Congenital Titinopathy

Comprehensive characterization and pathogenic insights

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Congenital Titinopathy: Comprehensive Characterization and Pathogenic Insights

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**Objective:** Comprehensive clinical characterization of congenital titinopathy to facilitate diagnosis and management of this important emerging disorder.

**Methods:** Using massively parallel sequencing we identified 30 patients from 27 families with 2 pathogenic nonsense, frameshift and/or splice site TTN mutations in trans. We then undertook a detailed analysis of the clinical, histopathological and imaging features of these patients.

**Results:** All patients had prenatal or early onset hypotonia and/or congenital contractures. None had ophthalmoplegia. Scoliosis and respiratory insufficiency typically developed early and progressed rapidly, whereas limb weakness was often slow progress, and usually did not prevent independent walking. Cardiac involvement was present in 46% of patients. Relatives of 2 patients had dilated cardiomyopathy. Creatine kinase levels were normal to moderately elevated. Increased fiber size variation, internalized nuclei and cores were common histopathological abnormalities. Cap-like regions, whorled or ring fibers, and mitochondrial accumulations were also observed. Muscle magnetic resonance imaging showed gluteal, hamstring and calf muscle involvement. Western blot analysis showed a near-normal sized titin protein in all samples. The presence of 2 mutations predicted to impact both N2BA and N2B cardiac isoforms appeared to be associated with greatest risk of cardiac involvement. One-third of patients had 1 mutation predicted to impact exons present in fetal skeletal muscle, but not included within the mature skeletal muscle isoform transcript. This strongly suggests developmental isoforms are involved in the pathogenesis of this congenital/early onset disorder.

**Interpretation:** This detailed clinical reference dataset will greatly facilitate diagnostic confirmation and management of patients, and has provided important insights into disease pathogenesis.

**T**TN (Online Mendelian Inheritance in Man database [OMIM] #188840) includes the most exons (364) and has the longest coding sequence (>100kb) of any human gene. It encodes titin, the largest protein in nature (maximum size = 4,200kDa).¹ In striated (cardiac and skeletal) muscle, each titin molecule pairs with a second antiparallel titin molecule to span the full length of the sarcomere like two “springs in series,”² forming a continuous elastic myofilament. These myofilaments provide a scaffold for sarcomere assembly during muscle...
Developmental skeletal and cardiac muscle isoforms have been reported. Previous cases were described as early onset muscular dystrophy with fatal cardiomyopathy. To date, 16 patients from 12 families with a recessive prenatal or infant onset form of titinopathy have been reported. Previous cases were described as early onset muscular dystrophy with fatal cardiomyopathy.

Autosomal dominant TTN mutations cause 2 adult onset skeletal muscle disorders: (1) tibial muscular dystrophy (TMD; OMIM #600334) and (2) hereditary muscular dystrophy with early respiratory failure (HMERF; OMIM #603689). All reported TMD mutations are within the final 6 (M-band) exons. HMERF is caused by missense mutations within exon 344. Heterozygous truncating TTN mutations are the most common genetic cause of dilated cardiomyopathy (DCM; OMIM #608807). Various homozygous and compound heterozygous TTN mutations have also been reported in patients with childhood, adolescent, and adult onset recessive distal titinopathy and childhood-juvenile onset Emery-Dreifuss-like titinopathy.

To date, 16 patients from 12 families with a recessive prenatal or infant onset form of titinopathy have been reported. Previous cases were described as early onset muscular dystrophy with fatal cardiomyopathy.
myopathy,\textsuperscript{22} core myopathy with heart disease,\textsuperscript{23} and arthrogryposis multiplex congenita with myopathy.\textsuperscript{24} We suggest the term “congenital titinopathy” be adopted for this disorder.\textsuperscript{25} Previously reported patients had a range of different causative mutations, including more difficult-to-interpret missense changes. Clinical features included neck, axial, and limb weakness, joint contractures, spinal and chest wall deformities, early onset respiratory insufficiency, mild facial weakness and ptosis. Congenital cardiac anomalies and/or childhood or adolescent onset DCM were common (10/16 patients). Creatine kinase (CK) levels were normal to moderately elevated. Muscle biopsies showed increased internalized and central nuclei, minicores and/or dystrophic lesions.

To facilitate diagnostic assessments and to better understand the natural history of congenital titinopathy, we analyzed the clinical, muscle pathology and imaging findings in a large international cohort with recessively inherited nonsense, frameshift and/or splice site \textit{TTN} mutations; that is, the most pathogenically-convincing subset of mutations. Patients with difficult-to-interpret missense variants were excluded, to gain the clearest possible clinical picture of this disorder.

**Subjects and Methods**

**Ethical Approval and Consent**

The project was approved by the Human Research Ethics Committee of the Sydney Children’s Hospitals Network and by other researchers’ relevant institutional review boards. Informed consent for research participation and use of clinical photographs, including 3 non-obscured facial photographs, was obtained from patients/parents/legal guardians.

**Recruitment**

Patients were ascertained from neurology clinics. The study included published and unpublished clinical data from Families 13 to 16, reported previously as 314-1, 979-1, 1044-1, and
1093-1 respectively. The Family 10 proband has also been described.

**Mutation Detection, Confirmation, and Annotation**

The type of massively parallel sequencing (MPS) technology used to identify each causative mutation (panel, whole exome sequencing, whole genome sequencing) is shown in Supplementary Table 1. Bioinformatics analysis pipelines focused on rare compound heterozygous or homozygous variants within known neuromuscular disease genes that were likely to be pathogenic. Variants in genes associated with cardiac abnormalities in the absence of neuromuscular pathology were not systematically analyzed in most cases.

Each mutation was Sanger-confirmed and reported according to Human Genome Variation Society recommendations (http://varnomen.hgvs.org/) using the inferred complete TTN metatranscript as reference (NM_001267550.1: LRG391_t1). Exons were numbered 1 to 364 according to the LRG schema. Family members were Sanger sequenced to confirm segregation and carrier status.

The Leiden Muscular Dystrophy (http://www.dmd.nl/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and Cardiodb mutation databases (http://cardiodb.org/titin/) were interrogated to identify previously reported mutations. The Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org/) was used to determine the frequency of each mutation in the general population. Alamut® Visual (Interactive Biosoftware, North Seattle, WA) was used to predict the impact of splice site mutations.

**Clinical Features Analysis**

The clinical analysis cohort included patients with 2 Sanger-confirmed nonsense, frameshift and/or splice site mutations shown to be in trans by segregation studies. The single exception was the Family 7 proband, who had a homozygous mutation and history of parental consanguinity; however, parental DNA was unavailable. The data from clinical analysis cohort members (henceforth referred to as patients) were collected from primary medical records and managing clinicians, tabulated, and the percentage of patients with each feature calculated. The denominator for each calculation (shown in Table 1 and Supplementary Table 2) was the number of patients for whom data were available for that feature, rather than total cases. As patients “unknown” for any feature are more likely to have been negative than positive for that feature, this approach may have resulted in a small overestimation of prevalence.

Four additional segregation-inconclusive families (Families 28–31) were studied, but not included in the clinical analysis cohort.

**Cardiac Isoform Analysis**

The Cardiodb database (http://cardiodb.org/titin) was interrogated to determine which mutations were predicted to alter the 2 most abundant adult cardiac isoforms, N2BA and N2B. Families were stratified according to the cardiac status of affected members (“Yes” = cardiac involvement in at least 1 member), and whether each had (1) 2 mutations predicted to alter both N2BA and N2B or (2) other combinations of mutations. Significance was determined using Fisher’s Exact Test.

**Western Blot Analysis**

Western blot analysis of 5 patient muscle samples (Families 4, 5, 13, 15 and 16) and 2 segregation-inconclusive cases (Families 28 and 29) was undertaken. Biopsy protein extracts were electrophoresed on 0.8% agarose gels and transferred to polyvinylidene difluoride membranes using a semidy transfer system (Bio-Rad Laboratories, Hercules, CA). Affinity-purified rabbit polyclonal antibodies specific for the titin N-terminus (α-Z1Z2; Myomedix, Neckargemünd, Germany) and C-terminus (α-M8M9; Myomedix) were used (1:2,500 dilution). Fluorescent secondary antibodies with infrared excitation (LI-COR Biosciences, Lincoln, NE) were used (1:20,000 dilution). Blots were scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

**Splicing, cDNA and RNAseq Analysis**

Direct analysis of the impact of each mutation was beyond the scope of this study; however, an in vitro hybrid minigene splicing assay was previously used to evaluate the transcriptional consequences of 2 splice site changes.

The impact of an extended splice site deletion (Family 6: exon 317: c.66769+4_66769+7del) was evaluated using cDNA analysis. cDNA was synthesized from 1 μg of total skeletal muscle RNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) for reverse transcription polymerase chain reaction (PCR), according to the manufacturer’s protocol (90 minute cDNA synthesis). RNA was primed with oligo(dt) or random primers. One microliter of the resulting cDNA was used in PCR reactions. The primers were: forward 5’-ACCCGCTCTTGAAGATGGA-3’ (NG_011618.3 exon 315) and reverse 5’-TGGAGCCTCAGTTGTCAC-3’ (exon 319).

**Results**

**Demographic Information**

The study cohort consisted of 30 patients from 27 families. The demographic features are described in Supplementary Table 2. Four patients were deceased at ascertainment, and 1 died during the study. Cause of death was (1) early respiratory failure (2 patients: Family 6 proband on day 1 (38 weeks gestation) and Family 18 proband at 3 months of age); (2) sudden death at age 8 years in the context of mild–moderate DCM, with arrhythmia suspected (Family 7 proband); (3) pneumonia at age 13 years (Family 10 proband); and (4) bowel cancer during early 30s (Sibling 2/Family 1). Of the 25 surviving patients, age at ascertainment ranged from 9 months to 34 years.

As only 5 patients were ≥18 years old (see Supplementary Table 2), it was not possible to comprehensively characterize the features or natural history of this disorder.
TABLE 1. Summary of common and clinically significant features

More detailed information regarding overall findings is provided in Supplementary Table 2.

| Onset                                      | 17/29 | 59% | Face                                      | 26/26 | 100% |
|--------------------------------------------|-------|-----|-------------------------------------------|-------|------|
| In utero (reduced fetal movements)         | 9/30  | 30% | Absence of ophthalmoplegia                | 19/27 | 70%  |
| Congenital                                 | 4/30  | 13% | Facial weakness (typically mild to moderate)| 19/27 | 73%  |
| Infancy or very early childhood (3,3,8 & 14m)| 2/27  | 7%  | High arched palate                        | 7/21  | 33%  |
| Pregnancy & delivery                       | 4/23  | 17% | Ptosis                                    | 17%   | -    |
| Abnormal liquor volume                     | 2/27  | 7%  | One or more additional dysmorphic facial fx|       |      |
| Limb contractures noted on prenatal ultrasound| 6/27  | 22% | Vocal cord / bulbar involvement           | 8/21  | 38%  |
| Other prenatal ultrasound anomalies        | 3/28  | 11% | Neck                                      | 7/27  | 26%  |
| Preterm delivery (34/40, 35+6/40, 36+6/40)  | 7/27  | 26% | Current requirement for tube or PEG feeding?| 4%    | -    |
| Breech or other abnormal presentation      |       |     | Voice / vocal cord abnormalities          |       |      |
| Neonatal features (excluding cardiac)      |       |     | Axial features                            |       |      |
| Congenital hypotonia / weakness            | 19/27 | 70% | Neck flexion weakness                     | 20/21 | 95%  |
| One or more congenital limb contractures   | 17/28 | 61% | Neck extension weakness                   | 15/18 | 83%  |
| Arthrogryposis multiplex congenita          | 12/28 | 43% | Limited range of neck movement (rigidity) | 3/18  | 17%  |
| Congenital scoliosis                       | 2/26  | 8%  | Kyphosis                                  | 3/24  | 13%  |
| Congenital fractures                        | 2/27  | 7%  | Scoliosis (typically early-onset & progressive)| 16/28 | 57%  |
| Cleft palate                               | 3/23  | 13% | Spinal rigidity                           | 6/22  | 27%  |
| Neonatal respiratory difficulties          | 12/29 | 41% | Lumbar hyperlordosis                      | 6/22  | 27%  |
| Neonatal feeding difficulties              | 17/27 | 63% | Chest wall deformity                      | 10/23 | 43%  |
| One or more additional congenital abnormalities | 13/23 | 57% | Scapular winging                          | 9/20  | 45%  |
| Early motor development and ambulation     |       |     | Respiratory involvement                   |       |      |
| Delayed sitting (> 9m of age)              | 11/20 | 55% | Respiratory insufficiency/18, 19          | 17/27 | 63%  |
| Delayed walking (> 18m of age)             | 9/19  | 47% | Nocturnal or full time ventilation requirement| 11/25 | 44%  |
| Currently or previously able to walk independently | 19/27 | 70% | Frequent / severe respiratory infections | 5/25  | 20%  |
| Young & gaining motor skills: may still walk| 3/27  | 11% | Paradoxical breathing pattern/20          | 2/12  | 17%  |
| Not walking and unlikely to ever walk      | 5/27  | 19% | Cardiac involvement                       |       |      |
| Lost ability to walk once achieved         | 1/19  | 5%  | Congenital cardiac abnormalities          | 13/28 | 46%  |
| Stable/slow loss of limb strength / walking ability/10 | 9/19  | 47% | Non-congenital cardiac abnormalities      | 9/29  | 31%  |
| Moderate loss of limb strength / walking ability/11 | 4/19  | 21% | Foot features                             | 5/28  | 18%  |
| Rapid loss of limb strength / walking ability/12 | 1/19  | 5%  | Pes planus                                | 5/21  | 24%  |
| Still gaining limb strength / walking ability| 4/19  | 21% | Pes cavus                                 | 1/22  | 5%   |
| Ever able to run?                          | 8/19  | 42% | Other foot abnormalities                  | 9/21  | 43%  |
| Ever able to jump?                         | 4/19  | 21% | Bone features                             |       |      |
| Muscle bulk                                |       |     | Generalised muscle hypotrophy / wasting  | 15/20 | 75%  |
| Calf hypertrophy / pseudohypertrophy       | 1/21  | 5%  | Osteopaenia                               | 4/13   | 31%  |
| Pattern & severity of limb weakness        |       |     | Pathological fracture(s)                  | 2/18  | 11%  |
| Symmetrical                                | 15/22 | 68% | Additional features/findings              |       |      |
| Mild to moderate weakness (MRC 3-4/5/13)   | 21/26 | 81% | Joint hypermobility/ligamentous laxity    | 16/22 | 73%  |
| Severe weakness (MRC 2/5 or less)          | 5/26  | 19% | Height and/or weight all/below 3rd percentile | 5/19  | 26%  |
| Upper & lower limb weakness approx. equal   |       |     | Fatigability                               | 11/16 | 69%  |
| Upper limb predominant                     |       |     | Intellectual disability                   | 2/27  | 7%   |
| Lower limb predominant                     |       |     | One or more disease-related surgeries/23  | 10/26 | 38%  |
| Proximal & distal weakness approximately equal |       |     | Creatine kinase (CK) level: normal        | 23/26 | 88%  |
| Distant predominant (P > D)                |       |     | CK level: elevated                        | 3/26  | 12%  |
| Distal predominant (D > P)                 |       |     | Electromyogram: normal                    | 10/20 | 50%  |
| Proximal predominant (P > D)               |       |     | Electromyogram: myopathic                 | 8/20  | 40%  |
| Acquired limb contractures                 |       |     | Electromyogram: neuropathic/24            | 1/20  | 5%   |
| One or more acquired limb contractures     | 16/24 | 67% | Electromyogram: equivocally abnormal: NOS | 1/20  | 5%   |
| Two or more acquired limb contractures     | 10/22 | 45% | Acquired limb contractures                |       |      |
| Deep tendon reflexes                       |       |     | Normal/14                                 | 1/21  | 5%   |
| Reduced / trace                            |       |     | MRI brain: mild structural abnormalities  | 3/12  | -    |
| Absent                                     | 9/21  | 43% | MRI brain: hypoxia / birth trauma-related abn. | 2/12  | -    |
| MRI lower limb: distinct pattern of muscle involvement/25 | 11/21 | 52% | MRI lower limb: distinct pattern of muscle involvement/25 | 3/3   | -    |
during adulthood. Younger patients were well represented.

Mutations

The 45 different mutations identified in clinical analysis cohort members (from Families 1-27) are shown in Figure 1 and detailed in Supplementary Table 1. Thirty-three mutations were novel. Fifteen mutations were nonsense (Family 19 proband). Many cohort members had not been screened for osteopenia at the time of ascertainment.

Four mutations from 8 unrelated families (Families 20–27) were within the inferred complete TTN metatranscript (NM_001267550.1) but not within exons that encode the N2A mature skeletal muscle isoform (NM_133378.4) or the multiple mature cardiac isoforms. In support of the pathogenicity of these "metatranscript-only" mutations, the exon 163 nonsense mutation (p.Glu11932?) was a recurrent mutation present in 5 unrelated cohort families (Families 20, 21, 23, 24, and 25) and 1 segregation-inconclusive family (Family 31).

Two additional mutations were also recurrent mutations present in 2 unrelated cohort families (p.Val10952-Leu exonic splice mutation present in Families 4 and 13, and c.15496+1G>A present in Families 1 and 14; see Supplementary Table 1).

Family History

Five probands had 1 affected sibling. Clinical data from 3 of these siblings (from Families 1, 20, and 26) were available for inclusion in the analysis (see Supplementary Table 1). There was no history of weakness or other
neuromuscular abnormalities among the first-degree relatives of cohort patients.

Only 19 of the carrier-confirmed parents from 11 of 27 clinical analysis cohort families and an even smaller subset of extended family members had undergone cardiac screening (shown in Supplementary Table 1). Of specific note is that two families (Families 9 and 13) had first- and/or second-degree relatives with DCM. All 3 Family 13 maternal members with DCM were heterozygous carriers. The maternally inherited mutation from this family was novel. In a third family (Family 12), a sibling pregnancy had been terminated due to congenital heart disease (fetal mutation status unknown). There was also a strong family history of cardiomyopathy (both parents and a paternal uncle) in 1 segregation-inconclusive family (Family 29).

The 77-year-old Family 1 father and 2 older paternal mutation-carrier relatives from Family 13 had additional cardiac abnormalities of unknown significance (atrial fibrillation in all 3 and mitral valve changes in 2 of 3; details in Supplementary Table 1).

Five parents (from Families 3, 4, 5, 13, and 25) carried a mutation previously reported in the DCM literature (DCM-mutation) but had normal cardiac findings (2 parents) or had not yet had cardiac screening (3 parents; additional details in Supplementary Table 1). The Family 5 maternal carrier of a previously reported congenital titinopathy-cardiac-mutation (p.Gln35278*) had no cardiac involvement on recent screening.

**Clinical Features**

Common and significant clinical features, and the prevalence of each feature, are summarized in Table 1, with more detailed clinical information provided in Supplementary Table 2. Selected features are illustrated in Figure 2.

**PREGNANCY/CONGENITAL FEATURES.** Almost 60% of patients had reduced fetal movements. Features of the disorder developed in utero or were present at birth in all but four cases (exceptions: symptoms/signs first noted at 3 months (two patients), 8 and 14 months). One patient (Family 19 proband) had congenital finger contractures and unilateral talipes, but no apparent weakness at birth and remains strong (current age = 21 years).

Congenital limb contractures were common (61%), and often distal (involved joint[s] are shown in Supplementary Table 2). Twelve patients (43%) had congenital contractures involving ≥ 2 areas consistent with the clinical diagnosis of arthrogryposis multiplex congenita (AMC). Congenital scoliosis and fractures were each present in 2 patients (congenital scoliosis was present in both Family 1 siblings; congenital fractures were present in Family 6 proband and Sibling 1/Family 26).

Neonatal feeding and respiratory difficulties were common (63% and 41%). Most patients required only a brief period of respiratory support after birth. Three patients (Family 12, 17, and 18 probands) had required nocturnal or all-day ventilation from the neonatal period onward.

**LIMB WEAKNESS, CONTRACTURES, MUSCLE BULK AND MOTOR DEVELOPMENT.** In most cases, limb weakness was predominantly proximal and symmetrical, and affected both upper and lower limbs. Severity was typically mild to moderate (Medical Research Council [MRC] 3–4/5), but sometimes severe (MRC 2/5 or less). Generalized muscle wasting was common (75%). Calf hypertrophy was rare (Family 13 proband only). Acquired limb contractures were frequent (67%), affected both proximal and distal joints, and were often multiple (see Supplementary Table 2).

A total of 19 of 30 patients could, or had previously been able to walk. Three (11%) were younger than 5 years and might still walk. Nine patients could walk fast or run, and 4 could jump. Rate of ambulatory loss was often slow (defined as "still ambulant or predicted to be still ambulant after 20 years of age"; 9/19 patients), and 4 of 19 patients were still gaining rather than losing walking ability (see Supplementary Table 2). Only 1 patient had lost the ability to walk independently (without aids) once achieved (Family 2 proband; unable to walk from age 7 years). Another initially walked with calipers but became wheelchair dependent at age 4 years (Family 5 proband). Three were experiencing increasing walking difficulties due to progressive weakness, pain, contractures, foot deformities, and/or fatigue (Family 7, 8, and 15 probands).

**AXIAL FEATURES AND RESPIRATORY INSUFFICIENCY.**

Neck flexion weakness was present in all but 1 of the 21 patients for whom data were available. Neck extension weakness was also common (83%). Three patients had a striking “dropped head” phenotype (Sibling1/Family 1, Sibling 1/Family 26, and Family 2 proband).27

Many patients (63%) had objective evidence of respiratory impairment, and 44% required nocturnal or full-time ventilation. Scoliosis was present in 57% of patients and was typically early-onset, rapidly progressive, and had required (or was likely to require) intervention. Chest wall deformities and scapular winging were present in 43% and 45% of patients respectively. Scoliosis, chest wall deformities, and respiratory insufficiency were often concurrent. Spinal rigidity, kyphosis, and lumbar
hyperlordosis were sometimes present (6, 3, and 6 cases respectively).

**CARDIAC ABNORMALITIES.** Thirteen patients (46%) had congenital and/or early onset cardiac pathology (see Supplementary Tables 1 and 2). Nine had congenital cardiac abnormalities. Five had early onset cardiac complications including dilated cardiomyopathy (onset at 18 months and 9 years), left ventricular dysfunction (onset at 3 years and “during childhood”), or a dilated right ventricle (autopsy finding at age 13 years). Some cardiac abnormalities progressed rapidly and/or were associated with life-threatening arrhythmias.

Two patients with structural cardiac abnormalities (Family 4 and 25 probands) had 1 reported DCM-mutation in combination with 1 novel mutation (see Supplementary Table 1). The patient with the most severe congenital anomaly (Family 5 proband: aortic coarctation) was the only patient to have 2 previously reported cardiac mutations (a DCM-mutation and a congenital titinopathy-cardiac-mutation). The remaining 10 patients with cardiac involvement had no previously reported cardiac mutations.

**FACIAL FEATURES, FEEDING ABNORMALITIES AND OTHER NOTABLE FINDINGS.** No patient had ophthalmoplegia (based on data for 26/29 patients who survived beyond day 1 of life). High-arched palate and mild to moderate facial weakness were common (73% and 70%). Ptosis was present in 33%, and was occasionally congenital, asymmetrical or fluctuating. A submucous cleft palate was present in 2 patients (Family 19 and 22 probands). A third patient had Pierre Robin sequence (Family 5 proband). Growth abnormalities, torticollis, facial asymmetry, and a range of dysmorphic features were noted in more than one patient (see Supplementary Table 2).

Several patients were reported to have a Noonan-like facial appearance, sometimes in combination with other Noonan-associated features (low posterior hairline, neck webbing, short stature); however, ascertainment of these features was incomplete.

Sucking, chewing, and/or swallowing difficulties affected 38% of patients, and 26% required supplemental tube or gastrostomy feeding.

Joint hypermobility was present in 73% of patients. Only 2 siblings had a history of learning difficulties (mild: Family 26). Four patients had documented osteopenia, 2 with pathological fractures. None had experienced malignant hyperthermia during exposure to anesthetic agents.

**CK Levels and Electrophysiology Results**
CK levels were typically normal but occasionally elevated (400–2,324 IU/L). Nerve conduction velocities were normal. Electromyography was usually normal (50%) or myopathic (40%).

**Magnetic Resonance Imaging**
Three of the 12 patients with magnetic resonance imaging (MRI) brain results had minor structural abnormalities of unknown significance (see Supplementary Table 2). Two severely affected infants had birth hypoxia or birth trauma-related features, but normal cerebral morphology.

Descriptions of pelvic and lower limb MRI findings were available for 3 patients (at ages 3, 9, and 15 years), with original images for 1 of the 3 (Family 26, 15 years; see Fig 2L–N). All 3 cases showed paraspinale, gluteal, and global hamstring atrophy. The adductors, sartorius, and gracilis were spared or hypertrophied. In the lower limb, the gastrocnemius and soleus were variably involved. Muscle MRI signs associated with clinically similar disorders, for example *SEPN1* and *COL6*-myopathies were absent.

Original images from a segregation-inconclusive case at age 29 years (Family 31; see Fig 2O–Q) showed severe involvement of the gluteal and anterior compartment muscles and all 3 components of the visible hamstring muscles, with clear adductor sparing and marked calf atrophy. This case may represent a more severe and/or more advanced case.

**Muscle Pathology**
One patient from each family had undergone at least 1 muscle biopsy (source of data, age, site, and findings are listed in Table 2), with the single exception of Family 19. Examples of biopsy features are shown in Figure 3.

All muscle biopsies were abnormal. The histopathologic changes fell into 3 main patterns: (1) increased fiber size variation (FSV; present in ≥1 biopsies from 89% of families), (2) increased internalized nuclei (IN; 59%), and (3) cores (48%), with additional structural abnormalities in some cases. Seven biopsies had FSV alone, 1 had IN alone, and 1 had only cores (multiminicores). The remaining biopsies had a combination of ≥2 main patterns with or without additional structural abnormalities (see Table 2, Fig 3M).

Where fiber type information was available, the pattern was of type 1 fiber type disproportion (small predominant type 1 fibers and fiber size disproportion of >12%; see Fig 3H). One biopsy (see Fig 3I; Family 2 proband) fulfilled histopathological criteria for congenital fiber type disproportion.
A subset of biopsies with increased IN (present in $\geq 3\%$ of fibers) had nuclei in the geographical center of the fiber (see Fig 3A,C). Seven biopsies had been given the histopathological diagnosis of centronuclear myopathy (CNM). When the IN were not centralized, and there was significantly increased fibroadipose tissue, fiber splitting, and/or additional architectural abnormalities, the biopsies had typically been reported as “dystrophic” or “severely myopathic” (see Fig 3L). Signs of regeneration and/or degeneration were minimal or absent.

Multiminicores (see Fig 3D–F) were more common than centrally placed cores (see Fig 3G). Centrally placed...
cores typically had the appearance of larger, perhaps merged minicores. Central cores extending down the majority of the longitudinal fiber axis were not seen on available electron micrograph images.

Rarer structural abnormalities seen (see Table 2) included cap-like regions (see Fig 3), K), ring, coiled, and whorled fibers, and striking central and peripheral mitochondrial accumulations. A subset of biopsies showed variable fiber type grouping.

Additional features associated with MTM1-, DNM2- and RYR1-centronuclear myopathy, such as central accumulation of oxidative stains, radial arrangement of sarcomeres, or necklace fibers, were not observed. Rimmed vacuoles (described in HMERF and TMD) were absent.

**Autopsy Findings**

The main features of the 2 autopsied deceased patients are shown in Table 3. Brain and spinal cord were normal in both patients.

**Subgroup Analysis Of Patients with Mutations that are predicted to have no Impact on the N2A Skeletal Muscle Isoform Transcript**

We compared the clinical features of the 10 patients with metatranscript-only mutations to those seen in the combined clinical analysis cohort (see Supplementary Table 3). Respiratory involvement appeared less common in this subgroup; however, this subgroup was younger at ascertainment (average age = 9 years vs 12.5 years). Cardiac involvement was also rare (only mild pulmonary stenosis in the Family 25 proband, who also had a DCM-mutation). The 2 patients with mild intellectual disability and 2 of the 3 patients with structural brain abnormalities were in this subgroup. Internalized nuclei were often present; however, no subgroup member had a centronuclear myopathy biopsy picture. The remaining clinical and histopathological features were similar.

**Cardiac Isoform Analysis**

As shown in Table 4, cohort families with 2 mutations predicted to impact both N2BA and N2B cardiac isoforms showed a trend toward an increased likelihood of cardiac involvement (\(p_{\text{Fisher}} = 0.087\), odds ratio [OR] = 6.0, 95% confidence interval [CI] = 0.78–78). This achieved statistical significance with the inclusion of 5 previously published congenital titinopathy families with 2 truncating mutations (from Carmignac et al, Chauveau et al, and Fernandez-Marmiesse et al) in the analysis (\(p = 0.009\), OR = 11, 95% CI = 1.6–130). The pattern held with the addition of the 2 published families with 1 truncating and 1 missense mutation (from Chauveau et al; \(p = 0.002\), OR = 13, 95% CI = 2.0–150).

**Western Blotting**

All 7 muscle biopsies analyzed by western blot showed a band corresponding to full-length or nearly full-length titin skeletal muscle isoform; N2A (Fig 4). A full-length/nearly full-length band was seen with the N-terminal antibody in all 7 cases, and with the C-terminal antibody...
TABLE 2. Summary of skeletal muscle histopathology and ultrastructural (EM) features

| Family | Source of data | Age at Bx, Site | Major pattern(s), structural abnormalities &/or atypical fibre-types |
|--------|----------------|-----------------|---------------------------------------------------------------------|
| 1      | Pathology report & review of LM/EM by AC&SB | S1: 14y: Q  
S2: 10y: Q&B | FSV + IN + MMC + CC + cap-like regions |
| 2      | Pathology report (translated from French by EO) & review of subset of LM images by AC | 3y: U | FSV (CFTD) |
| 3      | Pathology report & review of LM by AC | 5y: Q | IN (CNM) + MMC |
| 4      | Pathology report & review of LM/EM by AC&SB | 11m: Q | FSV + MMC + CC |
| 5      | Pathology report & review of Bx2 LM/EM by AC&SB | Bx1: 3m corrected: Q  
Bx2: 14y10m: Q | FSV + IN (Bx1: CNM pattern) + MMC + CC |
| 6      | Pathology report & review of LM/EM by AC&SB | 38/40 years Bx: Q | FSV |
| 7      | Pathology report | 1y: Q | FSV + IN |
| 8      | Pathology description (original in French) | 3y8m: U | FSV + IN + MMC |
| 9      | Pathology description (original in French) | Bx 1: 5y: Q  
Bx 2: 7y: Q | MMC |
| 10     | Pathology description (original in Danish) | 7y: U | IN (CNM) |
| 11     | Pathology report & some original images | 1y: Q | FSV |
| 12     | Pathology report | 10m: Q | FSV + IN + coiled fibres + whorled fibres |
| 13     | Pathology description | 14m: D | FSV + IN (CNM) + |
| 14     | Pathology description | 3m: Q | FSV + IN (CNM) + Cores |
| 15     | Pathology description | 4y3m: Q | FSV + IN (CNM) + Cores |
| 16     | Pathology description | 10y: leg? Q | FSV + IN (CNM) |
| 17     | Pathology report, images & additional review/opinion by RP | 2m13d: Q  
Bx1: 6y: Q  
Bx2: 6y: Q | FSV + IN + Cores + striking central & circumferential peripheral mitochondrial accumulations |
| 18     | Pathology report & some original images | 7d: Q | FSV |
| 19     | Pathology description | S1: Bx1: 1y: Q  
S1: Bx2: 3y: G | FSV + Cores + whorled fibres + ring fibres + |
| 20     | Pathology description & limited report | 1y: site Q | FSV |
| 21     | Pathology report | 7y: Q | FSV + IN + whorled fibres |
| 22     | Pathology description | Bx1: 3y: Q  
Bx2: 4y: Q | FSV + MMC |
| 23     | Pathology report & review of LM/EM by AC&SB | 2y8m: Q  
(prior G Bx showed mature adipose tissue only) | FSV |
| 24     | Pathology report | 2y6: Q | FSV + IN + ring fibres |
| 25     | Pathology description (original in French) | S1: Bx1: 2y: U  
S1: Bx2: 15y: U | FSV + IN + Cores + |
| 26     | Pathology description (original in Spanish) | Bx1: age not known: Q  
Bx2: 8y: Q | FSV |
| 27     | Pathology report & review of LM/EM by AC&SB | 13y6m: PS&TA | FSV + IN + MMC |
| 28     | Pathology report & review of Bx3 LM by AC and Bx2 & Bx3 EM by SB | Bx1: 2y3m: Q&D  
Bx2: 2y: Q  
Bx3: 3y: Q | Bx1: IN (CNM) + MMC  
Bx2: FSV + IN (CNM) + MMC + cap-like regions + ring fibres  
Bx3: FSV + IN (CNM) + MMC + CC + cap-like regions + ring fibres + rods |
| 29     | Pathology report | 5y: U | FSV + MMC |
| 30     | Pathology reports | Bx1: 1y7m: Q  
Bx2 6y6m: T | FSV + IN + Cores |

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in 6 of the 7 cases. The absence of predicted truncated proteins in samples with nonsense and frameshifting mutations may be due to nonsense-mediated decay, or protein degradation eliminating the N- and C-termini. However, western blots with antibodies raised to either titin’s I-band (9D10) or A-band (MIR) regions did not detect truncated titin proteins (data not shown). An exception is the sample from Family 13 (with 2 splice site changes, 1 of which is predicted to result in in-frame exon skipping), which contained an additional band that was Z1Z2-positive but M8M9-negative (see Fig 4, asterisk). This might represent a truncated protein derived from the mutant TTN allele that is predicted to produce a truncated ~1.5MDa protein. Several other samples contained barely detectable Z1Z2-positive M8M9-negative bands (see Fig 4, + symbols) that are not the size of predicted truncation proteins. These might represent mutant titin degradation and/or alternatively spliced products. The reduced band intensity may be due to lower levels of protein production and/or the loss of smaller degradation/splicing products that were not retained on the gels.

**Splice Site Mutation Analysis**

A minigene splicing assay showed that the exon 220 c.40558G>C exonic splice site mutation shared by Families 4 and 13 results in in-frame exon skipping, and that the Family 13 exon 244 c.44816-1G>A mutation results in frameshift.22

cDNA analysis showed that the Family 6 paternally inherited extended splice site deletion resulted in in-frame skipping of exon 317. No other sample from 70 patients with neuromuscular disease or 184 GTEx (http://gtexportal.org/home/) control adult skeletal muscle samples showed skipping of this exon.

**Discussion**

Since MPS “opened the door” to the comprehensive genetic analysis of TTN, congenital titinopathy has emerged as an important cause of early onset myopathy. Using MPS, we identified an international cohort of 30 individuals with early onset muscle weakness and/or contractures, and recessively inherited nonsense, frameshift, and/or splice site TTN mutations. The clinical features, muscle histopathology, autopsy, and imaging results of cohort members were analyzed to identify features with clinical and/or diagnostic utility.

One of the most striking findings was the degree of axial involvement, which was present in all patients, manifesting as one or more of the following features: neck weakness, early onset progressive scoliosis, chest wall deformities and early onset respiratory insufficiency. Axial complications, including scoliosis and respiratory impairment, typically progressed rapidly, whereas limb weakness was often stable or only slowly progressive, and insufficient to prevent acquisition of independent walking in the majority of patients. This truncal-predominant phenotype is reminiscent of SEPN1- and severe nemaline myopathy, and COL6-, LAMA2- and LMNA-congenital muscular dystrophy.32,33 Congenital contractures were also common, affected distal as well as proximal joints, and often
involved two or more areas of the body, leading to the diagnosis of AMC. Acquired limb contractures were common, as was joint hypermobility, features shared with COL6-myopathy.\textsuperscript{33}

Respiratory complications were the most common cause of death. Progressive respiratory insufficiency typically developed within the first 12 years of life, often necessitating assisted ventilation. With 1 exception, respiratory insufficiency occurred in patients with concomitant spinal and/or chest wall deformities. Many patients with significant respiratory impairment remained ambulant, a
feature also shared with early onset SEPN1-, NEB-, TPM3-ACTA1-myopathy and COL6-muscular dystrophy.

Almost 50% of patients had congenital or early onset cardiac abnormalities. Cardiomyopathy is sometimes associated with muscle disorders caused by MYH7, LMNA, FKRP, FKTN, SPEG, ACTA1 and TAZ mutations; however, it is not typically associated with other forms of early onset muscle disease. Congenital cardiac defects and a history of dilated cardiomyopathy in heterozygous relatives are not typical features of other congenital myopathies. Congenital titinopathy should be considered in the differential diagnosis of patients with any of these cardiac/family history features.

It has recently been shown that heterozygous TTN truncating and essential splice site mutations that alter both N2BA and N2B, the 2 longest and most abundant adult cardiac isoforms, increase the risk of adult onset DCM. Truncating mutations that alter fetal and/or the smaller novex cardiac isoforms, or that impact only the N2BA isoform (N2BA-only mutations) are not associated with DCM in the heterozygous state. This is presumably because the predominant adult cardiac isoform (N2B) can still be transcribed from the truncating allele. Missense variation in TTN is common, and although some individual variants cause cardiomyopathy, it is not currently possible to interpret the DCM risk associated with the vast majority of these variants.

Our data suggest that congenital titinopathy patients with 2 mutations predicted to impact both N2BA and N2B cardiac isoforms (N2BA/N2B mutations) are significantly more likely to have cardiac involvement than those with other combinations of mutations. With the available cohort and previously published cases, there was insufficient power to determine whether patients carrying 1 N2BA/N2B mutation are at higher cardiac risk than those with biallelic N2B-only mutations (ie, 2 mutations that alter N2BA but spare N2B), as would be expected by extrapolating from the TTN DCM literature. There were also insufficient data to confirm whether the pattern of familial risk is highest for relatives with N2BA/N2B mutations, although both families with a maternal family history of DCM had a maternal N2BA/N2B mutation.

If it is confirmed that having biallelic N2BA/N2B mutations confers the highest risk of cardiac involvement in congenital titinopathy, this might explain why the cohort members with 1 metatranscript-only mutation (all of which spare N2BA and N2B) had fewer cardiac abnormalities than other patients (only 1/10 had a cardiac anomaly: mild pulmonary stenosis).

Until the cardiac risk factors associated with this disorder are better understood, and the association between risk and cardiac isoform involvement is confirmed, cardiac screening is strongly recommended for all congenital titinopathy patients, and should be considered in heterozygous carrier relatives, particularly those carrying truncating or splice-altering variants in cardiac constitutive exons.

In combination, the muscle biopsy results of this study confirm that congenital titinopathy is a pathological "chameleon," presenting with a wide range of structural abnormalities: cap-like regions, ring, coiled, and whorled fibers, and central and peripheral mitochondrial accumulations. Black circles indicate patients with two N2A mutations. White circles indicate patients with 1 N2A mutation and 1 metatranscript-only mutation.
structural abnormalities. Central/internalized nuclei and cores have been reported in other congenital titinopathy cases\textsuperscript{21–23}; however, a typical congenital fiber type disproportion muscle picture, cap-like regions, rods, ring, whorled, and coiled fibers, and central and circumferential peripheral mitochondrial accumulations have not been reported previously. Overall, fiber size variation, cores, and internalized nuclei, either alone, or in combination, were the most common histopathological abnormalities.

| TABLE 3. Summary of autopsy findings |
|--------------------------------------|
| **Family** | **Cause of Death and Data Source** | **Autopsy Findings** |
| 6 | Died on day 1 of life (38/40\textsuperscript{a}) due to worsening respiratory distress and poor prognosis. Complete autopsy report and review of LM/EM by ACh & SB. | External: Small for gestational age (weight < 3rd percentile), small placenta, normal fetal/placental ratio, myopathic and dysmorphic facial features, multiple bilateral upper limb contractures (shoulders, elbows, wrists, fingers), reduced palmar creases, bilateral talipes equinovarus, congenital femoral and humeral fractures, thin ribs, undescended testes. Internal (macroscopic and microscopic): Normal brain, spinal cord and heart; pulmonary hypoplasia (lung-body weight ratio = 0.6%; < 1.2\% indicates hypoplasia). |
| 10 | Died at age 13 yr from pneumonia. Autopsy description (original in Danish). | External: Height and weight < 3rd percentile, retrognathia, muscle wasting, limb contractures (elbows, knees), evidence of previous scoliosis surgery, asymmetrical rib positioning, PEG tube in situ. Internal (macroscopic and microscopic): No brain or spinal cord abnormalities, nerve normal; pneumonia, left lower lobe compressed by scoliosis, dilated hypertrophic right ventricle. |

\textsuperscript{a}38 weeks gestation.
ACh = Amanda Charlton (coauthor); EM = electron microscopy; LM = light microscopy; PEG = percutaneous endoscopic gastrostomy tube; SB = Susan Brammah (coauthor).

| TABLE 4. Cardiac Isoform Analysis |
|---------------------------------|
| Isoform | Cardiac Involvement | Fisher’s Exact Test |
|        | Yes | No | p | OR (95% CI) |
|-----------------|-----|----|---|------------|
| Clinical analysis cohort members only |     |    |   |            |
| 2 N2BA/N2B\textsuperscript{a} | 6   | 2  | 0.087 | 6.01 (0.78–78.21) |
| Other\textsuperscript{b} | 6   | 13 |   |            |
| Cohort + published families with 2 truncating muts\textsuperscript{21,23,24} |     |    |   |            |
| 2 N2BA/N2B\textsuperscript{a} | 10  | 2  | 0.009 | 10.66 (1.59–129.40) |
| Other\textsuperscript{b} | 6   | 14 |   |            |
| Cohort + published families with 2 truncating muts\textsuperscript{21,23,24} or 1 truncating & 1 missense mut\textsuperscript{23} |     |    |   |            |
| 2 N2BA/N2B\textsuperscript{a} | 12  | 2  | 0.002 | 12.75 (1.97–152.30) |
| Other\textsuperscript{b} | 6   | 14 |   |            |

Table shows the 2 × 2 Fisher’s Exact Test tables used to analyze the association between carriage of 2 mutations (in trans) that impact both N2BA and N2B cardiac isoforms, and the presence of cardiac pathology in one or more affected family members.
\textsuperscript{a}Patients with 2 mutations predicted to alter both N2BA and N2B cardiac isoforms (see Supplementary Table 1).
\textsuperscript{b}Patients with other combinations of mutations.
CI = confidence interval; mut = mutation; OR = odds ratio.
Muscle from congenital titinopathy patients sometimes showed a typical CNM pattern. The absence of ophthalmoplegia may be helpful in discriminating between congenital titinopathy and other CNM genetic subtypes.

A subset of muscle biopsies had a dystrophic appearance, sometimes in association with elevated CK, suggesting histopathological overlap with congenital muscular dystrophies. Congenital titinopathy is also an important diagnosis to consider in genetically unresolved core and cap myopathy cases.
Ten patients from 8 families had 1 mutation that spares the N2A skeletal muscle isoform: a metatranscript-only mutation. These mutations were within 1 of 4 exons (163, 172, 181, and 201) included within the I-region PEVK segment of the established complete TTN metatranscript. In further support of the pathogenicity of these mutations, 1 of these mutations (exon 163: c.35794G>T; p.Glu11932*) was shared by 5 unrelated cohort families and 1 segregation-inconclusive case. The utility of a subgroup analysis of clinical features associated with patients carrying 1 metatranscript-only mutation was limited given the small subgroup size; however, the clinical features were largely similar to other cohort members. Furthermore, Fernandez-Marmiesse et al recently described an AMC case with typical congenital titinopathy clinical and histopathological features (without cardiac involvement), and a homozygous truncating mutation within an additional metatranscript-only exon.24 Together, these findings suggest that the metatranscript-only mutations identified in our cohort patients are “true” congenital titinopathy-causing mutations.

Titin developmental isoforms have been characterized in multiple species, including humans, mice, and rabbits.9,10 They are longer than their mature muscle counterparts, due to an elongated I-band PEVK spring segment that includes exons absent from mature isoform transcripts. Human fetal skeletal muscle isoforms have not been formally characterized; however, 3 of the 4 metatranscript-only PEVK exons (163, 172, and 181) were originally detected in a human fetal skeletal muscle cDNA expression library,36 and 3 (163, 172, and 201) have been detected in mouse fetal skeletal muscle.10 Overall, these findings suggest that the pathogenesis of congenital titinopathy can result from mutations in exons that are present in one or more developmental isoform(s).

All muscle samples analyzed by western blot showed full-length or nearly full-length titin. This finding raises the possibility that the presence of a near-normal sized protein might be essential to survival. This is supported by mouse studies that show that Tim-deficient mice are embryonic lethal due to abnormal cardiac morphogenesis and dysfunction.37,38 Interestingly, all families had at least 1 mutation predicted to result in production of a near-normal sized titin. Most had at least 1 C-terminal M-line exon mutation, or at least 1 splice site mutation predicted or shown to cause in-frame loss of a single exon, which should result in a near-normal protein product. All remaining patients had 1 metatranscript-only mutation whose impact is unknown but might not affect transcript length or protein size in mature muscle. Some natural read-through of nonsense mutations might also be responsible for minor amounts of full-length protein seen on western blot. Protein analysis of muscle from additional patients will shed further light on whether the presence of a full, or near full-length protein band is a consistent finding in patients who do not succumb to the disorder during development.

Congenital titinopathy increasingly appears an important, common, and potentially severe form of axial-predominant congenital myopathy. Analysis of the clinical, histopathological, imaging, and autopsy features of this 30-member truncating/splice mutation cohort significantly expands our understanding of the clinical features and natural history of this disorder. This study will facilitate the diagnosis and management of affected individuals, inform cardiac surveillance in heterozygous relatives, and guide clinical decision making around severely affected infants. In addition, the unexpected discovery of metatranscript-only mutations in a significant subset of patients suggests that yet-to-be-characterized developmental skeletal muscle isoform(s) are involved in the pathogenesis of this disorder. Studies are currently underway to further improve cardiac risk prediction, gain a molecular-level understanding of disease-associated mutations at the RNA and protein levels, and extend our understanding of the pathological mechanisms involved in this emerging disorder.

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**Author Contributions**

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**Potential Conflicts of Interest**

Nothing to report.

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