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Review

Increasing Role of Titin Mutations in Neuromuscular Disorders

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Abstract. The *TTN* gene with 363 coding exons encodes titin, a giant muscle protein spanning from the Z-disk to the M-band within the sarcomere. Mutations in the *TTN* gene have been associated with different genetic disorders, including hypertrophic and dilated cardiomyopathy and several skeletal muscle diseases.

Before the introduction of next generation sequencing (NGS) methods, the molecular analysis of *TTN* has been laborious, expensive and not widely used, resulting in a limited number of mutations identified. Recent studies however, based on the use of NGS strategies, give evidence of an increasing number of rare and unique *TTN* variants. The interpretation of these rare variants of uncertain significance (VOUS) represents a challenge for clinicians and researchers.

The main aim of this review is to describe the wide spectrum of muscle diseases caused by *TTN* mutations so far determined, summarizing the molecular findings as well as the clinical data, and to highlight the importance of joint efforts to respond to the challenges arising from the use of NGS. An international collaboration through a clinical and research consortium and the development of a single accessible database listing variants in the *TTN* gene, identified by high throughput approaches, may be the key to a better assessment of titinopathies and to systematic genotype–phenotype correlation studies.

Keywords: *TTN*, titin, neuromuscular disorders, Limb-girdle muscular dystrophy (LGMD), Hereditary myopathy with early respiratory failure (HMERF), Late-onset autosomal dominant tibial muscular dystrophy (TMD), Congenital centronuclear myopathy (CNM), Early-onset myopathy with fatal cardiomyopathy (EOMFC), Multi-minicore disease with heart disease (MmDHD), Childhood-juvenile onset Emery-Dreifuss-like phenotype without cardiomyopathy

INTRODUCTION

With its 363 coding exons and a full-length transcript of more than 100 kb [1] *TTN* gene encodes titin, the by far longest known polypeptide in nature. The longest human theoretical isoform of TTN would produce a protein of 3,960 kDa containing 35,991 amino acids, although this isoform has not been observed [1].

Titin acts as a scaffold protein aiding in myofibrillar assembly during myogenesis [2], as a molecular spring determining the passive elasticity of the muscle [3, 4], and as a mechanosensor serving various signaling functions [5, 6].

TTN mutations have to date been reported to cause various cardiomyopathies [7, 8] and a range of skeletal muscle diseases and phenotypes listed below:

– Late-onset autosomal dominant tibial muscular dystrophy (TMD) (MIM #600334);
– Young or early adult onset recessive distal titinopathy;

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– Limb-girdle muscular dystrophy type 2J (LGMD2J; MIM #608807);
– Congenital centronuclear myopathy (CNM; MIM #255200);
– Early-onset myopathy with fatal cardiomyopathy, EOMFC (MIM #611705);
– Multi-minicore disease with heart disease (MmDHD) including clinical variations;
– Childhood-juvenile onset Emery-Dreifuss-like phenotype without cardiomyopathy;
– Hereditary myopathy with early respiratory failure (HMERF; MIM #603689);
– Adult onset recessive proximal muscular dystrophy.

Mutations in titin will probably prove to be the cause of many additional phenotypes of muscular disorders in the coming years.

Due to its huge size, it has not been possible to sequence the entire *TTN* gene routinely in research and diagnostic laboratories until recently. Thus, before implementation of the next generation sequencing (NGS) methods, only a limited amount of *TTN* mutations were identified. NGS sequencing has enabled the rapid and thorough investigation of genetic material [9] and has resulted in an explosion in the identification of new *TTN* variants. However, their clinical interpretation is a challenge.

Here, we focus on the current understanding of the titin gene and protein from a human disease perspective. In particular, we provide an overview of the different neuromuscular disorders caused by mutations in the *TTN* gene, reviewing the molecular findings as well as the clinical data. Finally, we highlight the difficulties related to the interpretation of the clinical significance of *TTN* variations and the need for further functional studies and bioinformatics tools.

THE TITIN GENE, ISOFORMS AND PROTEIN

The titin gene (MIM #188840), is located on the short arm of chromosome 2 (chromosomal band q31.2). It contains 363 coding exons and an additional first non-coding exon [1]. The longest theoretical transcript (variant IC, NM_001267550.2), virtually obtained by the transcription of all the coding exons (excluding the alternative C-terminal Novex-3 exon) and called "meta isoform", has been adopted as the gold standard for describing *TTN* variants, and will be used as reference for cDNA and protein numbering in this review. Exon numbering will be according to the HGVS recommendations [10] and to the current Leiden database (LOVD) numbering (modified on 11th October 2013, changing exon 47b to exon 48 and adding +1 to all subsequent exon numbers) [11].

The titin protein spans from the Z-disk to the M-band [12]. Its modular structure is composed of four main parts (Fig. 1): the amino-terminal Z-disc region, the I-band and A-band regions, and the carboxyl-terminal part spanning the M-band. Titin is composed of repeated immunoglobulin-like (Ig) and fibronectin type 3-like (FN3) domains, interspersed by unique sequence regions [1]. It also contains the repetitive PEVK region, rich in proline (P), glutamate (E), valine (V), and lysine (K) residues, in the I band, and a serine/threonine kinase (TK) domain in the M-band.

More than 1 million splice variants could be generated theoretically by the *TTN* gene [13]. Indeed, extensive alternative splicing results in a remarkable diversity of titin isoforms that can be divided into three main classes based on the presence of the N2A and N2B elements in the I-band region [1, 14, 15] (Fig. 1). Skeletal muscles express so-called N2A isoforms, characterized by the inclusion of the N2A element and exclusion of the cardiac-specific N2B element. In the heart, N2BA isoforms include both the N2B and N2A elements, while N2B isoforms use the N2B element only. The aforementioned isoforms also differ in the lengths of the proximal tandem-Ig and PEVK regions, which are longest in the N2A isoforms and shortest in the N2B isoforms. Within each isoform class, the tandem-Ig and PEVK regions also show variable expression in different muscles, and across developmental and physiological states. Moreover, the second last *TTN* exon 363 (Mex5), coding for the is7 domain located in the M-band, is differentially spliced, producing is7– and is7+ isoforms [16]

The major isoform classes are represented in the NCBI RefSeq database by the entries NM_133378 (N2A; NP_596869:3,680 kDa and 33,423 aa), NM_001256850.1 (N2BA; NP_001243779:3,780 kDa and 34,350 aa), and NM_003319 (N2B; NP_003310:2,960 kDa and 26,926 aa) [1, 14, 15].

The Novex-1 (NM_133432; NP_597676) and Novex-2 (NM_133437; NP_597681) isoforms are similar to N2B, but they also include further 125 and 192 amino acids encoded by the Novex-1 and Novex-2 exons in the I-band. Finally, the much smaller Novex-3 isoform (NM_133379; NP_596870:616 kDa and 5604 aa) only contains the N-terminal part of the protein. This isoform, expressed on a low level in all

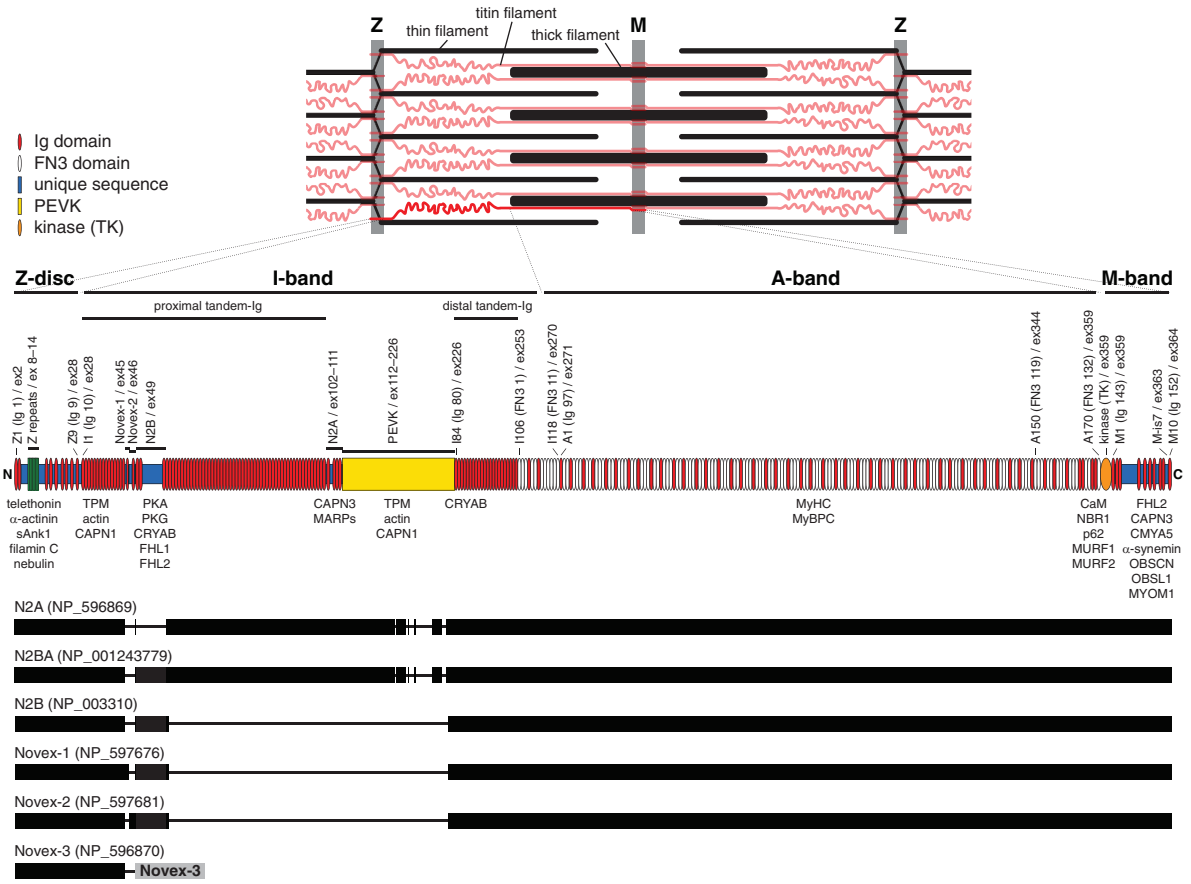

Fig. 1. Top: A schematic view of the sarcomere, with titin filaments shown in red. One titin molecule, extending from the Z-disc to M-band, is highlighted. Middle: The modular structure of the titin protein (theoretical meta isoform). Titin is mostly comprised of repeated immunoglobulin-like (Ig; red) and fibronectin type 3-like (FN3; white) domains. Selected domains are labeled above the diagram with the classical titin nomenclature (sarcomere region Z/I/A/M+domain number; Bang et al. 2001), followed in parentheses by the alternative numbering scheme (domain type Ig/FN3 + domain number), and with the corresponding exon number. Also indicated are other structural features: the Z-repeats, the Novex-1 and Novex-2 exons, the N2B and N2A elements, the PEVK (proline/glutamate/valine/lysine-rich) region, and the alternatively spliced M-band is7 (M-is7) region. Protein interactions of the different parts of titin are summarized below the diagram. Bottom: Exon inclusion in NCBI RefSeq database sequences representing the main titin isoform classes. The bars align to the protein diagram of the meta isoform above, except for the alternative C-terminal exon of the Novex-3 isoform (grey). Note that within each class there is further developmental, anatomical, and physiological variation in exon inclusion, mostly in the proximal tandem-Ig and PEVK regions.

striated muscles, results from inclusion of the Novex-3 exon encoding an alternative C-terminus [1].

The best characterized titin function is that of a scaffold protein aiding myofibrillar assembly during myogenesis [17]. However, it is also the backbone for the positioning of myosin filaments in the center of the sarcomere, and a molecular spring responsible for the passive elasticity of the muscle [3, 4]. The passive force of the muscle cells is, in fact, largely due to the elastic properties of I-band titin, allowing shortening of the sarcomere in contraction and extension when stretched. A crucial role in the myofibrillar signal transduction pathways has also been demonstrated [18]: titin seems to integrate or coordinate multiple signaling pathways related to gene activation and/or to protein folding, quality control and degradation [6, 19].

INTERACTIONS OF TTN WITH OTHER PROTEINS

The versatile roles played by titin in cardiac and skeletal muscles are enabled and facilitated by a high (or presumably very high) number of different protein ligands.

The search for TTN interactors in large public databases (PSICQUIC (20), IntAct [21], BioGRID [22]) results in a list of more than 170 putative ligands, as a product of large-scale studies of protein-protein interactions.

Even if a detailed discussion of titin interactions is not the main aim of this review, a summary of the best characterized ones is provided below and in Fig. 1.

Several reports have confirmed that telethonin (also named Titin-cap or T-cap) and α-actinin bind to the N-terminal portion of titin [23–27]. Moreover, Kontrogianni-Konstantopoulos et al. [28] demonstrated that the small ankyrin-1 (sAnk1) and the two most N-terminal Ig domains of titin form a three-way complex with telethonin.

Similarly, the actin binding proteins, filamin C and nebulin, have been shown to interact with titin in the Z-region [15, 29].

The central I-band region of titin has been widely studied, and several interactors identified, including tropomyosin [30], αB-crystallin [31], FHL1 [32] and FHL2 [33], two members of the four-and-a-half-LIM-only protein family, calpains 1 and 3 [34–36], and muscle ankyrin repeat proteins (MARPs) [37]. Protein kinases A and G (PKA and PKG) phosphorylate the N2B region, reducing the passive tension [38, 39]. Furthermore, PEVK region interacts with Ca2+, actin and S100A1, which is able to control the PEVK/actin interaction in a Ca2+-dependent manner [40].

The A-band region of titin, tightly associated with thick filaments, binds myosin heavy chain and MyBP-C [41].

The M-band region of titin has several interactors. The domains at the A-band/M-band boundary bind the ubiquitin ligases MURF1 and MURF2 (muscle RING finger 1 and 2) [42, 43]. The titin kinase (TK) domain, located at the M-band periphery, interacts with calmodulin [44], as well as with the signalosome composed of nbr1 p62, and MURF2 [33]. FHL2, expressed predominantly in the heart, binds to the is2 region [45]. The alternatively spliced is7 region binds calpain 3 (CAPN3) [35], the calcium-dependent protease involved in the pathogenesis of LGMD2A. The M10 domain interacts with the giant structural and signaling protein obscurin and its smaller homologue obscurin-like 1 (OBSL1) [46], and the A-kinase anchoring intermediate filament protein alpha-synemin [47]. Finally, several of the C-terminal titin domains can bind the multifunctional docking protein myospryn (CMYA5) [48].

TITIN VARIANTS AND DISEASES

Mutations in the *TTN* gene have been associated with several different muscle diseases, cardiomyopathies and combinations of these. The latter include dilated cardiomyopathy (DCM, MIM#604145), familial hypertrophic cardiomyopathy (HCM; MIM #613765), arrhythmogenic right ventricular cardiomyopathy (ARVC; MIM #602087) and monogenic restrictive cardiomyopathy (RCM). In this review, however, we focus on the large spectrum of skeletal muscle diseases caused by *TTN* mutations (Fig. 2), since other reviews provide a more detailed description of cardiac phenotypes linked to *TTN* variants [7, 8].

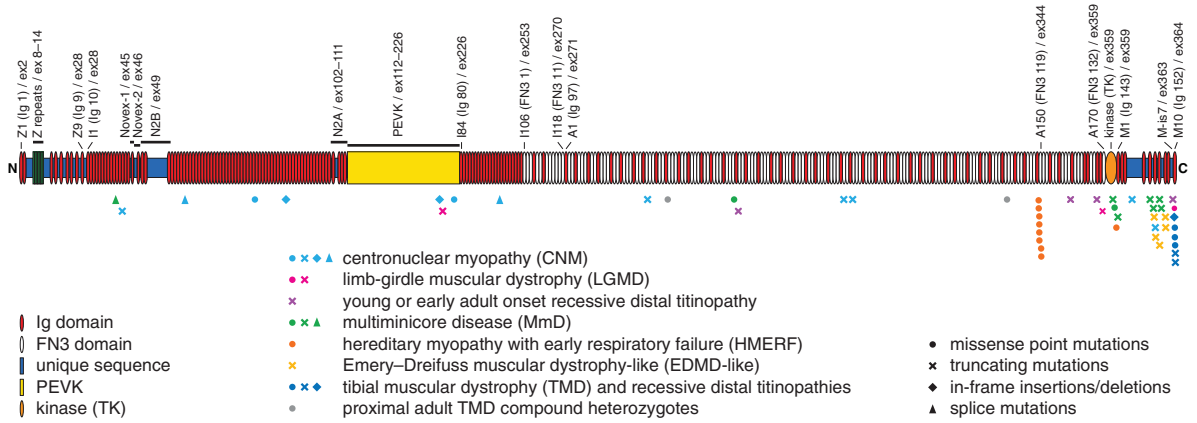

Fig. 2. Skeletal muscle disease mutations in titin. Symbols below the diagram depict mutations associated with neuromuscular diseases, with the symbol shape indicating mutation type and symbol color indicating the predominant clinical phenotype.

Late-onset autosomal dominant tibial muscular dystrophy (TMD)

Tibial muscular dystrophy (TMD; MIM #600334]) is a mild autosomal dominant distal myopathy involving the anterior compartment muscles of the lower legs but sparing of the short toe extensor digitorum brevis muscles [49].

It is characterized by a late onset (>35 years), a slow progression, normal or slightly increased values of serum creatine kinase (CK) and a myopathic EMG pattern [49, 50]. Biopsy findings in the target muscles include fiber size variability, central nuclei, necrosis, presence of fibroadipose tissue and rimmed vacuoles. Electron microscopy showed autophagic vacuoles without membrane and very rare inclusions of 15–18-nm filaments [51]. Muscle imaging (CT or MRI) is very informative with selective fatty replacement in the muscles of the anterior compartments of the lower legs starting in the anterior tibial muscle and representing a useful clinical tool to address the diagnosis.

In 2002, the first *TTN* mutation associated with human skeletal-muscle disease and causing TMD was reported [52] (Table 1). This dominant founder mutation, termed FINmaj, is responsible for the high prevalence (2/10.000) of TMD observed in Finland [49]. FINmaj is an 11-bp insertion-deletion in the last exon (exon 364 or Mex6) of the gene, changing four amino acids in the C-terminal Ig domain M10 of M-band titin. Three affected members of a French TMD family showed a different missense mutation in the same exon (c.107867T>C p.Leu35956Pro) [52].

One year later, a dominant missense variant in the exon 364 (c.107840T>A p.Ile35947Asn) was also identified in a Belgian family with a similar phenotype [51]. In 2008, three novel truncating variants (two deletions – c.107647delT p.Ser35883Glnfs*10 and c.107889delA p.Lys35963Asnfs*9 – and a nonsense mutation – c.107890C>T p.Gln35964*) were identified in two French families and a Spanish kindred [50] and a further missense mutation in exon 364 was then described in a large Italian family [53].

Young or early adult onset recessive distal titinopathy

More recently, Evilä et al. [54] described four patients with a more severe distal phenotype, resulting in a young or early adult-onset recessive distal titinopathy: all these patients were compound heterozygotes for described TMD mutations and novel frameshift variants (Table 1). Two French patients, previously reported with a more severe distal phenotype compared to TMD, had a second causative mutation that explains the peculiar phenotype. Similarly, a 36-year-old Spanish female with a similar distal phenotype and an early onset had the previously described Iberian *TTN* mutation (c.107889delA) combined with a second frameshift mutation. Homozygosity for the Iberian mutation was the cause of the early distal progressive disease observed in a female Portuguese patient.

In the reported patients, biopsy findings are highly variable, from mild myopathy to severe dystrophic changes usually with rimmed vacuoles, depending on the site of biopsy and the disease duration.

Muscle imaging shows an early (already at age 20) fatty degeneration of the anterior compartment, frequently combined with a similar degree of involvement of soleus, which is unusual in TMD.

Limb-girdle muscular dystrophy type 2J (LGMD2J)

Limb-girdle muscular dystrophies (LGMD) are Mendelian disorders affecting the voluntary muscles in proximal limbs of the hip and shoulder areas [55]. LGMDs includes more than 30 different diseases with different but often overlapping clinical pictures [56]. LGMD2J represents a recessive disease with an early age of onset (<12 y.o.) [50, 57]. The first Finnish patients described were homozygous for the FINmaj mutation, presenting with a very different and much more severe phenotype than TMD [50, 57]. In addition, in a French family with a dominant TMD phenotype due to a nonsense mutation in the last exon (c.107890C>T p.Gln35964*), one deceased patient with a more severe generalized muscle weakness proved in retrospect to be homozygous for the mutation [58].

More recently, Evilä et al. described three further LGMD2J patients [54]. Three Finnish patients, heterozygous for the FINmaj variant and presenting with an early onset LGMD or generalized muscle weakness phenotype, were clinically and molecularly re-evaluated. In two out of three patients, a second frameshift variant was detected in the other *TTN* allele (Table 1), and in the third patient the changes on the protein level were identical to FINmaj homozygous LGMD2L, suggesting an undetected truncating mutation on the other allele. Moreover, Zheng et al. identified, by exome sequencing, a homozygous missense mutation in the last exon (c.107788T>C

Table 1
Mutations causing TMD, young or early adult onset recessive distal titinopathy, LGMD or adult proximal phenotype

		Allele 1			Allele 2		
		Mutation[§]	Exon[†]	Domain	Mutation[§]	Exon[†]	Domain
TMD	Finnish TMD	FINmaj mutation	364 (363[#])	M10 (Ig 152)			
	de Seze 1998/Hackman 2002 (French family A)	c.107867T>C (p.Leu35956Pro)	364 (363[#])	M10 (Ig 152)			
	Hackman 2008 (French family B)	c.107890C>T (p.Gln35964*)	364 (363[#])	M10 (Ig 152)			
	Hackman 2008 (Albacete family)	c.107889delA (p.Lys35963Asnfs*9)	364 (363[#])	M10 (Ig 152)			
	Hackman 2008 (Barcelona family)	c.107889delA (p.Lys35963Asnfs*9)	364 (363[#])	M10 (Ig 152)			
	Van den Bergh 2003 (Belgian family)	c.107840T>A (p.Ile35947Asn)	364 (363[#])	M10 (Ig 152)			
	Pollazzon 2010 (Italian family)	c.107837A>C (p.His35946Pro)	364 (363[#])	M10 (Ig 152)			
Young or early adult onset recessive distal titinopathy	Hackman 2008 (French family C - Proband) and Evila2015 (pt.5b)	c.100558-100561dup (p.Gly33521Aspfs*25)	358 (357[#])	A169 (Ig 141)	c.107647delT (p.Ser35883Glnfs*10)	363 (362[#])	M-is7
	Hackman 2008 (French family C - Mother) and Evila2014 (pt.5a)	c.98105delC (p.Pro32702Leufs*15)	353 (352[#])	A160 (Ig 139)	c.107647delT (p.Ser35883Glnfs*10)	363 (362[#])	M-is7
	Evila2014 (pt.6)	c.67089delT (p.Lys22364Argfs*24)	319 (318[#])	A55 (FN3 50)	c.107889delA (p.Lys35963Asnfs*9)	364 (363[#])	M10 (Ig 152)
	Evila2014 (pt.7)	c.107889delA (p.Lys35963Asnfs*9)	364 (363[#])	M10 (Ig 152)	c.107889delA (p.Lys35963Asnfs*9)	364 (363[#])	M10 (Ig 152)
LGMD	Finnish original cases	FINmaj	364 (363[#])	M10 (Ig 152)	FINmaj	364 (363[#])	M10 (Ig 152)
	Penisson-Besnier 2010 (French family B - pt.IV-5)	c.107890C>T (p.Gln35964*)	364 (363[#])	M10 (Ig 152)	c.107890C>T (p.Gln35964*)	364 (363[#])	M10 (Ig 152)
	Evila 2014 (pt.1)	c.101113delT (p.Ser33705Leufs*4)	359 (358[#])	A170 (FN3 132)	FINmaj	364 (363[#])	M10 (Ig 152)
	Evila 2014 (pt.2)	c.39492dupT (p.Glu13165*)	208 (207[#])	PEVK	FINmaj	364 (363[#])	M10 (Ig 152)
	Evila 2014 (pt.3)	?			FINmaj	364 (363[#])	M10 (Ig 152)
	Zheng 2015	c.107788T>C (p.Trp35930Arg)	364 (363[#])	M10 (Ig 152)	c.107788T>C (p.Trp35930Arg)	364 (363[#])	M10 (Ig 152)
Proximal adult TMD compound heterozygotes	Evila 2014 (pt4)	c.92167C>T (p.Pro30723Ser)	340 (339[#])	A140 (FN3 112)	FINmaj	364 (363[#])	M10 (Ig 152)
	Evila 2015 (pt t13)	c.60494A>G (p.His20165Arg)	305 (304[#])	A32 (Ig 106)	c.107837A>C (p.His35946Pro)	364 (363[#])	M10 (Ig 152)

[§]Reported according to the longest theoretical transcript (NM_001267550). [†]Numbered according to the HGVS recommendations (ref.10) and to the current Leiden database (LOVD) numbering (modified on 11th October 2013) (ref.11). [#]Numbered according to the old numbering (before 11th October 2013).

p. Trp35930Arg) in a Chinese Han consanguineous family with a LGMD phenotype [59].

On the protein level LGMD2J shows a secondary CAPN3 defect [60] and loss of titin C-terminus on Western blots and immunofluorescence microscopy with antibodies against several C-terminal domains [50, 61]. Most biopsied muscles of patients homozygous for the FINmaj variant show dystrophic findings with end stage pathology without rimmed vacuoles [48]. However, rimmed vacuolar pathology was reported in a recently described case compound heterozygous for the FINmaj mutation and a truncating mutation [52].

Muscle imaging shows a progressive fatty degeneration of skeletal muscles. Muscles are relatively well preserved in young patients. A small degree of fatty degeneration can be observed 10 years after onset of muscle weakness, and fatty replacement is usually total after 40 years [48, 52].

Congenital centronuclear myopathy

Centronuclear myopathies (CNMs) are congenital myopathies characterized by the presence of centralized nuclei in the muscle fibers [62]. The term has also been used for myopathies with less specific increase of internalized nuclei. Mutations in four different genes have been reported to cause CNM: *DNM2* causes an autosomal dominant form [63]; *BIN1* and *RYR1* mutations may cause autosomal dominant or recessive forms [64, 65]; and the X-linked myotubular myopathy (XLMTM) is due to mutations in the *MTM1* gene [66]. Recently, Ceyhan-Birsoy et al. described five patients with generalized infantile muscle weakness and muscle biopsy findings compatible with CNM [67]. Using next generation sequencing of whole exomes and genomes, recessive truncating *TTN* mutations were identified in all the five patients (Table 2).

Fattori at al. described a further CNM patient with two *TTN* nonsense mutations (Table 2) in the *TTN* gene, resulting in a severe reduction of titin C-terminus at protein level [68]. The latter Italian case provided a further proof of the correlation between specific titin variants and the CNM.

Early-onset myopathy with fatal cardiomyopathy (EOMFC) and multi-minicore disease with heart disease (MmDHD)

In 2007, Carmignac et al. reported a novel recessive titinopathy involving both heart and skeletal muscle, in two consanguineous families of Moroccan and Sudanese origin [69]. The disease was characterized by early onset, slowly progressive, muscle weakness (1 y.o.); conversely, a severe dilated cardiomyopathy with rhythm disturbances developed later and resulted in a premature sudden death before adulthood. Skeletal muscle biopsies showed minicore-like lesions, centralized nuclei and type 1 fiber predominance.

In 2014, Chauveau and collagues described four other families with congenital core myopathy and primary heart disease associated with *TTN* mutations (Table 2) and suggested the inclusive name of multiminicore disease with heart disease (MmDHD) for all these clinically heterogeneous congenital diseases [70].

Chauveau described a wide range of phenotypes, spanning from an Emery-Dreifuss-like form to an unusual, severe condition with distal arthrogryposis multiplex congenita (AMC), congenital muscle weakness, kyphosis, and neonatal cardiac failure. All of them are congenital or infantile muscle conditions, characterized by weakness with rigid spine, distal or elbow joint contractures, impaired respiratory function and mild hyperCKemia (<5x).

Childhood-juvenile onset Emery-Dreifuss-like phenotype without cardiomyopathy

Emery-Dreifuss muscular dystrophy has been associated with several genes: *EMD* and *FHL1* for X-linked forms [71, 72], LMNA [73, 74] with both an autosomal dominant and recessive inheritance, and *SYNE1* and *SYNE2* [75] both causing a dominant phenotype. Recently, De Cid et al. reported on three patients with a peculiar phenotype, including limb-girdle weakness, high CK levels, early-onset contractures and a progressive course with permanent loss of ambulation during adolescence or early adulthood [76]. The clinical phenotype of the patients resembled EDMD, albeit with no cardiac abnormality. Novel truncating mutations in the C-terminus of titin segregating with the disease in all the three unrelated families were identified (Table 2), and they all had a secondary CAPN3 defect indicating a novel recessive titinopathy phenotype.

Congenital centronuclear myopathy [67, 68], early-onset myopathy with fatal cardiomyopathy (EOMFC) [69], multi-minicore disease with heart disease (MmDHD) [70], and childhood-juvenile onset Emery-Dreifuss-like phenotype without

Table 2
Mutations causing congenital centronuclear myopathy, multiminicore disease and an Emery-Dreifuss-like phenotype

		Allele 1			Allele 2		
		Mutation[§]	Exon[†]	Domain	Mutation[§]	Exon[†]	Domain
Congenital centronuclear myopathy	Ceyhan-Birsoy 2013 (pt.314-1)	c.40558G>C (p.Val13520Leu?)	220 (219[#])	PEVK	c.44816-1G>A (p.[?])	243i (242i[#])	–
	Ceyhan-Birsoy 2013 (pt.966-1)	c.24863_24877del (p.Asp8288_Ile8293delinsVal)	87 (86[#])	I65 (Ig 62)	c.103846_103849dup (p.Pro34617Glnfs*3)	359 (358[#])	M-is2
		c.39201_39203dup (p.Pro13068dup)	204 (203[#])	PEVK			
	Ceyhan-Birsoy 2013 (pt.979-1)	c.15496 + 1 G>A (p.[?])	53i (52i[#])	–	c.76393_76396del (p.Asn25465*)	327 (326[#])	A86 (FN3 73)
					c.106019delG (p.Gly35340Valfs*65)	359 (358[#])	M-is4
	Ceyhan-Birsoy 2013 (pt.1044-1)	c.77764C>T (p.Gln25922*)	327 (326[#])	A91 (FN3 76)	c.107889delA (p.Lys35963Asnfs*9)	364 (363[#])	M10 (Ig 152)
	Ceyhan-Birsoy 2013 (pt.1093-1)	c.21961G>A (p.Glu7321Lys)	76 (75[#])	I54–I55 (Ig 51–52)	c.58620delA (p.Val19541Phefs*22)	299 (298[#])	A26 (FN3 29)
	Fattori 2015 (pt.38)	c.9577C>T (p. Arg3193*)	41	I13	c.105832C>T (p.Gln35278*)	359 (358[#])	M-is4
Multiminicore disease	Carmignac 2007 (family 1)	c.106571delA (p.Lys35524Argfs*22)	361 (360[#])	M8 (Ig 150)	c.106571delA (p.Lys35524Argfs*22)	361 (360[#])	M8 (Ig 150)
	Carmignac 2007 (family 2)	c.105528_105535del (p.Gln35175Hisfs*9)	359 (358[#])	M5 (Ig 147)	c.105528_105535del (p.Gln35175Hisfs*9)	359 (358[#])	M5 (Ig 147)
	Chauveau 2014 (family 1)	c.106407_106408del (p.Glu35470Argfs*11)	360 (359[#])	M7 (Ig 149)	c.106407_106408del (p.Glu35470Argfs*11)	360 (359[#])	M7 (Ig 149)
	Chauveau 2014 (family 2)	c.102523C>T (p.Arg34175*)	359 (358[#])	M1 (Ig 143)	c.105832C>T (p.Gln35278*)	359 (358[#])	M-is4
	Chauveau 2014 (family 3)	c.66695T>A (p.Val22232Glu)	317 (316[#])	A53 (FN3 49)	c.102057delT (p.Asn34020Thrfs*9)	359 (358[#])	TK
	Chauveau 2014 (family 4)	c.9163 + 1 G>C (p.?)	38i	–	c.102214T>C (p.Trp34072Arg)	359 (358[#])	TK
Emery-Dreifuss-like phenotype	De Cid 2015 (pt.1)	c.106959T>A (p.Tyr35653*)	361 (360[#])	M8 (Ig 150)	c.106959T>A (p.Tyr35653*)	361 (360[#])	M8 (Ig 150)
	De Cid 2015 (pt.2)	c.106051delT (p.Glu35351Asnfs*54)	359 (358[#])	M6 (Ig 148)	c.106978C>T (p.Gln35660*)	361 (360[#])	M8 (Ig 150)
	De Cid 2015 (pt.3)	c.105910_105914del (p.Thr35304Cysfs*3)	359 (358[#])	M-is4	c.106422delG (p.Phe35475Serfs*4)	360 (359[#])	M7 (Ig 149)

[§]Reported according to the longest theoretical transcript (NM_001267550). [†]Numbered according to the HGVS recommendations (ref.10) and to the current Leiden database (LOVD) numbering (modified on 11th October 2013) (ref.11). [#]Numbered according to the old numbering (before 11th October 2013).

Table 3
Mutations causing hereditary myopathy with early respiratory failure (HMERF)

	Mutations[§]	
Palmio 2014 (fam A)	c.95126C>G (p.Pro31709Arg)	
Pfeffer 2012 (fam A/B/C)	c.95134T>C (p.Cys31712Arg)	
Ohlsson 2012 (fam A/B/C)	c.95134T>C (p.Cys31712Arg)	
Pfeffer 2013 (fam 1/2/3/4/5)	c.95134T>C (p.Cys31712Arg)	
Toro 2013 (fam B/C)	c.95134T>C (p.Cys31712Arg)	
Palmio 2014 (fam B/C/D/E/F/G)	c.95134T>C (p.Cys31712Arg)	
Yue 2015 (pt 2)	c.95134T>C (p.Cys31712Arg)	
Izumi 2013	c.95186G>T (p.Trp31729Leu)	
Palmio 2014 (fam H)	c.95185T>C (p.Trp31729Arg)	
Palmio 2014 (fam I)	c.95187G>C (p.Trp31729Cys)	
Pfeffer 2013 (fam 6)	c.95195C>T (p.Pro31732Leu)	
Palmio 2014 (fam L)	c.95195C>T (p.Pro31732Leu)	
Yue 2015 (pt 1)	c.95195C>T (p.Pro31732Leu)	
Palmio 2014 (fam J/K)	c.95195C>T (p.Pro31732Leu)	c.95195C>T (p.Pro31732Leu)
Pfeffer 2013 (fam 7)	c.95358C>G (p.Asn31786Lys)	
Toro 2013 (fam A)	c.95372G>A (p.Gly31791Asp)	

[§]Reported according to the longest theoretical transcript (NM_001267550).

cardiomyopathy [76] (Table 2) represent a group of TTN-related recessive disorders characterized by an early onset. Most of the patients described are homozygous or compound heterozygous for truncating variants. The causative mutations in CNM patients are mainly localized in I- or A-bands but frameshift variants in M-band titin have been found in 3/6 patients [67, 68]. On the contrary, almost all the patients with an EDMD-like phenotype or an EOMFC/MmDHD described so far have truncating mutations in the M-band, but these variants do not involve the last exon, previously associated to TMD/LGMD phenotypes [69, 70, 76]. Interestingly, despite the location of all these M-band truncating variants in proximity to each other, the clinical pictures and the histological findings are heterogeneous and, above all, a cardiac phenotype is only reported in a subset of patients.

Hereditary myopathy with early respiratory failure (HMERF)

Hereditary myopathy with early respiratory failure (HMERF) is an adult-onset autosomal dominant myopathy with respiratory muscle involvement that may lead to a fatal respiratory crisis if not treated [77].

In 2005, Lange et al. identified a *TTN* mutation (c.102271C>T p.Arg34091Trp, also known as R279W according to residue numbering of the isolated TK structure) in the TK domain of two Swedish families and a third unrelated Swedish patient sharing the same haplotype [33]. The variant affects the interaction between nrb1 and the TK domain, disrupting the signaling pathway that involves p62/SQSTM1, MURF2 and SRF.

With the exception of the TK-R279W mutation, all other mutations reported as being causative of HMERF are localized in the *TTN* exon 344 (Table 3) and most of them seem to hamper the correct folding of the A150 domain (119th Fn3 domain) [78–84]. The most common mutation (c.95134T>C p.Cys31712Arg, first reported as p.Cys30071Arg) has been found in more than 20 families [78, 79, 82], most of which share some markers in the haplotype, except one Indian [83] and one Chinese family [85]. The high number of mutations identified in exon 344 confirms the presence of a mutational hotspot region. Until recently, all the variants associated with HMERF seemed to be dominant and fully penetrant. In 2014, Palmio et al. reported a missense mutation (c.95195C>T p.Pro31732Leu, first published as p.Pro30091Leu) in three different families [82]. The variant has been defined "semidominant" or "semirecessive" since it is fully penetrant in homozygosity causing a more severe phenotype. The heterozygous carriers may have a subclinical disease or may manifest a less severe disease.

In the affected members of the original Swedish family described by Lange et al. [33], Hedberg et al. reported the presence of a second variant, the "recessive" p.Pro31732Leu change [86]. A suggestive hypothesis is that the co-inheritance of both p.Pro31732Leu and p.Arg34091Trp may cause the disease by a fully penetrant bi-mutational dominant allele [87].

Other titinopathies

In two families, one Finnish and one Italian, with well-known dominant TMD disease one individual in each family developed a different phenotype: adult onset proximal lower limb weakness without the normal ankle dorsiflexion weakness [54, 88]. Muscle MRI consistently showed significant dystrophic changes in the thigh muscles, and in the Finnish patient marked soleus muscle involvement. Both patients proved to have a second recessive mutation inherited from the healthy non-TMD parent. In particular, the Finnish patient showed a missense mutation (c.92167C>T p.Pro30723Ser) in the exon 340 [54] and in the Italian patient a missense mutation (c.60494A>G p.His20165Arg) was identified in the exon 305 [88].

Exome sequencing detected two variants in compound heterozygosity (c.45599C>G p.Ala15200Gly and c.106154 $A>C$ p.Lys35385Thr) in a male with Romanian and Hungarian origin with adult onset proximal weakness and an initial clinical suspicion of inflammatory disease [89]. Western blots with C-terminal titin antibodies showed significant reduction of identified protein, suggesting truncating mutations rather than the identified missense variants.

ANIMAL MODELS OF TITINOPATHIES

Several spontaneous and induced animal models with titinopathy have been described so far.

"Runzel" ("ruz") is a dystrophic zebrafish mutant with a reduced expression of certain *TTN* isoforms [90]. Poor swimming ability and decreased birefringence at 5 dpf are the first signs of a progressive myofibrillar disorganization, resulting in a premature death (10–12 dpf) for homozygous animals.

A spontaneous mouse model with a complex rearrangement causing the loss of 83 amino acids from the N2A region exhibits a recessive muscular dystrophy with myositis (mdm) [91]. The homozygous mice show a progressive muscle degeneration involving prominently distal skeletal muscles such as the tibialis and a reduced expression (50–60%) of CAPN3.

Mice carrying the FINmaj mutation [92] in homozygosity develop a progressive muscular dystrophy as well as a dilated cardiomyopathy, whereas heterozygotes only show a mild, later onset restricted phenotype. Interestingly, crossing the FINmaj model with CAPN3-deficient mice attenuates the muscular disorder in double heterozygotes, although not in the FINmaj homozygotes, suggesting a role for CAPN3 in the pathogenesis.

Similarly, other models were characterized and studied to focus on the *TTN*-related cardiomyopathies.

"Pickwick" ("pik") is a zebrafish mutant, carrying a *TTN* variant causing an alternative N2B exon splicing [93]. Pik heart is thin-walled, dilated and poorly contractile, resembling the human DCM phenotype.

A mouse lacking the cardiac N2B element was generated to study the role of this element in systole and diastole [94]. The shorter protein is correctly integrated into the sarcomere, but causes a restrictive diastolic dysfunction.

A murine conditional knock-out for the first two M-line exons (exons 359-360 or Mex1 and Mex2) has been produced to study the role of these domains during heart development [95, 96].

Finally, *Drosophila melanogaster* mutants have been characterized to study the role of *D-TTN* gene that shows a homology to vertebrate *TTN* [97]. In particular, D-Titin plays a crucial role into the formation of multi-nucleate syncytia and the organization of actin-myosin filaments in the skeletal muscle.

GENOTYPE-PHENOTYPE CORRELATION

As described above, variants in the *TTN* gene may cause different diseases, including several muscular disorders. Deducing a genotype–phenotype correlation has, however, so far been possible only to a limited extent, although the concentration of the currently confirmed mutations in the C-terminal (M-band) part of the gene is apparent. For instance, dominant late onset tibial muscular dystrophy is caused by mutations in the last exon 364 (Mex6) [51–53, 98]. Young or early adult onset recessive distal titinopathy is based either on one mutation in exon 363 or 364 (Mex5 or Mex6) combined with a truncating mutation on the other allele [54], or both mutations in exons 363 or 364 (Mex5–6). LGMD2J presentation may occur from similar mutational sites but is more typical with FINmaj on one or both alleles (Table 1) [50, 57].

Homozygous or compound recessive truncating mutations in the first four M-line exons (exons 359–362 or Mex1–4) cause a range of severe congenital or very early onset muscle diseases with or without cardiomyopathy [67–70, 76]. The reason why some truncations in the same exon cause cardiomyopathy and others not is unexplained.

All truncated transcripts do not undergo nonsense-mediated decay and some read-through occurs, which may lead to very variable amounts of titin protein available. Cardiomyopathy could be associated with lower amounts of protein, but this has not been conclusively confirmed. Compound heterozygosity including a missense mutation with a truncating change leads to functional homozygosity of the missense transcript due to nonsense-mediated decay of the truncated transcript. Moreover, a compound heterozygous patient (patient IV in ref [54]), carrying the FINmaj variant and a missense change in the A-band (p.Pro30723Ser), shows an atypical, adult onset, proximal lower limb titinopathy that spares the anterior tibial muscle [54]. An identical phenotype occurs with compound heterozygosity of the Italian TMD mutation (p.His35946Pro) and another missense A-band mutation (p.His20165Arg) [88].

The different molecular mechanisms underlying LGMD2J, the recessive distal titinopathy, or specific atypical phenotypes have not been clarified so far.

Finally, HMERF represents a unique, well recognized, phenotype (Table 3). Even if mutations in the exon 344 are the only changes confirmed to cause fully dominant HMERF, the recent finding of the more recessive change (p.Pro31732Leu) [82] highlights the possibility that a a second variant *in cis* may play an important role in the pathogenesis of a clinical phenotype [82, 87].

In the pre-NGS era, the low number of described patients, as well as the positional bias caused by the extensive scanning of M-band exons as compared to other *TTN* portions have prevented dissection of the genotype–phenotype correlations. Novel data arising from NGS projects, together with further functional studies, will be useful to get a more clear picture of *TTN* mutations and their associated phenotypes.

CHALLENGES ARISING FROM NGS PROJECTS

In the last few years, the next generation sequencing approaches have demonstrated to be extremely useful in research and diagnostic testing for various hereditary conditions, including neuromuscular disorders [99]. Whole exome (WES) [100], whole genome (WGS) [9], and targeted sequencing approaches [101] have been utilized to identify causative mutations in already known or novel disease genes. All these strategies are revealing a high number of novel and rare variants in the *TTN* gene. More than three rare non-synonymous titin variants are identified in any individual and this is of course partly due to the mere size of the gene.

In 2012, Herman et al. developed an affinity capture for the sequencing of the titin exons [102]. In this way, they identified 72 loss-of-function variants, indicating the important role of titin in the development of dominant dilated cardiomyopathy. Later studies have shown many truncating variants to be too common for fully penetrant dominant effects [103].

Several custom enrichment assays, including MyoCap [88], MotorPlex [104, 105], and others [106–108], have been developed to sequence specific genes of interest related to neuromuscular disorders.

The use of comprehensive NGS tools allows the analysis of almost all the coding regions of *TTN* gene, overcoming challenges related to its size.

On the other hand, novel challenges arise from the NGS data. *TTN* variants identified in NGS studies are rarely already known and characterized. Moreover, recent papers underline that a single heterozygous truncating mutation in the titin gene usually does not cause any relevant muscular phenotype, and a second mutation is necessary to generate a recessive condition [54, 59, 70, 76, 89].

Most of the patients analyzed by NGS strategies show previously undescribed rare missense variants. The clinical interpretation of missense variants in the *TTN* gene represents one of the most significant challenges related to NGS investigation in the field of neuromuscular disorders. In silico predictions are questionable and a careful approach in the interpretation of missense variants should include a comprehensive segregation analysis and mandatory functional assays. Unfortunately, functional validations of missense changes in the *TTN* gene are notoriously difficult, as the huge size of titin prevents the cloning and expression of the full-length protein in *in vitro* systems. So far, for *in vitro* experimentation, titin has been dissected into more manageable protein constructs, which have been used for testing the effects of mutations on protein–protein interactions in various assays, or on structural stability of the protein. For example, the missense variant p.Trp34072Arg has been proven to abolish the interactions of titin's kinase domain (TK) with its known ligands and to reduce the TK stability, providing a robust proof of its pathogenicity [70]. Similarly, some of the TMD-causing missense mutations have been shown to destabilize the titin M10 domain and to affect its binding to obscurin and/or obscurin-like 1 [109–111].

According to a recent large-scale study, most disease-associated variants perturb protein–protein interactions without causing misfolding [112]. Moreover, each variant may affect only specific interactions while leaving most other interactions unperturbed [112], explaining how different variants in the same gene can cause different phenotypes. These notions likely hold true also for *TTN* variants, and for the related phenotypes. For most of titin's domains, interaction partners and biological functions remain unknown. Their elucidation will be a prerequisite for the correct interpretation of rare missense changes identified as well as for the identification of therapeutic targets for innovative drug therapies for titinopathies.

Meanwhile, the research community has responded to the challenges arising from the use of NGS through the formation of clinical and research consortia [113, 114]. These collaborations take advantage of the power of shared resources and expertise, and particularly the benefit of combining cohorts of patients into larger groups. This greatly increases the likelihood of success of NGS projects and enhances the impact of these projects in terms of the clinically relevant data that is associated with them. To reach this goal, there is an urgent need to collect all reported, novel detected and rare *TTN* variants from patients all over the world and combine them into a single accessible database, in order to better be able to compare the results and draw conclusions on genotype–phenotype correlations. As discussed in the recent 219th ENMC workshop on titinopathies, an international database of *TTN* mutations, variation and their clinical phenotypes, could help all the researchers and clinicians to assess the pathogenicity of found variants in NGS analyses and would promote significant advances in the understanding of titinopathies (Ms in preparation).

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