) Axial HASTE images in another 35 weeks' foetus demonstrate small cerebral and cerebellar biometrics, abnormal gyral pattern with poor developed frontal lobes, dysmorphic ventricles and suspicion of hyperintense white matter. (F). Midsagittal HASTE view also remarks the dysgenetic corpus callosum (small and thin, with absent rostrum).

Figure 4. Postnatal brain MRI of individuals with pathogenic variants *RNU4-2*. **Patient 1 (A-D).** Cranial MRI performed at 9-months of age. (A) Sagittal T1-weighted image shows dolicocephaly and thin corpus callosum. (B, C) Axial T1-weighted images demonstrate the diminished white matter, small cavum vergae, ventriculomegaly, and prominence of cortical sulci. (D) Coronal T2-weighted image also shows prominent ventricles and cortical sulci related to parenchymal volume loss, as well as hippocampal malrotation. **Patient 2,** postnatal MR follow-up at 6 months of age (E-H). (E) Sagittal T2- and (F) T1-weighted images show the abnormal thin corpus callosum. (G) Axial T2- and (H) T1-weighted images demonstrate prominent cortical sulci, as well as simplified gyral pattern in frontal lobes. **Patient 3.** (I-N). Cranial MRI performed at 9 months of age. (I) Axial T2-weighted images demonstrate a left

temporal arachnoid cyst, (J) ventriculomegaly with prominent cortical sulci, simplified frontal gyral pattern and (K) presence of cavum vergae. (L) Corresponding axial T1-weighted image better demonstrated delayed White matter myelination. (M) Sagittal T1-weighted image shows dolicocephaly as well as a peculiar, thin corpus callosum. (N) A notorious arterial tortuosity was displayed in the reconstructed axial image from angio-MRI TOF 3D. **Patient 4** (O-S). Cranial MRI performed at 4 years of age. (O) Axial T1-weighted image demonstrates the slight dysmorphic ventricles with simplified gyral pattern in frontal lobes. (P) Axial FLAIR image better depicts the periventricular white matter lesions. (Q) Sagittal T1-Weighted image with a peculiar shape of corpus callosum with thin posterior portion. (R) Axial FLAIR image at upper level demonstrates several hyperintense foci within the white matter, presumed gliosis. (S) Some white matter lesions and simplified frontal gyral pattern on axial T2-weighted image. **Patient 9** (T-X). Cranial MRI performed at 11 months of age. (T) Axial T2-weighted image shows the abnormal ventricular system, with enlarged cortical sulci, thin anterior corpus callosum and simplified frontal gyral pattern. (U) Sagittal T1-weighted image demonstrates the microcephaly, as well as dysgenetic, very thin and short corpus callosum. (V) Axial T2-weighted image shows ventriculomegaly with prominent cortical sulci. (W) Axial T1-weighted image better demonstrates the delayed myelination, the simplified gyral frontal pattern, as well as the ventriculomegaly. (X) Angio-MRI with TOF 3D reconstructed image in axial plane depicts an abnormal tortuosity of intracranial arteries.

Figure 5. X-rays of individuals with pathogenic variants *RNU4-2*. Patient 11 (A-G). (A) Lateral chest x-ray shows pectus carinatum, generalized osteopenia, along with increased radiolucency of vertebral bodies and radiodensity of the cortical rim, and mild thoracic hyperkyphosis. (B) Frontal view demonstrates a right dorsolumbar scoliosis. (C, D) AP x-rays of lower limbs depict generalized osteopenia, with cortical thinning of long bones, as well as some metaphyseal dense striations and sclerotic bands. (E) Similar findings in x-rays of the right arm and forearm, with slight bone bowing. (F) Chest x-ray frontal view shows a non-specific increase of bronchovascular tracks, and mild dorsal scoliosis, together with generalized osteopenia. (G) Chest x-ray lateral frontal view with dorsal hyperkyphosis, osteopenia and slight decrease in height of dorsal vertebral bodies. Patient 6 (H-J). (H) Left arm x-ray shows a healed spiral diaphyseal fracture of the humerus with bone remodelling. Osteopenia and hyperlordosis is

demonstrated in the (J) lateral lumbar spine and (J) in the right arm and elbow. Patient 5 (K-L). (K) Total spine x-ray demonstrates significant right convexity thoracolumbar scoliosis. (L) Teleradiography of the lower extremities demonstrates slight hypoplasia of iliac bones, gracile long bones with bilateral genu valgus and some degree of tibial bowing.