Received: 5 June 2020 | Revised: 7 November 2020 | Accepted: 4 December 2020

DOI: 10.1002/humu.24151

BRIEF REPORT

Human Mutation HGVS HUMAN GENOME VARIATION SOCIETY WILEY

Expanding the phenotype of X-linked SSR4–CDG: Connective tissue implications

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Abstract

Signal sequence receptor protein 4 (SSR4) is a subunit of the translocon-associated protein complex, which participates in the translocation of proteins across the endoplasmic reticulum membrane, enhancing the efficiency of N-linked glycosylation. Pathogenic variants in *SSR4* cause a congenital disorder of glycosylation: SSR4–congenital disorders of glycosylation (CDG). We describe three SSR4–CDG boys and review the previously reported. All subjects presented with hypotonia, failure to thrive, developmental delay, and dysmorphic traits and showed a type 1 serum sialotransferrin profile, facilitating the diagnosis. Genetic confirmation of this X-linked CDG revealed one de novo hemizygous deletion, one maternally inherited deletion, and one de novo nonsense mutation of *SSR4*. The present subjects highlight the similarities with a connective tissue disorder (redundant skin, joint laxity, blue sclerae, and vascular tortuosity). The connective tissue problems are relevant, and require preventive rehabilitation measures. As an X-linked disorder, genetic counseling is essential.

KEYWORDS
congenital disorders of glycosylation, connective tissue disorders, SSR4, translocon associated complex, TRAP complex

Congenital disorders of glycosylation (CDG) are a growing number of more than 130 disorders due to defects in protein and lipid glycosylation. CDG present with varying degrees of neurological involvement, and, depending on the specific type, can have additional organ involvement (Ferreira et al., 2018). Most known CDG alter the N-linked glycosylation pathway, an essential process for protein folding and stability, protein-protein complex formation, protease resistance, intercellular and intracellular transport, cell-cell recognition, and signaling (Freeze et al., 2014).

A useful, inexpensive, and often reliable biomarker for detecting N-linked related CDG is isoelectrofocusing of serum transferrin (Trf) (Bruneel et al., 2018). An abnormal Trf profile is designated as type 1 (cytosolic or endoplasmic reticulum defect; CDG-I) or type 2 (Golgi defect; CDG-II) (Jaeken, 2010). Molecular studies are needed to achieve a genetic diagnosis (Bruneel et al., 2018). Within CDG-I are several disorders involving two functionally and structurally linked complexes required for correct N-glycosylation. The oligosaccharyltransferase complex, plays an important role in the transfer of newly synthesized oligosaccharides from dolichol to nascent polypeptide chains (Shrimal et al., 2013), and the translocon associated protein (TRAP) complex participates in the translocation of proteins across the endoplasmic reticulum (ER) membrane (Braunger et al., 2018). The family of signal sequence receptor proteins (SSRs) are essential components of the TRAP complex, and are needed for the discrimination of ER membrane substrates that must be translocated (Braunger et al., 2018).

Pathogenic variants causing CDG have been identified in three subunits of the TRAP complex: *SEC61* (MIM# 617056; Bolar et al., 2016), *SSR4* (MIM# 300934; Losfeld et al.,2014; Medrano et al., 2019; Ng et al., 2015), and *SSR3* (MIM# 606213) (Dittner-Moormann et al., 2020; Ng et al., 2019). The phenotypes show some similarity, such as intrauterine growth restriction, but many more differences: complex phenotypes have been related to *SSR3* and *SSR4*, and, in contrast, others restricted to kidney and blood cells in *SEC61* patients have been described. Fewer than a dozen subjects have been reported for each of these very rare disorders (only two for *SSR3* deficiency), so presumably many aspects of the phenotypes remain to be described.

As with many inborn errors of metabolism, most CDG are autosomal recessive, whereas mutations in *ALG13* (Tima et al., 2012), *ATP6AP1* (Jansen et al., 2016), *OGT* (Pravata et al., 2020), *PIGA* (Swoboda et al., 2014), *SLC9A7* (Khayat et al., 2019), *SLC35A2* (Kodera et al., 2013), *MAGT1* (Blommaert et al., 2019), and *SSR4* (Losfeld et al.,2014; Medrano et al., 2019; Ng et al., 2015) are X-linked disorders.

We report three new unrelated SSR4–CDG subjects, bearing chromosomal deletions and one nonsense variant in the *SSR4* gene. We describe novel features and review the literature on this CDG.

Samples were obtained in accordance with the Helsinki Declaration of 1964, as revised in October 2013 in Fortaleza, Brazil. Written informed consent from the parents was obtained. The three subjects (S1, S2, and S3) belonged to different families. Cranial magnetic resonance imaging (MRI) studies included T1- and T2-weighted images and FLAIR, diffusion-weighted images, and for S1, arterial and venous angiographic study. To evaluate the skull size and proportions, the summation index and the cranial index of Cronqvist were evaluated (Austin & Gooding, 1971). To evaluate the joint hypermobility, the Beighton scale score was applied considering a positive test when the subject scored more than 6/9 (Remvig et al., 2007).

Subjects and parental blood samples were obtained from each proband for the preparation of sera and nucleic acids. The separation and relative quantification of serum Trf glycoforms were undertaken using the capillary zone electrophoresis method (two different kits) for the three individuals (S1: CEofix -CDT kit; S2-S3: Capillarys CDT kit). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot using specific antibodies against two serum N-glycoproteins, that is, transferrin (Trf) and haptoglobin (Hpt), was used for S2 and S3 samples, as previously described (Seta et al., 1996). To analyze identical glycoprotein amounts and avoid overloading, the serum levels of Trf and Hpt were determined (VISTA 1500; Siemens HealthCare diagnostic) before western blot analysis. To test the loss of the cellular SSR4 protein we performed western blot analysis. Fibroblasts of the subjects and two different controls were scrapped with lysis buffer (NaCl 150 mM; Tris 50 µM pH 7,4; EDTA 2 mM; glycerol 10%; Triton X100 1%), and 20 µg of total protein was loaded in a Nupage 4%–12% gel (Invitrogen ref NP0323). After SDS-PAGE separation and transfer on a nitrocellulose sheet, the SSR4 protein was detected using rabbit anti-human SSR4 antibodies (dilution 1/100 in TTBS-milk; Novus biologicals ref NBP1-92390), anti-rabbit immunoglobulin G horseradish peroxidase–tagged antibodies (dilution 1/5000 in TTBS-milk; GE Healthcare ref NA934V), ECL substrate (GE Healthcare, ref RFN 2106) and the XRS camera from Bio-Rad. Second, on the same blot, beta-actin was detected using a mouse monoclonal antibody directly tagged with horseradish peroxidase (dilution 1/10 000 in TTBS-milk, Santa Cruz ref sc-47778). Genomic DNA was extracted from whole blood. The genetic protocol included chromosomal microarray analysis (CMA) using a comparative genomic hybridization oligonucleotide microarray (aCGH, 180K Agilent) and the Cytoscan 750K array (Affymetrix, including 200,000 SNP markers). Exome sequencing was performed on S3. Maternal studies were performed to determine whether the conditions were de novo, as female carriers with oligosymptomatic phenotypes have been described (Ng et al., 2015). The variants were submitted to ClinVar Submission Wizard (www.ncbi.nlm.nih.gov/clinvar/).

Table 1 shows the clinical and molecular data of three boys affected by SSR4–CDG. All three presented with perinatal hypotonia

TABLE 1 Molecular and clinical characteristics of subjects

Subject/gender	S1 Male	S2 Male	S3 Male
Age/origin	2 years 2 months/Chilean	2 years 10 months/French	12 years/French
Age at diagnosis	9 months	6 months	8 years
GA (weeks)/intrauterine growth retardation	32/IUGR	37 + 1/IUGR Small placenta, C-section	38/No IUGR
Perinatal Period	Hypotonia, feeding difficulties, failure to thrive, nasogastric feeding tube respiratory distress, jaundice	Hypotonia respiratory distress, jaundice	Hypotonia, feeding difficulties, failure to thrive, parenteral nutrition
Weight, g/height, cm/OFC, cm at birth	1400 (−3.6 *SD*)/38 (−6.2 *SD*)/28.5 (−3.4 *SD*)	2235 (−1.6 *SD*)/43 (−3.0 *SD*)/30 (−2.2 *SD*)	3050 (−0.3 *SD*)/48 (−0.9 *SD*)/36 (1.3 *SD*)
Weight, kg/height, cm/OFC, cm at 6 months[a]	5.0 (−2.9 *SD*)/59 (−3.3)/38 (−4.9)	4.9 (−3.0 *SD*)/58 (−3.7 *SD*)/38.5 (−4.5 *SD*)	5.0 (−2.9 *SD*)/61 (−2.6 *SD*)/42 (−1.7 *SD*)
Weight, kg/height, cm/OFC, cm/age	9.6 (−2.5 *SD*)/84.5 (−1.8 *SD*)/45.5 (−3.3 *SD*)/26 months	6.6 (−3.3 *SD*)/66.0 (−3.8 *SD*)/42 (−3.8 *SD*)/11.5 mo 8.8 (−3.3 *SD*)/75 (−6.4 *SD*) /NA/34 months	22.0 (−2.6 *SD*)/137.0 (−2.6 *SD*)/51 (−2.9 *SD*)/12 years 9 months
Neurodevelopment, milestones, other neurological, clinical features	Head control: 10 months, sitting position: 18 months, no independent walk, quiets or smiles in response to sound or voice: 5 months, Absence of speech, gurgling, no babbling, hypotonia, no epilepsy, no ASD traits, good social interaction	Head control: 12 months, Able to bring hands to mouth: 18 months, Standing with support: 21 months, no independent walk, Quiets or smiles in response to sound or voice: 6 months, Babbling: 21 months, Some single words, no sentences Hypotonia, no epilepsy, no ASD traits	Independent walk at 5 years, speech delay, autistic features, hypotonia, no epilepsy, severe aggressive behavior and lack of impulse control
Neuroimaging	MRI (5 months)[a]: No structural abnormalities, slight blood vessel tortuosity	MRI (8 months): septal callosal dysplasia, abnormal temporal lobe gyration	MRI (7 months): delayed myelination, no structural abnormalities
Gastrointestinal involvement	Gastroesophageal reflux, gastrostomy, difficulties/aversion to food with certain textures	Gastroesophageal reflux, no dysphagia	Feeding difficulties but no need of parenteral nutrition, no dysphagia
Hearing, ophthalmological, other sensorial abnormalities	Normal hearing and visual acuity	Convergent strabismus	Hypermetropia, strabismus, astigmatism
Dysmorphic features and other signs	Blue sclerae, high and broad nasal bridge, anteverted nares, long philtrum, thin upper lip, hypoplastic vermillion of upper lip, micrognathia, large ears. Fifth finger clinodactyly, joint laxity, pes planus valgus, redundant skin, cryptorchidism. Positive Beighton scale score	Blue sclera, small lacrimal, broad nasal bridge, anteverted nares, long philtrum, thin upper lip, hypoplastic vermillion of upper lip, micrognathia, large asymmetric ears. Superposition of the second toe without syndactyly, joint laxity, redundant skin	Broad nasal bridge, long philtrum, thin upper lip, hypoplastic vermillion of upper lip, downturned corners of the mouth, large ears, hypomimia. Joint laxity, severe deformation of the feet in valgus, orthopedic surgery (11.5 years). Positive Beighton scale score
Coagulation (normal values)	APTT = 11.2 (24.8–33.2 s); PT = 97% (80%–120%)	NA	FVIII = 48% (60%–150%); vWF 34% (50%–150%)

TABLE 1 (Continued)

Subject/gender	S1 Male	S2 Male	S3 Male
Transferrin values & profile (normal values)	Type 1: 4-sialo 74.4% (71%–79%), 3-sialo 2.2% (<6.5%), 2-sialo **4.58**% (<1.78%)	Type 1: 4-sialo 80.8% (78%–86%), 3-sialo 2.1% (<6%), 2-sialo **3.9**% (<1.6%) Type 1 by SDS-PAGE	Type 1: 4-sialo 78.6% (78%–86%), 3-sialo 4.2% (<6%), 2-sialo **5.0**% (<1.6%) Type 1 by SDS-PAGE
Molecular findings/ inheritance	arr(GRCh37) Xq28(153011909_153063825)x0 dn/de novo	arr(GRCh37) Xq28(153060022_153063888)x0 mat/maternal	NM_001204526.1: c.274C>Tp. Gln92[a]/ de novo

Note: Abnormal values are highlighted in bold letters.

Abbreviations: APTT, activated partial thromboplastin time; ASD, autism spectrum disorder; F VIII, factor VIII; GA, gestational age; IUGR, intrauterine growth restriction; MRI, magnetic resonance imaging; NA, not available; OFC, occipito-frontal circumference; PT, prothrombin time; vWF, von Willebrand factor.

[a]For Subject 1, due to prematurity, corrected age has been considered for tests and evaluations during the first years.

and two with intrauterine growth restriction, feeding difficulties at birth, respiratory distress, and jaundice.

Feeding difficulties and failure to thrive led to enteral (nasogastric tube and gastrostomy) and parenteral nutrition in S1 and S3, respectively. Feeding problems were associated not only with hypotonia or dysphagia but also with sensorial disturbances such as aversion to food with certain textures.

Length and height were under normal range parameters for all individuals but occipito-frontal circumference was the most affected parameter as a sign of disharmonic microcephaly.

The three boys presented a marked global developmental disability but no epileptic seizures. Behavior disturbances with lack of impulse control and aggressiveness were presented by the older boy (S3).

Facial dysmorphism (Figure 1a,b) comprised a high and broad nasal bridge, blue sclerae, large mouth with long philtrum, thin upper lip, hypoplastic vermillion of upper lip, micrognathia, and large ears. S3 also showed hypomimia and downturned corners of the mouth. All three subjects presented clinically relevant joint laxity, two of them showing redundant skin and a positive Beighton scale score (Figure 1e). S1 showed a flexible pes planovalgus and S3 had severe valgus feet requiring orthopedic surgery at the age of 11 years.

All subjects underwent cranial MRI during infancy. S1's MRI showed slight tortuosity of blood vessels (Figure 1d), S2's showed callosal dysplasia and abnormal temporal lobe gyration, and S3's had a myelination delay without structural abnormalities.

Regarding ocular findings, internal strabismus was evident in two individuals. No clinical or radiological signs of kidney or heart disease were found. In S1 a skeletal X-ray at the age of 7 months revealed an asynchrony and delay of bone maturation and mild disproportionate shortening of long bones, with a mesomelic pattern. In addition to microcephaly, there was a small face with a summation index of 354 (normal range: 421 ± 42; Figure S1).

Regarding laboratory studies, the three subjects presented similar discrete type 1 Trf profiles combined in S2 and S3, with haptoglobin (Hpt) hypoglycosylation (Figures 1f, g, and l). Genetic confirmation revealed a de novo hemizygous deletion in S1, and S2 harbors a maternally inherited deletion, both deletions encompass all coding exons of SSR4 (Table 1). S3 presented one unreported nonsense variant (NM_001204526.1: c.274C>T(p. Gln92*) de novo). Western blot analysis of SSR4 protein expression in fibroblasts from S2 and S3 showed undetectable SSR4 protein expression compared to controls (Figure 1h).

We describe three male individuals with X-linked SSR4–CDG harboring an unreported *de novo* point mutation, a de novo SSR4 deletion, and one maternally inherited deletion. Since the first subject was reported by Losfeld et al in 2014, only nine additional individuals have been reported to date (Medrano et al., 2019, Ng et al., 2015), indicating the extreme rarity of the condition. However, the associated abnormal Trf profile facilitates diagnosis, and the widespread use of CMA and next generation sequencing may prove essential to increase diagnoses.

A review of all reported individuals (Figure S2) shows some constant phenotypic characteristics, such as developmental delay, intellectual disability, and microcephaly, in the context of a small global somatometry.

From the neurodevelopmental point of view, there is a delay in motor milestone acquisition that could be partially explained by central hypotonia but also by joint laxity. To assess this joint hypermobility we selected the Beighton score and found positive results in two out of the three boys, both also affected by foot malposition: pes planovalgus in S1 and severe valgus in S3 leading to orthopedic surgery. These joint findings, together with the presence of redundant skin, blue sclerae, and slight vessel tortuosity in the MRI, force a differential diagnosis with connective tissue diseases. Moreover, skeletal findings in S1 (Figure S1) also support the hypothesis of connective tissue implications. In one of the two reported individuals with a TRAP defect, SSR3-CDG, joint laxity was described (Ng et al., 2019), and other CDG related to Golgi trafficking abnormalities, associated joint hypermobility, and cutis laxa, such as COG7-CDG, ATP6V0A2-CDG, and MAN1B1-CDG, were also described (Rymen & Jaeken, 2014). One of the limitations to verify our hypothesis is that we have not performed pathological studies of the subjects' skin, which may be very informative. However, based on

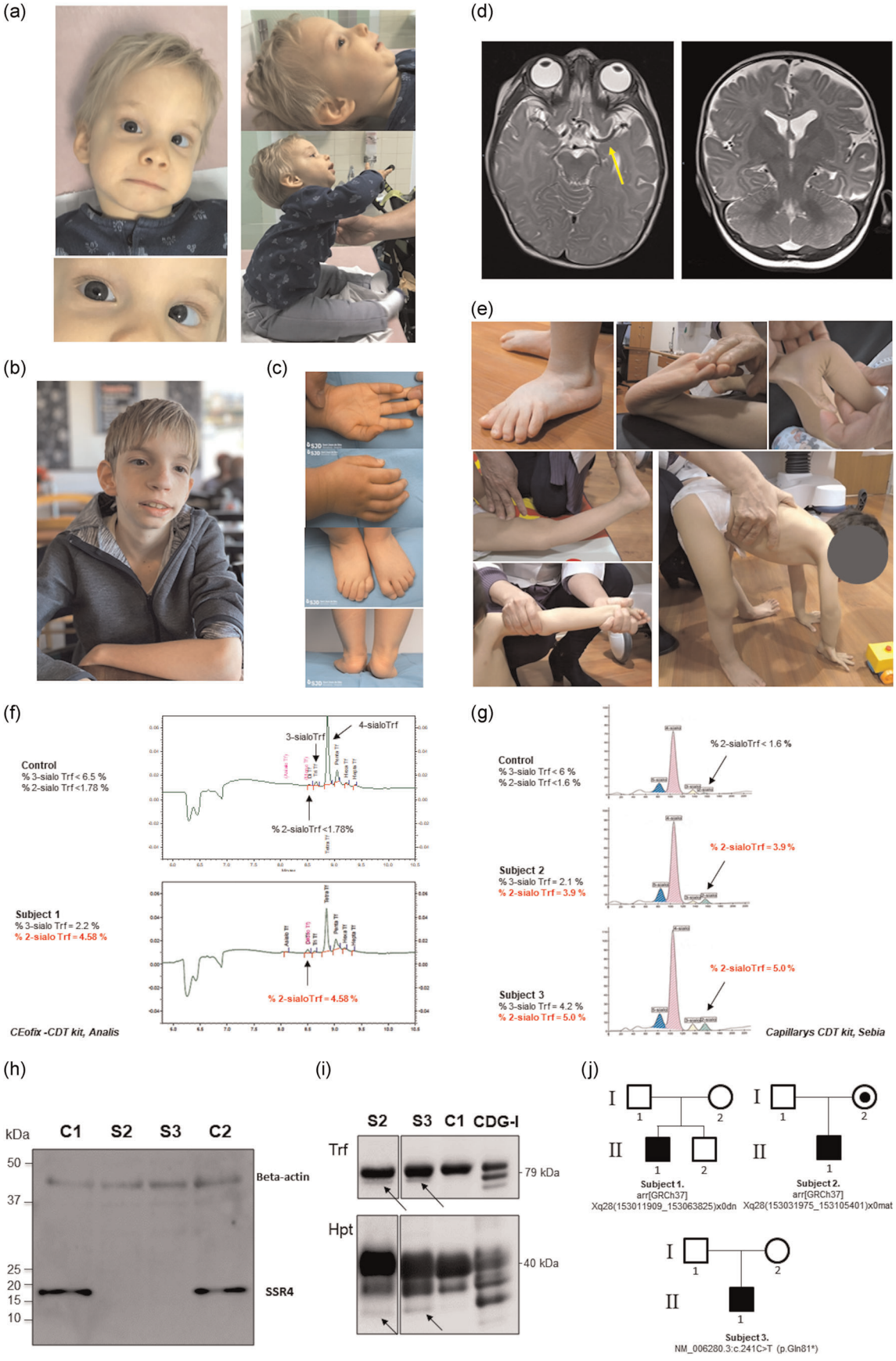

FIGURE 1 (See caption on next page)

our series and on previous reports, from a therapeutic point of view special preventive measures should be taken with SSR4–CDG subjects, as joint dislocations may occur and orthopedic surgery would be required (Ng et al., 2015).

Epilepsy and febrile seizures have been reported in six SSR4–CDG subjects (Losfeld et al., 2014; Medrano et al., 2019; Ng et al., 2015), but not in any in our series. In the description of Losfeld et al, good evolution was reported, and antiepileptic drugs could be stopped with continued good evolution, while in the Ng et al. series, epilepsy was briefly described. It is likely that epilepsy is not a determining factor in the neurological prognosis of SSR4–CDG subjects.

Other frequent medical problems are feeding difficulties. In our experience they may be explained not only by swallowing difficulties due to hypotonia and dysphagia, but also by some texture restrictions, without the presence of other sensorial restrictions and denoting a behavioral disturbance. Gastroesophageal reflux is also a prevalent comorbidity that should be considered in the face of failure to thrive (Losfeld et al., 2014; Ng et al., 2015).

There is probably a typical dysmorphic gestalt that is not well defined, but it may help clinicians to suspect the diagnosis, together with the other clinical signs. Midface features identified in our series were wide nasal bridge, anteverted nares, long and flat philtrum with hypoplastic vermillion of the upper lip, and thin upper lip and micrognathia, together with large ears. At present, new technologies for facial pattern recognition have demonstrated their usefulness in a wide number of genetic conditions, previous to or following whole exome sequencing, and they can be harnessed to identify new genetic disorders at different ages by using a large series of individuals (Gripp et al., 2016; Martínez-Monseny et al., 2019). Unfortunately, a minimum number of facial pictures of different subjects and ages, not available for this extremely rare condition, are needed to train these artificial intelligence platforms.

Regarding neuroimaging, cranial MRI showed unspecific findings in most individuals, such as abnormalities in the white matter, global volume loss, thin corpus callosum, and absence of septum pellucidum. In our series other unspecific signs were evidenced in neuroimaging, such as myelination delay. Unfortunately, the increase in vessel tortuosity found in one of the subjects in our series, could not be compared to images of previous reports.

Regarding laboratory studies, the three boys presented similar type 1 Trf profiles showing an increase in disialotransferrin using capillary zone electrophoresis. The increase in disialotransferrin was clear (two- to three-fold over the upper limit) but mild compared to the values seen in other classical CDG-I deficiencies, such as PMM2-CDG (Bruneel et al., 2020).

Concerning inheritance, of the thirteen individuals from ten different families, including the subjects reported here, three of them presented oligosymptomatic or healthy mothers and carrier women in the family, and seven were de novo mutations or mutations due to germline mosaicism in one family (two affected brothers). This is a high de novo ratio in contrast to the ratios for other X-linked diseases with a 1/3 ratio (Duchenne muscular dystrophy, Lowe syndrome, ornithine transcarbamylase deficiency, etc.).

Molecular studies revealed high impact point mutations in six out of the ten pedigrees, corresponding to frameshift mutations, stop codon mutations, and mutations at canonical donor or acceptor splice sites (Figure S2). Missense mutations have not been reported to date. It is difficult to establish a reliable genotype-phenotype correlation due to the small number of subjects described.

Finally, in the context of TRAP complex protein deficiencies, two subjects affected by SSR3–CDG have been reported (Dittner-Moormann et al., 2020; Ng et al., 2019), showing many similarities between both SSR4–CDG and SSR3–CDG. They have been related to complex multiorgan clinical pictures with intrauterine growth retardation, perinatal respiratory distress, early feeding difficulties and failure to thrive, moderate-to-severe developmental delay, intellectual disability, and joint laxity. However, global clinical severity appears to be greater in SSR3-CDG subjects.

In conclusion, the present patients broaden and consolidate the SSR4–CDG clinical picture, of a disease in which the Trf profile facilitates the diagnosis. Clinical symptoms, such as developmental delay, intellectual disability, hypotonia, microcephaly, and dysmorphic features are consistent among all the subjects reported.

FIGURE 1 Neuroradiology findings, dysmorphic features and laboratory studies. (a) Pictures show dysmorphic features of Subject 2 at two years of age: wide nasal bridge, anteverted nares, long, and flat philtrum with hypoplastic vermillion of the upper lip, and thin upper lip (above left). Blue sclerae (below left), micrognathia and redundant skin (above right). Hypotonic posturing (below right). (b) Picture from Subject 3 at 12 years of age showing broad nasal bridge, long philtrum, thin upper lip, hypoplastic vermillion of upper lip, downturned corners of mouth, and large ears. (c) Pictures from Subject 1 show fifth finger clinodactyly, joint laxity, and pes planus valgus. (d) Magnetic resonance imaging obtained at 5 months of age (3 months of corrected age due to prematurity) showing subtle blood vessel elongation (see arrow) and appropriate white matter myelination for age. Axial T2 (left) and coronal T2 (right). (e) Pictures showing Subject 1 joint hypermobility and some of the Beighton score maneuvers assessed at 3 years of age: flexible pes planovalgus, passive flexion of feet backwards, passive apposition of the thumbs to the flexor aspects of the forearm, hyperextension of the knee and elbow joints, and ability to put hands on the floor with the knees extended. (f, g) Capillary zone electrophoresis (CZE) profiles of serum transferrin (Trf) of subject 1 (a) and subjects 2 and 3 (b) both showing (arrows) increases of the 2-sialo Trf glycoform compared to the controls. (h) Western blot analysis of SSR4 protein expression in fibroblasts from S2 and S3 compared to two different control fibroblasts. SSR4 protein is undetectable in the cell lysate of the affected individuals. (i) Western blot against transferrin (Trf) and haptoglobin (Hpt), for samples of subjects 2 and 3 (S2 and S3), one control (Ctrl) and a type 1 CDG individual (CDG-I) to detect additional bands (arrows) corresponding to the loss of entire N-glycan chain(s). (j) Family pedigrees. Squares denote men, circles women. Affected individuals are shown as filled symbols and unaffected individuals as unfilled symbols. Circles with a dot represent carrier women

Connective tissue problems seem relevant, and special preventive rehabilitation and orthopedic measures should be taken, as joint dislocations are frequent, and orthopedic surgery would be required. As this is an X-linked disorder, genetic counseling is essential, since asymptomatic carrier mothers have been reported. Apart from clinical suspicion and Trf profile, the widespread use of chromosome microarray analysis and next-generation sequencing may prove essential to increase the number of individuals diagnosed.

ACKNOWLEDGMENTS

The authors would like to thank the patients families for their kind collaboration. M.S. is supported by the Generalitat de Catalunya (PERIS SLT008/18/00194) and National Grant PI17/00101 from the National R&D&I Plan, cofinanced by the Instituto de Salud Carlos III (Subdirectorate-General for Evaluation and Promotion of Health Research) and FEDER (European Regional Development Fund). TD, NS, ABR, and SVB were supported (i) by grant ANR-15RAR3-0004-06 under the frame of E-RARE-3, the ERA-Net for Research on Rare Diseases, (ii) by the European Union's Horizon 2020 research and innovation program under the ERA-NET cofund action N° 643578. MS is supported by the Generalitat de Catalunya (PERIS SLT008/18/00194) and National Grant PI17/00101 from the National R&D&I Plan, cofinanced by the Instituto de Salud Carlos III (Subdirectorate-General for Evaluation and Promotion of Health Research) and FEDER (European Regional Development Fund).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The genetic variants have been uploaded to ClinVar Submission Wizard (https://www.ncbi.nlm.nih.gov/clinvar/) with submissions ID SUB8461253, SUB8461427, and SUB8480802.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Castiglioni C, Feillet F, Barnerias C, et al. Expanding the phenotype of X-linked SSR4–CDG: Connective tissue implications. *Human Mutation*. 2021;42:142–149. https://doi.org/10.1002/humu.24151