Recessive variants in *COL25A1* gene as novel cause of arthrogryposis multiplex congenita with ocular congenital cranial dysinnervation disorder

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Abstract

A proper interaction between muscle-derived collagen XXV and its motor neuron-derived receptors protein tyrosine phosphatases σ and *δ* (PTP σ/*δ*) is indispensable for intramuscular motor innervation. Despite this, thus far, pathogenic recessive variants in the *COL25A1* gene had only been detected in a few patients with isolated ocular congenital cranial dysinnervation disorders. Here we describe five patients from three unrelated families with recessive missense and splice site *COL25A1* variants presenting with a recognizable phenotype characterized by arthrogryposis multiplex congenita with or without an ocular congenital cranial dysinnervation disorder phenotype. The clinical features of the older patients remained stable over time, without central nervous system involvement. This study extends the phenotypic and genotypic spectrum of *COL25A1* related conditions, and further adds to our knowledge of the complex process of intramuscular motor innervation. Our observations indicate a role for collagen XXV in regulating the appropriate innervation not only of extraocular muscles, but also of bulbar, axial and limb muscles in the human.

Key words: arthrogryposis; axon guidance; *COL25A1*; congenital cranial dysinnervation disorders; distal arthrogryposis.

1. Introduction

Arthrogryposis multiplex congenita (AMC) is a clinical entity encompassing an extremely heterogeneous group of disorders characterized by multiple joint contractures at birth (Cachecho et al., 2019). The common denominator in all is prenatal hypokinesia/akinesia. The underlying defects that lead to AMC can be categorized into four large groups: muscular disorders, neurological disorders (central and/or peripheral, and neurometabolic), connective tissue disorders and environmental disorders (uterine space limitations, maternal diseases, drugs use) (Ravenscroft et al., 2011; Hall, 2014; Hall et al., 2019). Defects in intramuscular innervation are an established cause of AMC, with variants in *ECEL1/DINE* being the only described cause of AMC due to this pathophysiological mechanism (McMillin et al., 2013; Dohrn et al., 2015).

COL25A1 encodes a transmembrane-type collagen reported to be indispensable for intramuscular motor innervation (Tanaka et al., 2014). Previous studies in mice indicated that *Col25a1* is transiently highly transcribed in developing skeletal muscle cells, but not detected in adult skeletal muscles (Tanaka et al., 2014; Gonçalves et al., 2019). Intramuscular innervation requires proper interaction between muscle-derived collagen XXV and its motor neuron-derived receptors protein tyrosine phosphatases σ and *δ* (PTP σ/*δ*) (Munezane et al., 2019). This interaction is crucial for the navigation of motor axons into the developing muscle (Munezane et al., 2019), and *Col25a1* knockout (KO) mice exhibited neonatal lethality due to lack of intramuscular innervation (Tanaka et al., 2014). Despite the widespread skeletal muscle involvement in *Col25a1* KO mice, thus far recessively inherited pathogenic variants in human *COL25A1* have only been associated with ocular congenital cranial dysinnervation disorders (CCDDs) in two families (Khan and Al-Mesfer, 2015; Shinwari et al., 2015) that had no features suggestive of a more widespread neuromuscular involvement. CCDDs are congenital disorders resulting in absent or aberrant innervation of

extraocular and/or cranial musculature. These are typically caused by variants that affect either motor neuron specification or motor nerve development, and include involvement of the kinesin motor protein KIF21A; the tubulin isotypes TUBB3, TUBB2B, or TUBA1A; or transcription factors that, when mutated, cause syndromic conditions with associated cranial nerves axonal guidance dysfunction (Whitman and Engle, 2017; Jurgens et al., 2021).

Here we present five patients from three unrelated families with recessive *COL25A1* variants presenting with an arthrogryposis/AMC phenotype with or without an ocular CCDD. Our findings expand the phenotypic spectrum of *COL25A1*-related conditions and broaden the molecular etiologies of AMC.

2. Patients and Methods

2.1 Clinical features

2.1.1 Family A

The proband (individual A1) is a now 16 year-old British female of Pakistani origin whose parents are first cousins. She was delivered at term after an uneventful pregnancy, including normal ultrasound scans and normal intrauterine movements. Examination at birth revealed hypotonia, bilateral ptosis (more evident on the left), facial weakness with high-arched palate, micrognathia, and laryngeal stridor. She had joint contractures involving proximally and distally the upper and lower limbs, with limited extension of distal interphalangeal joints, elbows, shoulders, hips and knees, as well as club feet. Ophthalmic examination showed globe retraction and limited abduction in the left eye as well as full movements of the right eye, compatible with an esotropic Duane syndrome in the left eye (Figure 1, Table 1). Gross motor development was within the upper limit of normal; the patient first walked at 18 months of age. The clinical picture has remained stable over the

years. At last examination at age 16 years, she presented with bilateral ptosis, Duane syndrome in the left eye, AMC, proximal more than distal weakness, scoliosis, and vocal cord paralysis. She can walk independently for about 300-500 metres with a posture control walker to assist balance and uses a manual wheelchair for longer distances due to pain and fatigue. She cannot rise from the floor unassisted or climb steps without holding onto the railing with both hands. A left distal femoral and proximal tibia epiphysiodesis was performed at age 12 years to improve her posture. Spirometry at age 15 years showed a forced vital capacity (FVC) of 1.55 liters, with reduced FVC% predicted at 58%. Cognitive development is normal.

Creatine kinase (CK), radiographic skeletal survey and brain MRI performed during her first year of life were normal. Karyotype was 46,XX. Maternal anticholinesterase receptor antibodies were absent. Nerve conduction studies on medial plantar, medial popliteal, lateral popliteal and ulnar nerves, as well as EMG on tibialis anterior, vastus medialis and orbicularis oculi at 5 months and 3 years of age revealed no abnormalities and no neuromuscular junction abnormalities. Muscle MRI of the lower limbs showed diffuse muscle bulk atrophy in the anterior and posterior compartments, in keeping with disuse atrophy (Figure 2). Muscle biopsy at 5 years and 8 months (site unknown) showed mild fibre size variation (30-55 μm; normal range for age: 20-40 μm) . There were no overt chronic myopathic or neurogenic features. Oxidative stains showed subtle type I fibre predominance, and presence of ill-defined core-like lesions, more often seen in type I fibres. Myosin heavy chain immunohistochemistry confirmed mild overall slow fibre predominance. There were no excess hybrid fibres or aberrant expression of fetal/embryonic myosin heavy chains (Figure 3).

Individual A2 was the elder brother of patient A1. He was born at 36 weeks of gestation after an unremarkable pregnancy, with no concerns regarding fetal movement or

amniotic fluid volume. His birth weight was 2.1 kg. He was admitted to the neonatal unit due to respiratory distress and required nasal prong oxygen, nasogastric tube feeding and frequent suctioning for excessive oral secretions. Widespread limb contractures were noted from birth with few spontaneous arm movements. On examination at 4 months of age he was noted to have a hirsute forehead, synophrys, large underfolded ears with uplifted earlobes, a high-arched palate and a weak cry. Involvement of extraocular muscles or presence of ptosis was not reported in available clinical records. He held his neck in extension and had joint contractures involving both upper and lower limbs, with limited extension of the metacarpophalangeal and proximal interphalangeal joints, elbows, wrist, hips and knees. He had large cutaneous dimples at the elbow with early soft tissue web formation, and absent distal phalangeal creases. In addition, he had an unusual fat distribution over the buttocks and low-lying nipples. His tone was globally reduced and he had a poor sucking reflex. He remained globally developmentally delayed and had a continuous requirement for nasal prong oxygen. He developed recurrent lower respiratory infections, thought to be related to aspiration of secretions, which led to collapse of the left lung. The latter remained uninflated, despite bronchoscopy and lavage, which led to multiple episodes of respiratory sepsis requiring paediatric intensive care unit admission. He died at the age of 2 years and 5 months as a result of a further respiratory complications.

Clinical tests in the first few months of life in individual A2 were uninformative. Extensive metabolic and infectious testing were negative, as well as genetic studies, including a karyotype and screens for myotonic dystrophy and Prader Willi syndrome. No anomalies were noted on brain MRI, EEG, echocardiogram, renal ultrasound, EMG or nerve conduction studies (site unknown). An ophthalmology assessment included a normal electroretinogram (ERG), though the visual evoked potentials (VEP) indicated marked post-retinal dysfunction. Audiological assessment revealed mild bilateral conductive hearing loss. Maternal

anticholinesterase receptor antibodies were absent. No skeletal survey or muscle biopsy was performed.

2.1.2. Family B

The proband (individual B1) is an 11-year-old female of Palestinian origin whose parents are first cousins. She was born at 33 weeks gestation via cesarean section due to oligohydramnios, breech presentation and premature uterine contractions. She remained in the neonatal intensive care unit for 45 days after birth with severe gastroesophageal reflux (requiring nasogastric tube feeding) and hypoxia and respiratory distress (requiring transient oxygen supplementation). Examination at birth revealed right hip dysplasia and contractures in the right knee, clenched hands, and congenital vertical talus with bilateral club feet (Table 1). Echocardiogram revealed a patent foramen ovale and muscular ventricular septal defect that resolved without intervention. At 3 months of age she was re-hospitalized with chronic stridor and respiratory distress. Direct bronchoscopy revealed bilateral vocal cord paralysis for which she received a tracheostomy; she was decannulated at 4 years of age. Examination at 3 months old revealed micrognathia, low-set posteriorly rotated ears, contractures of the elbows, wrists, and right knee, camptodactyly, and overlapping fingers. Her right hip was dislocated. An incomitant large angle exotropia with left hypertropia and nystagmus were observed on initial ophthalmological exam. She was later noted to have severe bilateral adduction limitation and mild upgaze limitation, worse on the right, with full abduction and infraduction and no ptosis. Nystagmoid-like movements were considered to be secondary to restricted motility, dysinnervation, and fixation effort. She was diagnosed with the ocular CCDD referred to as congenital fibrosis of the extraocular muscles, but atypical due to the lack of ptosis. During her hospitalization at 3 months of age, conduction studies of median and left peroneal nerves, EMG of proximal and distal muscles of the left upper and lower extremities, and brain MRI were normal. MRI imaging was not optimized for visualization of

the cranial nerves, but third cranial nerves were visualized bilaterally. EEG demonstrated near continuous focal slowing over the right posterior quadrant, but no seizures. She underwent multiple orthopedic procedures, including right quadriceps lengthening, right hip open reduction, and right knee lengthening. At 11 years of age, her parents report she is able to walk without assistance, she is in regular school without extra support, and her speech is full but labored due to her vocal cord paralysis. Her orthopedic issues persist, and she has additional surgeries planned for her hand contractures. She is also followed by endocrinology for slow growth.

2.1.3. Family C

The proband (individual C1) is a 3-year-old Australian male of Caucasian origin who was delivered at term after an uneventful pregnancy. He presented at birth with bilateral hip dysplasia and AMC, bilateral camptodactyly, thumb adduction, limited knee extension and flexion and Achilles tendon shortening (Figure 1, Table 1). On examination, he had small hands and short fingers, short feet and curved short toes, and mild calf wasting. He also presented with ophthalmalogical features including a large exotropia, bilateral limitation of adduction (right worse than left) and limitation of left elevation with left upper eyelid blepaharoptosis, consistent with congenital fibrosis of the extraocular muscles. This resulted in marked compensatory head position that changed depending on fixation preference (Figure 1). The compensatory head position was dramatically improved following two surgical procedures at age 27 and 37 months (Figure 1). Facial dysmorphism was not observed and cognitive function was normal. An isolated delay in gross motor skills was detected, with first unassisted steps at 2 years old.

Brain and orbital MRI at 24 months old showed marked thinning of the extraocular muscles, in particular of the medial rectus muscles bilaterally, more obvious on the right, and

milder thinning also of the left superior rectus muscle (Figure 4). Intracranial lesions were not observed. Spinal MRI was normal.

Individual C2 is a now 3-month-old female, younger sister of individual C1. She was born at term after an uneventful pregnancy, except for the reduced hand movements that were observed by prenatal ultrasound. She presented at birth with bilateral clasped thumbs, but without lower limb involvement (Figure 1, Table 1). Failure to thrive and poor feeding were observed, currently requiring nasogastric tube feeding. On examination, her tongue is thin with reduced muscle bulk and its range of movement is reduced, resulting in poor suck. Ophthalmic features at 3 months of age were characterized by left exotropia and hypotropia with markedly reduced left adduction and elevation. No ptosis was observed and right eye movements were full. Both eyes were frequently observed to be held in down and left gaze. Findings were thought to be consistent with congenital fibrosis of the extraocular muscles, similar to her brother (Figure 1, Table 1).

2.2. Whole exome sequencing and variant analysis

DNA was extracted from peripheral blood samples of individuals A1, A2, C1, and their unaffected parents, and DNA from A1 and C1 and their parents underwent trio whole exome sequencing (WES). To capture the coding regions, we used an Illumina Twist exome capture (~38 Mb target). All sequencing was performed by the Genomics Platform at the Broad Institute of MIT and Harvard (Boston, MA, USA) to cover >80% of targets at 20x with a mean target coverage of >100x. Exome sequencing data was processed through a pipeline based on Picard (https://broadinstitute.github.io/picard/) and mapping done using the BWA aligner (http://bio-bwa.sourceforge.net/bwa.shtml) (Li and Durbin, 2010) to the human genome build 38. Variants were called using Genome Analysis Toolkit (GATK) Haplotype Caller package version 3.5 (Poplin et al., 2017).

DNA from individual B1 and her parents was extracted from saliva using the prepIT.L2P solution (DNA Genotek, Ottawa, ON, Canada). Sanger sequencing of coding exons and intron-exon boundaries of *TUBB3*, *CHN1*, *PHOX2A*, and *ECEL1* and of *KIF21A* mutation hotspot exons 8, 20, and 21 was uninformative. WES of the trio was performed at the Ocular Genomics Institute (OGI, Massachusetts Eye and Ear Infirmary, Boston, MA, USA). DNA libraries were prepared using the Agilent SureSelect Human v4 kit and sequenced on an Illumina HiSeq 2000. 98.6% of captured regions had ≥10X coverage. Exome findings were inconclusive, so subsequent whole genome sequencing (WGS) of the trio was performed at the Baylor College of Medicine Human Genome Sequencing Center (Houston, TX, USA) as part of the NIH Gabriella Miller Kids First Research Program. DNA libraries were prepared using KAPA Hyper PCR-free library prep kit (KAPA Biosystems Inc., Wilmington, MA). Sequences were obtained on an Illumina HiSeq X to a 30X mean coverage using 150 bp paired-end reads. Raw data were transferred to the Broad Institute of MIT and Harvard for realignment to the human reference genome (GRCh38; https://www.ncbi.nlm.nih.gov/grc) (Schneider et al., 2017) using BWA-MEM (http://bio-bwa.sourceforge.net/bwa.shtml), (Li and Durbin, 2010), and reprocessed using Picard (https://broadinstitute.github.io/picard/). Joint variant calling was conducted with the Genome Analysis Toolkit (GATK 4.0 Haplotype Caller) alongside >20,000 reference genomes assembled by the Broad Institute (McKenna et al., 2010; Depristo et al., 2011). Variant filtering was conducted using the GATK Variant Quality Score Recalibrator. Variants were annotated using a custom version of the Ensembl Variant Effect Predictor and uploaded into seqr, a web-based variant analysis tool (https://seqr.broadinstitute.org/) (McLaren et al., 2016). A detailed description of the variants analysis is provided as supplementary material. For the missense changes, several standard in silico tools (Polyphen: http://genetics.bwh.harvard.edu/pph2 ; Mutation taster: http://www.mutationtaster.org/);

GERP: https://cadd.gs.washington.edu/snv; and CADD: https://cadd.gs.washington.edu/snv were used to assess deleteriousness (Davydov et al., 2010; Kircher et al., 2014).

In individuals A1, A2 and C1, copy-number variants (CNVs) were analyzed from whole-exome sequencing following GATK-gCNV best practices, as follows: read coverage was first calculated for each exome using GATK CollectReadCounts. After coverage collection, all samples were subdivided into batches for gCNV model training and execution; these batches were determined based on a principal components analysis (PCA) of sequencing read counts. After batching, one gCNV model was trained per batch using GATK GermlineCNVCaller on a subset of training samples, and the trained model was then applied to call CNVs for each sample per batch. Finally, all raw CNVs were aggregated across all batches and post-processed using quality- and frequency-based filtering to produce a final CNV callset. In individual B1, CNVs were analyzed via clinical chromosomal microarray.

2.3. Assessment of the impact of variants on splicing

Total RNA was extracted from frozen muscle tissue of individual A1 and a 2-year-old control individual using Qiagen RNeasy Kit and following the manufacturer's protocol. One microgram of RNA was reverse transcribed using High-Capacity RNA-to-cDNA Kit (ThermoFisher) and the synthesized cDNA was used in a PCR reaction amplifying region covering exon 23 to exon 30 of COL25A1 transcript (forward primer AGGAGCCACTGAGATCATAGA and reverse primer TAACCCTGGTTCACCCTTTG). PCR was performed with HotStart TaqDNA polymerase (Qiagen) at initial heating at 95ºC for 15 minutes followed by 32 cycles at 95°C for 40 seconds, 57°C for 40 seconds and 72°C for 3 minutes, and a last extension at 72ºC for 10 minutes. The PCR products were visualized by agarose gel electrophoresis, and were purified for Sanger sequencing.

3. Genetic Results

WES/WGS identified a homozygous *COL25A1* variant (NM_198721.4) c.1450A>G p.(Lys484Glu) in individual A1, a homozygous *COL25A1* variant c.1198G>A p.(Gly400Arg) in individual B1 and compound heterozygous *COL25A1* variants c.672+1del and c.672+1G>A in individual C1 (Figure 5). All variants are absent from population databases, with the exception of the c.672+1G>A variant, which is reported in heterozygosity in 2 individuals of African/South Asian descent in gnomAD v2.1.1 (Karczewski et al., 2020). All variants affected highly conserved residues of the gene and were predicted to be pathogenic by *in silico* predictors: variant c.1450A>G p.(Lys484Glu) has a CADD score of 26 and a GERP score of 5.24; variant c.1198G>A p.(Gly400Arg) has a CADD score of 31 and a GERP score of 5.89; while variants c.672+1del and c.672+1G>A, located at a canonical splice-donor site, are predicted to affect splicing according to Splice AI v1.3.1 (https://github.com/Illumina/SpliceAI) (Jaganathan et al., 2019). The c.1198G>A p.(Gly400Arg) variant affects the first nucleotide of exon 23 and *in silico* analyses provided conflicting predictions on its effect on splicing. In particular, Splice AI v1.3.1 predicts the variant not to affect splicing, whereas TraP v3 (http://trap-score.org) predicts it as probably damaging (date of access January 5, 2021). Finally, RESCUE-ESE and ESEfinder v3.0 predict that the variant to create a putative SF2/ASF exonic splicing enhancer site, with scores of 1.0 and 2.49, respectively (date of access January 5, 2021). No aberrant splicing events were detected in individual A1 (data not shown), demonstrating that variant c.1450A>G p.(Lys484Glu) does not lead to COL25A1 splicing abnormalities.

No additional variants of interest (defined as variants classified as pathogenic in ClinVar) in relevant disease-causing genes were found in any analysed individual from the three families. Likewise, no other homozygous variants were identified in additional genes in the two consanguineous families (family A and family B). Germline copy number variation (CNV) analysis, performed in families A and C, identified no CNV in *COL25A1* or other

phenotype-relevant genes. No clinically significant CNVs were detected by clinical microarray in individual B1.

Segregation analysis was performed by Sanger sequencing in the three families. The analysis identified the c.1450A>G p.(Lys484Glu) variant in homozygosity in A2, the affected brother of A1. Variants c.672+1del and c.672+1G>A were found in compound heterozygosity in C2, affected sister of individual C1 (Figure 5). Unaffected parents of the three families and one unaffected sibling of individual B1 were heterozygous carriers. Segregation analysis was not possible for a further unaffected sibling of individuals C1 and C2 as his DNA is not available.

4. Discussion

Here we describe a novel contractural phenotype in three unrelated families, characterized by variable severity of arthrogryposis, ranging from mild distal upper limb involvement to AMC, and ocular CCDD in four patients, associated with homozygous or compound heterozygous putatively deleterious variants in the *COL25A1* gene. This, to our knowledge, is the first association of *COL25A1* variants with syndromic AMC, and our finding further expands the phenotypic spectrum associated with this gene. The *COL25A1* variants are distinct from those reported previously and result in a more severe phenotype compared to that observed with the three previously reported CCDD pathogenic variants (Khan and Al-Mesfer, 2015; Shinwari et al., 2015), further expanding current genotype knowledge.

The phenotypic analysis of our cohort of individuals with *COL25A1* variants indicates the most common clinical findings are finger and knee contractures and restrictive paralytic strabismus (consistent with a congenital cranial dysinnervation disorder), present in the three

families. Eye movements were often asymmetrically affected. Individual A2, who died at 2.5 years, was not reported to have an ocular motility defect. Shoulder and elbow contractures, respiratory muscle involvement (in 3 individuals each), hip dysplasia, congenital vertical talus, vocal cord paralysis, ptosis, and bulbous tip of the nose (in 2 individuals each), were also common. Further features observed were micrognathia and scoliosis. It is remarkable that vocal cord paralysis has also been described in a relevant proportion of individuals with *CHRNG*-related AMC, but whether this is caused by a primary disorder is unknown (Carrera-García et al., 2019). From a musculoskeletal point of view, all three living individuals over 1 year old were ambulant at last examination (age range 3-17 years), with delay in independent walking in all (range 18-36 months). Our results also provide relatively long-term follow up data, with the oldest individual being ambulant for short distances at age 18 years, and without relevant cardiac complication. Of note, while her older brother died at age 29 months due to severe respiratory complications and recurrent aspirations, individual A1 shows moderate reduction of lung volumes.

Recessive pathogenic variants in *COL25A1* were previously identified in four individuals from two families with ocular CCDDs (Khan and Al-Mesfer, 2015; Shinwari et al., 2015). No joint contractures or symptoms related to fetal akinesia were present in any of these patients. Three siblings carried the homozygous variant NM_198721.3 c.1144G>A; p.(Gly382Arg), while the fourth unrelated individual was compound heterozygous for the NM_198721.3 c.1489G>T; p.(Gly497*) variant and a 12.4-kb deletion spanning exons 4–10 (chr4:109,852,901–109,976,457, UCSC GRCh37/hg19) (Khan and Al-Mesfer, 2015; Shinwari et al., 2015). Functional studies supported the notion that these variants significantly reduce the ability of collagen XXV to interact with PTP σ/δ and highlighted the importance of the C-terminal half of the COL3 domain in PTP σ/δ interaction (Munezane et al., 2019). It is puzzling how the novel variants described in our report may cause a more

complex phenotype with associated AMC, compared to those observed in previously reported CCCD patients. We may hypothesise that the novel variants lead to a greater destabilization of the COL25 triple-helix structure than those previously reported, or alternatively they lead to a more severe disruption in the interaction with PTP σ/*δ,* which is essential for the motor innervation of skeletal muscles. However, given that at least one of the previously-reported individuals with an isolated ocular phenotype had a nonsense variant and a multi-exon deletion that would disrupt the reading frame, either of these hypotheses appears unlikely (Khan and Al-Mesfer, 2015; Shinwari et al., 2015). A further hypothesis would be that the novel variants identified in our patients affect splicing efficiency resulting in a partial or total loss of function of the protein. However, studies of cDNA from muscle and fibroblasts, as well as protein expression analysis in individual A1, indicate no impairment of splicing efficiency due to the c.1450A>G p.(Lys484Glu) variant. The observed intrafamilial variability in family C, with individual C1 presenting AMC and C2 only mild distal arthrogryposis, further complicates these observations. Description of further patients and long-term monitoring will be key to better understand the the basis for this intra- and inter-familial phenotypic variability, and delineate the phenotypic spectrum and long-term evolution of this new form of contractural phenotype.

During intramuscular innervation, motor nerves enter into the target muscle and then the incoming axons elongate toward the endplate as they concurrently defasciculate and arborize to form distal branches (Hughes et al., 2016; Munezane et al., 2019). So far, several proteins have been found to be involved in the peripheral motor axon branching process, including ECEL1 (Landmesser et al., 1990; Jaworski and Tessier-Lavigne, 2012; Matsumoto et al., 2016). Interestingly, the clinical picture of individuals with *ECEL1* variants strongly resembles what seen in our individuals: AMC with a marked distal component accompanied by a CCCD in a significant proportion of individuals (Khan et al., 2014; Ullmann et al.,

2018), and less commonly, severe respiratory complications (Shaheen et al., 2014). While scoliosis, with or without associated respiratory compromise, was common in individuals with *ECEL1* variants, this was only observed in one individual in our study with *COL25A1* variants (Table 1). However, it should be noted that most of the individuals described here are still young and scoliosis may still occur in the future. Muscle biopsies from *COL25A1*-related AMC showed variable myopathic features, similar to what was reported with *ECEL1*-related AMC, with no selective fibre type involvement. The EMG was normal, in spite of the expected disturbance in muscle innervation. A reasonable explanation for this is that the innervation defect is not a widespread phenomenon, and some muscles might be more affected than others. A neurophysiological study of the extraocular muscles, the most affected muscle type, would be of particular interest, but was not feasible for these three families. In keeping with the lack of a postnatal progressive myopathy or neurogenic condition, muscle MRI showed no features of selective muscle involvement, with a pattern characterized by a reduction in volume of muscles, similar to that observed in *CHRNG*-related AMC due to a neuromuscular junction defect (Vogt et al., 2012; Carrera-García et al., 2019). Neurophysiological investigations did not identify abnormalities in neuromuscular transmission.

In summary, we provide clinical and genetic evidence for expansion of the phenotypic spectrum associated with *COL25A1* variants. We describe a new genetic form of contractural phenotype and associated ocular CCDDs, with absence of central nervous system involvement and relatively stable/slow progression over time, with respiratory involvement in some patients. Our observations indicate a role for COLXXV in regulating innervation of human bulbar, axial and limb muscles in addition to extraocular muscles. Future investigations are warranted to fully clarify the pathophysiologic mechanisms leading to either AMC or isolated CCDDs.

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Conflicts of interest None of the authors has any conflict of interest to disclose.

Ethics/Consent to participation This study was performed in accordance with the Declaration of Helsinki and was approved by the NRES Committee East of England – Hatfield (REC reference: 13/EE/0398) and the Institutional Review Board of Boston Children's Hospital, Boston, MA (protocol 05-03-036R). Data were collected in accordance with ethical guidelines of each of the institutions involved. Written informed consent and age-appropriate assent for study participation was obtained by qualified clinicians.

Data availability Any data not published within the article will be shared from the corresponding author, upon reasonable request. Novel data variant were submitted to LOVD (https://databases.lovd.nl/shared/genes/COL25A1).

Web resources:

CADD, https://cadd.gs.washington.edu/snv

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/

ESEfinder v3.0, http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi

Franklin, https://franklin.genoox.com/clinical-db/home

Genomic Evolutionary Rate Profiling, http://mendel.stanford.edu/SidowLab/downloads/gerp/

GnomAD, https://gnomad.broadinstitute.org

Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php

HGVS nomenclature recommendations, http://www.hgvs.org/content/guidelines

LOVD, http://www.lovd.nl/

Mutalyzer, https://mutalyzer.nl/index

Mutation taster, http://www.mutationtaster.org

OMIM, https://www.omim.org

PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/index.shtml

Provean, http://provean.jcvi.org/genome_submit_2.php?species=human

RESCUE-ESE, http://hollywood.mit.edu/burgelab/rescue-ese/

Splice AI, https://github.com/Illumina/SpliceAI

Trap v3, http://trap-score.org

UCSC Genome Browser, https://genome-euro.ucsc.edu/index.html

Varsome, https://varsome.com

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Table 1. Clinical features of individuals with *COL25A1* gene variants. F female, M male, y years, m months, N.R. not reported. N.A. not acquired, GDD global developmental delay.

	Patient A1	*Patient A2*	*Patient B1*	*Patient C1*	*Patient C2*
Sex	F	M	F	M	F
Ethnicity	Pakistani	Pakistani	Palestinian	Caucasian	Caucasian
Variants	c.1450A>G p.(Lys484Glu)	c.1450A>G p.(Lys484Glu)	c.1198G>A p.(Gly400Arg)	c.672+1del	c.672+1del
	c.1450A>G p.(Lys484Glu)	c.1450A>G p.(Lys484Glu)	c.1198G>A p.(Gly400Arg)	c.672+1G>A	c.672+1G>A
Age at last examination	16 y	22 m	11 y	3 y	3 m
Gestational age at birth (in weeks)	38	36	33	Term	Term
Prenatal findings	No	No	Oligohydramnios, contractures	No	Reduced hand movements
Hip dysplasia	No	N.R.	Yes	Yes	No
Knee contractures	Yes	Yes	Yes	Yes	No
Congenital vertical talus	Yes	No	Yes	No	No
Limitation of shoulder movements	Yes	N.R.	No	No	No
Elbow contractures	Yes	Yes	Yes	No	No
Wrist contractures	Yes	Yes	Yes	No	No
Finger contractures	Yes	Yes	Yes	Yes	Yes
adducted thumbs					
Scoliosis	Yes	No	No	No	No
Micrognathia	No	No	Yes	No	No
Age at walking	18 m	N.A	3 y	2 y	N.A.
Ptosis	Yes	N.R.	No	Yes	No
Eye movements	Globe retraction and limited abduction left eye	N.R.	Incomitant large angle exotropia, left hypertropia, bilateral limited adduction, asymmetric limited upgaze	Incomitant large angle exotropia, bilateral limited adduction,	Left exotropia and hypotro with markedly reduced lef adduction and elevation. N

				limited left elevation and blepharoptosis	ptosis noted and right eye movements were full
Vocal cord paralysis	Yes	N.R.	Yes	No	No
Respiratory involvement	Yes	Yes	Yes	No	No
Intellectual impairment	No	GDD	No	No	N.A.

Figure legends

Figure 1. Clinical images of individuals with *COL25A1* variants

(a-i) Individual A1. Note ocular findings in individual A1 at age 3 months (a) and age 9 years (d and g), with asymmetric ptosis and limitation of upwards gaze in left eye, and bulbous tip of the nose. Note finger contractures at age 3 years 11 months (b) and 9 years (c, e and f). In (h) and (i) note ankle and toe contractures, in particular of first toe, at age 3 months and 3 years 11 months, respectively. **(j-n)** Individual C1. Note marked compensatory head position in individual C1 at age 14 months, which changed depending on fixation preference (j: patient is fixing right eye; k: patient is fixing left eye) and how it was dramatically improved after two surgical procedures at age 27 and 37 months (l, n). Also, note bulbous tip of the nose (l, n). **(o,p)** Individual C2. Note clasped thumb (o).

Figure 2. Muscle MRI images of an individual with *COL25A1* variants

T1-weighted MRI sequences of lower limb muscles in patient A1 at 8 years old showing diffuse reduction in volume of muscles, more marked in thighs (a) than in calves (b), especially in the posterior compartment, possibly suggestive of disuse atrophy. Fatty infiltration in muscles is not observed.

Figure 3. Muscle pathology from individual A1 with *COL25A1* variants

Muscle biopsy (site unknown) of individual A1 at 5 years 8 months of age. (a) Haematoxylin & Eosin; (b) NADH-TR; (c) COX-SDH; (d) slow myosin; (e) fast myosin. There is mild fibre size variation (a).Oxidative stains (b, c) show mild overall type I fibre predominance, and ill-defined core-like lesions in several fibres, more often type I (b, c, arrows). Immunolabeling with slow (d) and fast (e) myosins confirms mild slow fibre predominance. Scale bar: a,d,e: 250 μm; b,c: 100 μm

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Figure 4. Brain and orbital MRI image of an individual with *COL25A1* variants

Coronal PD-weighted TSE sequence in patient C1 at 24 months old revealed marked thinning of the medial rectus muscles bilaterally (discontinuous arrows) and the left superior rectus levator complex (thick arrows).

Figure 5. Schematic COL25A1 protein structure and location of *COL25A1* gene variants

Location of here-reported *COL25A1* variants is indicated with red (homozygous) and orange (compound heterozygous) arrows, while previously reported changes are indicated with black (homozygous) and grey (compound heterozygous) arrows and horizontal grey bar.