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Expanding the *MTM1* mutational spectrum: novel variants including the first multi-exonic duplication and development of a locus-specific database

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Myotubular myopathy (MIM#310400), the X-linked form of Centronuclear myopathy (CNM) is mainly characterized by neonatal hypotonia and inability to maintain unassisted respiration. The *MTM1* gene, responsible for this disease, encodes myotubularin – a lipidic phosphatase involved in vesicle trafficking regulation and maturation. Recently, it was shown that myotubularin interacts with desmin, being a major regulator of intermediate filaments. We report the development of a locus-specific database for *MTM1* using the Leiden Open Variation database software (http://www.lovd.nl/MTM1), with data collated for 474 mutations identified in 472 patients (by June 2012). Among the entries are a total of 25 new mutations, including a large deletion encompassing introns 2–15. During database implementation it was noticed that no large duplications had been reported. We tested a group of eight uncharacterized CNM patients for this specific type of mutation, by multiple ligation-dependent probe amplification (MLPA) analysis. A large duplication spanning exons 1–5 was identified in a boy with a mild phenotype, with results pointing toward possible somatic mosaicism. Further characterization revealed that this duplication causes an in-frame deletion at the mRNA level (r.343_444del). Results obtained with a next generation sequencing approach suggested that the duplication extends into the neighboring *MAMLD1* gene and subsequent cDNA analysis detected the presence of a *MTM1/MAMLD1* fusion transcript. A complex rearrangement involving the duplication of exon 10 has since been reported, with detection also enabled by MLPA analysis. It is thus conceivable that large duplications in *MTM1* may account for a number of CNM cases that have remained genetically unresolved.

INTRODUCTION

Congenital myopathies are a heterogeneous group of diseases, generally characterized by muscle weakness, and with onset at birth or during infancy. These myopathies have characteristic histological hallmarks in muscle biopsy allowing the differential classification in distinct entities: centronuclear myopathy (CNM), core myopathy (centralcore and minicore diseases) and nemaline rod myopathy.[1,2] In CNM, the most prominent histopathological features include hypotrophy of type 1 fibers and a high frequency of centrally located nuclei with perinuclear halos lacking myofilaments and occupied by mitochondrial and glycogen aggregates.[1] Several genes are reported to be associated with CNM; these include *MTM1* in the X-linked form,[3,4] *DNM2* and *MTMR14* in the autosomal dominant forms,[4–6] *BIN1* and *RYR1* associated with the autosomal recessive forms.[4,7–9]

X-linked myotubular myopathy (XLMTM; MIM 310400) has a prevalence of approximately 1/50 000 males and is characterized by severe hypotonia present at birth and inability to maintain sustained

spontaneous respiration.[10] Different authors have proposed that patients be classified according to their phenotype, as: (i) *severe* – characteristic facial features, markedly delayed motor milestones and requiring prolonged ventilatory support (>12 h); (ii) *moderate* – more rapid acquirement of motor milestones and independent respiration for >12 h per day; (iii) *mild* – motor milestones slightly delayed and independent spontaneous respiratory function achieved after the neonatal period.[11,12] Carrier females are usually asymptomatic, however there are several records of manifesting heterozygotes due to skewed X chromosome inactivation.[13–18]

The *MTM1* gene (in Xq28) is composed of 15 exons and has an open reading frame of 1.8 kb encoding the myotubularin protein. Structurally, myotubularins are constituted by four characteristic domains: the protein tyrosine phosphatase (PTP), the predicted GRAM (glucosyltransferases, Rab-like GTPases activators and myotubularins), the RID (Rac-induced recruitment domain) and SID (SET-interaction domain). Functionally, myotubularin is a

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phosphatase acting specifically on PtdIns3P and PtdIns(3,5)P2, two phosphoinositides (PIs). PIs participate in the regulation of various cellular mechanisms by direct binding to PI-binding domains of effector proteins (that control membrane/vesicular trafficking) and subsequent recruitment/activation of these at specific membrane sites. PtdIns3P and PtdIns(3,5)P2 have a direct role in the endosomal-lysosomal pathway.[19] The PTP-catalytic domain of myotubularins is responsible for the phosphoester hydrolysis of the 3-phosphate of PIs. This hydrolysis involves two residues of cysteine and arginine located on a Cys-X5-Arg motif, characteristic for the PTP domain.[20] The loss of phosphatase activity or the production of truncated proteins as a result of *MTM1* mutations could lead to abnormal dephosphorylation of PtdIns3P/PtdIns(3,5)P2 and subsequent abnormal trafficking of the effector proteins of the endosomal-lysosomal pathway.[19] Similar results were observed with mutations located in the GRAM domain of myotubularin. This domain of about 70 amino acids is responsible for PtdIns(3,5)P2 binding.[21] Recently mitochondrial homeostasis in muscle fibers and regulation of the desmin cytoskeletal system was attributed to mytobularin. It was experimentally demonstrated that myotubularin interacts with desmin, and that this complex is disrupted by specific mutations in the *MTM1* gene.[22]

It is recognized that, despite the genetic advances in this field and the large number of cases reported,[23] a significant number of CNM cases remain genetically unresolved. This may be explained either by the involvement of further gene *loci* or by the presence of mutations in known genes, that are not detectable by routine techniques. During the development of a locus-specific database (LSDB) for the *MTM1* gene we noticed that no large duplications (involving one or more exons) had been reported for this gene. This observation led us to investigate the possibility of their occurrence in molecularly unresolved CNM patients. Accordingly, we report the first multi-exonic duplication in *MTM1* (exons 1–5) detected in a male patient with a mild XLMTM phenotype. In line with this finding, a complex rearrangement involving the duplication of exon 10 was recently published.[24]

MATERIALS AND METHODS
MTM1-LOVD database development
The *MTM1* mutation database was implemented using LOVD v2.0 software,[25] and is integrated in Leiden Muscular Dystrophy pages (http://www.dmd.nl/). Currently (by 29th June 2012), this large database installation displays a total of 141 genes with 70 512 variants (9265 unique) described in 55 775 individuals. The *MTM1* database is subdivided into two main tables, for variant data ($n = 20$) and for patient/clinical items ($n = 22$), the latter being shared among the different LSDBs in the Leiden Muscular Dystrophy pages (example of an entry in Supplementary Figure S1). Data were retrieved from peer-reviewed literature and new variants were directly submitted by different sources (laboratories and clinicians). The curators' tasks included confirmation of information concerning the sequence variants, especially with regard to their descriptions following the recommendations of the Human Genome Variation Society (HGVS),[26] using the cDNA reference sequence NM_000252.2. Classification of the clinical phenotype (severe, moderate or mild) was added to the patient's entry when adequate information was reported or submitted.

New MTM1 variants
Variants submitted to MTM1-LOVD that had not been described previously, are presented here. These were reported from different centers (Belgium, Germany and Portugal) and submitted to MTM1-LOVD. Information regarding the patient's phenotype and the mutation origins was also collected. Bioinformatic analysis of sequence variants, in particular missense and splicing mutations, was performed using Polyphen v2 (http://genetics.bwh.harvard.edu/pph2/)[27] and the Human Splicing Finder v2.3 (http://www.umd.be/HSF/).[28]

Phylogenetic conservation analysis of the affected residues and population screening of variants were also performed.

Studies performed in patient 1
Clinical description. Patient 1 (P1) is a 7-year-old boy born to a non-consanguineous couple. He has a healthy younger sister. Prenatal manifestations included oligohydramnios and signs of premature birth. Delivery was at 35 weeks by cesarean section, with an Apgar score of 8/9, weight 2465 g, height 46 cm and head circumference 33 cm. During the neonatal period the only clinically relevant sign was facial paresis. There were no feeding or respiratory difficulties. During the first month his pediatrician noticed that he was hypotonic and started global stimulation. He started to walk at 21 months. At the age of two he was referred to a pediatric neurology clinic. He was shy and had poor language skills. There was limitation in the abduction of the right eye, facial diparesis with the left side more exacerbated, with a lagophtalmos. Proximal tetraparesis was detected; the patient could not raise his arms completely and was unable to stand up from the floor without bilateral help. He had a waddling gait and could not run. CK levels were normal (36 U/l). Biopsy was performed at age 3. Over the last 3 years muscle weakness progressed and there has been a gradual loss of motor skills.

Multiple ligation-dependent probe amplification (MLPA) analysis. Screening for duplications and deletions in *MTM1* was performed by the MLPA technique using the P309-A1 Probe Set (MRC-Holland, Amsterdam, The Netherlands). This contains 16 probes for the *MTM1* gene, 7 probes for the *MTMR1* gene, 3 probes located on Xq28 (*DKC1* and *FLNA* genes) and 11 reference probes for distinct regions on the X chromosome (Supplementary Table S2). gDNA samples of P1 and four healthy controls (150 ng each) were used in the procedure. Products were separated by capillary electrophoresis on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). Data analysis was conducted using GeneMarker software (SoftGenetics LLC, State College, PA, USA). Population normalization method was selected and data were plotted using probe ratio.

Southern blotting and hybridization. gDNA samples from P1, his mother and controls were digested with *Eco*RI (New England Biolabs, Beverly, MA, USA) and resolved on a 0.8% agarose gel. DNA fragments were vacuum-transferred to a nylon membrane using a saline method. A cDNA probe recognizing *MTM1* exons 2–7 was prepared using digoxigenin (DIG) DNA Labeling Kit (Roche Applied Science, Indianapolis, IN, USA) and incubated overnight using the Easy Hyb Buffer (Roche Applied Science). The membrane was prepared with DIG Wash and Block Buffer Set (Roche Applied Science), incubated with Anti-DIG-AP conjugate (Roche Applied Science), and the DIG-labeled probe detected with ready-to-use CDP-Star (Roche Applied Science).

cDNA analysis. Total RNA was extracted from muscle samples of P1 and controls using the PerfectPure RNA Fibrous Tissue Kit (5 PRIME, Hamburg, Germany) and converted to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). *MTM1* transcripts were subjected to PCR amplification of the region(s) encompassing exons 2–7, using the specific primers (2F: 5′-TC CAGGATGGCTTCTGCATC-3′ and 7R: 5′-CAAGCCCTGCCTCCTGTATTC-3′). For the detection of the *MTM1/MAMLD1* fusion transcript total RNA was extracted from blood using the PerfectPure RNA Blood Kit (5 PRIME). After cDNA conversion, PCR amplification was performed using the primer mentioned above for exon 2 of *MTM1* and a reverse primer for exon 5 of *MAMLD1* (cMAMLD1-5R: 5′-AGTCTGGCCTGAGTGTGAGAGG-3′). PCR products were purified, sequenced using BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems) and resolved on an ABI 3130xl genetic analyzer (Applied Biosystems).

Next generation sequencing. gDNA was Covaris (S-series) sheared to an average size of 300 to 400 bp. An Illumina sequencing library was prepared using the NEBNext kit (New England Biolabs) without modifications and using Illumina Truseq adapters. No library pre-amplification was performed. Sequencing data was generated on an Illumina Hiseq2000 (Illumina, San Diego, CA, USA) using standard instrument settings for flowcell clustering and paired-end sequencing of 2×100 bp reads on a flowcell with v3 reagents. Fastq

Table 1 Overview of mutations in MTM1-LOVD

DNA	RNA[a]					Protein[b]		del/dup/ins/delins				
Mutation type	r.(0?)	del/dup/ins/subs	r.(spl?)	r.(?)	p.0?	Missense	Nonsense	IF	OF	MP	p.(?)	Total
Substitutions	—	26	62	238	5	145	91	35	8	3	39	326 (152)
Deletions	—	2	7	63	—	—	3	10	52	—	7	72 (50)
Duplications	—	—	1	30	—	—	7	—	23	—	1	31 (23)
Insertions	—	1	—	5	—	—	—	1	5	—	—	6 (6)
Deletion/insertions	—	—	2	5	—	—	1	—	4	—	2	7 (7)
Large deletions[c]	15	2	—	13	18	—	—	10	1	—	1	30 (18)
Large duplications[c]	—	2	—	—	—	—	—	2	—	—	—	2 (2)
												474 (258)

Abbreviations: r.(0?), predictably no expression at RNA level; del, deletion; dup, duplication; ins, insertion; subs, substitution; r.(spl?), predicted to affect splicing; r.(?), RNA effect unknown; p.0?, predictably no protein production; delins, deletion and insertion; IF, in-frame; OF, out-of-frame; MP, multiple polypeptides; p.(?), effect at protein level unknown.
Data extracted from MTM1-LOVD (29/06/2012). Numbers between brackets indicate the total count of unique (different) mutations.
[a]Experimentally tested at RNA level (del, dup, ins, subs) or predicted (remaining columns).
[b]Predicted protein changes inferred from DNA or RNA sequence.
[c]Involving one or more exons.

files were generated using the Illumina Casava v1.8 pipeline. Stampy (v1.0.13) was used to align the sequence data to the human genome (Hg19) using standard parameters. The total number of aligned reads per 10 000 bp bins across the whole-genome was calculated using the Bedtools package.[29] Bins overlapping more than 1% with simple-repeat regions were excluded from further processing. For sample comparison of tag coverage in the *MTM1* locus, the bin counts were normalized relative to total number of reads aligned to the chromosome X. For visualization purposes, BED tracks were generated for the patient, control and the ratio patient/control, and were uploaded to the UCSC genome browser.[30]

Studies performed in patient 2

Clinical description. Patient 2 (P2) was the first child of healthy parents, with no consanguinity or family history of note. After delivery he was very hypotonic and required immediate respiratory support with continuous positive airway pressure and naso jujenal feeding. At 2 months of age he underwent tracheal endoscopy and aryepiglottoplasty after which he needed ventilatory support. After extubation he was transferred for intensive care and was diagnosed with congenital nystagmus and pyloric stenosis. At 3 months he was referred for a neurology opinion. He had a frog like posture, absent antigravity movement in the upper limbs, with some in the lower limbs and areflexia. At pyloromyotomy a quadriceps muscle biopsy was taken, which confirmed the diagnosis of CNM. From 5 months respiratory function improved and by 7 months he was off all respiratory support. He began to lift each leg off the cot for 1–2 min and the nystagmus disappeared, with normal eye movements, but he had persisting marked neck weakness. Following an episode of lung collapse he began to show restriction of lateral and vertical eye movements. He was able to lift his arms against gravity and bring his hands together, as well as to hold a rattle, and was able to lift his legs momentarily against gravity. He had a marked scoliosis, a bell shaped chest and there was early hip and knee flexion contractures. Placed prone he was unable to turn his head or push himself up on his arms. At 8 months he developed bronchiolitis and pulmonary collapse, as well as a brief cardiac arrest. Following this he was re-intubated and given ventilatory support. After recovery he was much less active and lost antigravity limb movement and eye movements. At the age of 10 months he became unwell again and died.

Molecular analysis. Initially gDNA was PCR amplified for subsequent sequencing. As no products were obtained for exons 3–14, a large hemizygous *MTM1* deletion was suspected. Deletion was confirmed by MLPA analysis, as described for P1. The breakpoints of the large deletion encompassing exons 3–14 were determined by long-range PCR. Amplification of gDNA was performed using the BIO-X-ACT Long DNA Polymerase Kit (Bioline, Taunton, MA, USA) and primers complementary to introns 2 and

14 of *MTM1* (2iF: 5′-GAAAGGTTGCTGAAGGACATACTG-3′ and 14iR: 5′-GCCTTGGGTATGAATGCTGG-3′). PCR products were resolved on a 2% agarose gel and purified with QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA), followed by sequencing.

RESULTS

MTM1-LOVD

A total of 496 variants are currently (June 2012) listed in the *MTM1* LSDB. Besides non-pathogenic variants ($n = 18$) or sequence changes with unknown significance ($n = 4$), this database includes 474 *MTM1* mutations identified in 472 XLMTM patients. The mutation profile of the *MTM1* gene, according to LOVD-MTM1 data, is summarized in Table 1 and Figure 1. The most represented group of mutations is single-nucleotide substitutions (68.8% of total mutations), which can be subdivided as: missense ($n = 145$), nonsense ($n = 91$), those affecting splicing ($n = 85$) or translation ($n = 5$). Deletions were identified in 102 patients. These include 'small' deletions reported in 72 patients (15.2% of total mutations) the majority of which (76.4%) predictably induce premature termination codons (PTC). Large deletions encompassing one or more *MTM1* exons account for 6.3% of mutations reported. The eighteen different large deletions reported to date (identified in 30 cases) are detailed in Figure 1b. Small duplications were identified in 6.5% of total mutations, all but one case predictably creating PTC. Mutations that consist in a deletion associated with sequence insertion (delins) were identified in seven cases (1.5%), whereas simple insertions were reported in six patients (1.3%). Only two cases (0.4%) involving large (exonic) duplications were reported to date. One patient was reported as having a complex rearrangement that involves the duplication of exon 10.[24] Patient P1 presented in detail in this work is the only recorded case with a multi-exonic duplication of *MTM1*. A total of 55 cases were not reported in the literature and were submitted directly to the MTM1-LOVD.

New MTM1 variants

The 25 novel point mutations identified in CNM male patients are described in Table 2. Among these, 11 are single-nucleotide substitutions located within exonic sequences: c.2T>A, c.32C>A, c.323G>A, c.469G>T, c.595C>A, c.637C>T, c.659G>C, c.1241T>C, c.1247A>G, c.1318C>T and c.1600T>C. With the exception of c.2T>A that predictably affects the initiation codon of
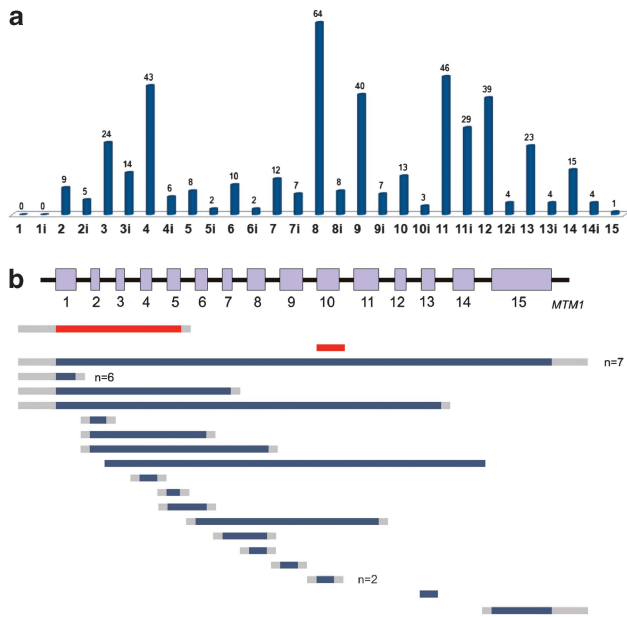
Figure 1 Mutations registered in MTM1-LOVD as distributed along the *MTM1* gene. (**a**) Total number of small mutations is shown for each exon and intron of *MTM1*. (**b**) Large deletions and duplications identified in *MTM1*. Solid bars represent regions involved (blue – deletions; red – duplications); gray bars represent undelineated breakpoints. In cases with more than one independent report the total number (*n*) of patients is indicated.

myotubularin (p.0?), the majority of these substitutions are predicted to be missense mutations (p.Gly108Asp, p.Pro199Thr, p.Leu213Phe, p.Arg220Thr, p.Phe414Ser, p.His416Arg and p.Trp534Arg). The remaining three are nonsense mutations (p.Ser11*, p.Glu157* and p.Gln440*). A condensed view of all data corroborating the pathogenicity of missense changes is presented in Supplementary Information (Supplementary Table S3). Briefly, missense variants were considered pathogenic when affecting phylogenetically conserved residues and/or were not detected in ethnically matched control chromosomes. Three single-nucleotide substitutions are located in intronic donor splice consensus sequences and predictably affect splicing: c.231 + 1G > T, c.342 + 5G > A and c.867 + 1G > A. In one of these changes (c.342 + 5G > A) cDNA studies demonstrated that it promotes exon 4 skipping (r.232_342del) leading to an in-frame deletion at the protein level (p.Ser79_Asp115del). In addition, four deletions (c.1088_1089del, c.1328_1331del, c.1509_1510del and c.1519_1522del), two duplications (c.509_528dup and c.596dup) and two deletion/insertion mutations (c.765_767delinsGG and c.1319_1321delinsTA) were also detected. All of these variants are predicted, at the RNA and protein level, to be frame-shift mutations that generate PTC. An insertion of 376 bp in exon 13 (c.1388_1389ins376, GenBank JQ403527) was detected in a patient with a severe phenotype. Using the Repbase database,[31] this insertion was identified as an *AluYa5* sequence. Further studies revealed that this alteration affects exon 13 splicing (Supplementary Figure S4), predictably resulting in an in-frame deletion of 38 amino acids (p.Phe452_Gln489del) located in the SID region of myotubularin. The remaining two novel mutations are the large deletion and duplication presented below in more detail.

Multi-exonic duplication in patient 1. Patients included in this work were initially studied by routine *MTM1* gene analysis. From our molecularly unresolved CNM patient cohort, six males and two females were selected for quantitative studies by MLPA analysis of *MTM1*, based on clinical and histological criteria that were compatible with CNM. Of these eight candidates, a single male patient (P1) was found to be positive, with a multi-exonic duplication in *MTM1*. A second male patient was subsequently found to carry a *DNM2* mutation and the remainder are as yet uncharacterized.

Muscle biopsy of P1 showed fiber size variation with atrophy, numerous central nuclei and endomysium fibrosis (Figure 2a). In some areas muscle was better preserved, with scanty central nuclei and atrophic fibers (Figure 2b). There was central dark staining with NADH-TR, SDH and PAS, reflecting aggregation of mitochondria and glycogen (Figures 2c and d). 'Necklace'-fibers were easily identified with routine histological stains (Figure 2e), PAS (Figure 2d) and histoenzimological stains. ATPase histoenzymology showed the presence of only type 1 fibers.

Following the detection of a large duplication spanning exons 1–5 of the *MTM1* gene (Figure 3a), the 3'-breakpoint of the duplication was narrowed down by Southern blot analysis (Figure 3b), which revealed the absence of a ∼10 kb band corresponding to exons 5 and 6 of the gene. cDNA studies were carried out on reminiscent muscle tissue, in order to further characterize the rearrangement and evaluate its impact at the mRNA level. This analysis revealed a residual amount of normal transcript and a predominant mutant isoform lacking exon 6 (r.343_444del) (Figure 3c). It is predictable that this abnormal transcript will originate an in-frame deleted myotubularin lacking 34 amino acids of the GRAM domain.

In order to gain more insight into the structural rearrangement at the *MTM1* locus, we performed a whole-genome low coverage analysis by next generation sequencing. After alignment, the total number of reads aligned per 10 kb interval was calculated for the patient and an unrelated normal control sample. The ratio of aligned reads in the 10 kb bins between patient and control is indicative for amplification (ratio > 1) or deletion (ratio < 1) events. Multiple 10 kb bins in the 5'-region of the *MTM1* locus showed amplification (Figure 3d). Interestingly, the amplification appeared to extend into the 3'-region of the neighboring *MAMLD1* gene. An in depth analysis of the orientation of the aligned read pairs, as well as several long-range PCR experiments, failed to provide further information regarding the breakpoints of the structural rearrangement. Considering the NGS results, we postulated that the duplication could originate a *MTM1/MAMLD1* fusion transcript. In order to test this possibility, additional expression experiments were performed using RNA obtained from whole blood. Results revealed that a *MTM1/MAMLD1* fusion transcript was indeed generated (Figure 3e). Based on these results, a schematic representation of the duplication is presented (Figure 3f).

Large deletion in patient 2. During routine *MTM1* gDNA sequencing in P2, no symmetrical PCR amplification was obtained for the majority of exons (3–14). This led us to suspect the presence of a large intragenic deletion, which was subsequently confirmed by MLPA (Supplementary Figure S5a). This mutation was further characterized by long-range PCR, in order to predict its impact at the protein level and to facilitate carrier screening and prenatal diagnosis. Sequencing of the junction fragment obtained by long-range PCR revealed breakpoints located in introns 2 and 14 (∼75 kb sized deletion) and enabled us to determine the full description of the mutation as c.63 + 834_1645-2104del (Supplementary Figure S5b). At the protein level, if efficient translation occurred, this would correspond to the

Table 2 Novel *MTM1* mutations submitted to the MTM1-LOVD

Patient ID	DNA mutation	Gene location	Type of mutation	cDNA effect	Protein effect	Origin of mutation	Phenotype	Geographic origin
18914	c.=/(?_-76)_342 +?dup	Exons 1–5	Large duplication	r.[=, 343_444del]	p.Asp115_Leu148del	*De novo*, somatic	Mild	Portugal
10959	c.63+834_1645-2104del	Intron 2–15	Large deletion	r.(?)	p.(Thr22_Gln548del)	Inherited (MC)	Severe	United Kingdom
24703	c.2T>A	Exon 2	Affects initiation codon	r.(?)	p.0?	Inherited (*de novo*, in mother)	Severe (at birth)	Germany
21000	c.32C>A	Exon 2	Nonsense	r.(?)	p.(Ser11*)	*de novo*	Severe	Germany
24706	c.231+1G>T	Intron 4	Donor splice site disruption[a]	r.(spl?)	p.(?)	Inherited (MGC)	Severe (died at 10 months)	Germany
20980	c.323G>A	Exon 5	Missense	r.(?)	p.(Gly108Asp)[b]	*de novo*	Mild (24 years old, still walking)	Germany
18861	c.342+5G>A	Intron 5	Donor splice site disruption	r.232_342del	p.Ser79_Asp115del	Inherited (MC)	Severe	Germany
20960	c.469G>T	Exon 7	Nonsense	r.(?)	p.(Glu157*)	Inherited (MC)	Severe	United Kingdom
20961	c.509_528dup	Exon 7	Out-of-frame duplication	r.(?)	p.(Gly177Trpfs*16)	Unknown	Severe (long-term survivor)	Germany
20933	c.595C>A	Exon 8	Missense	r.(?)	p.(Pro199Thr)[b]	Inherited (MC)	Unknown (neonatal death)	Portugal
24711	c.596dup	Exon 8	Out-of-frame duplication	r.(?)	p.(Ala200Cysfs*12)	Inherited (MC)	Severe	Germany
24702	c.637C>T	Exon 8	Missense	r.(?)	p.(Leu213Phe)[b]	Inherited (MC)	Mild (diagnosed at 24 years of age)	Germany/Albania
24707	c.659G>C	Exon 8	Missense	r.(?)	p.(Arg220Thr)[b]	Unknown	Severe (at birth)	Germany/Albania
20951	c.765_767delinsGG	Exon 9	Out-of-frame deletion	r.(?)	p.(Asp256Valfs*28)	Inherited (MC)	Severe	Turkey
11124	c.867+1G>A	Intron 9	Donor splice site disruption[a]	r.(spl?)	p.(?)	Inherited (MC)	Unknown	Portugal
16734	c.1088_1089del	Exon 11	Out-of-frame deletion	r.(?)	p.(Lys363Serfs*14)	Inherited (MC)	Severe	Germany
11170	c.1241T>C	Exon 11	Missense	r.(?)	p.(Phe414Ser)[b]	Unknown	Unknown	Portugal
12852	c.1247A>G	Exon 11	Missense	r.(?)	p.(His416Arg)[b]	Unknown	Severe	Germany
20944	c.1318C>T	Exon 12	Nonsense	r.(?)	p.(Gln440*)	Inherited (MC)	Severe	Germany
20955	c.1319_1321delinsTA	Exon 12	Out-of-frame deletion/insertion	r.(?)	p.(Gln440Leufs*24)	Inherited (MC)	Severe	Germany
24712	c.1328_1331del	Exon 12	Out-of-frame deletion	r.(?)	p.(Asp443Valfs*20)	Unknown	Severe	Germany
14208	c.1388_1389ins376, GenBank JQ403527	Exon 13	In-frame exon skipping	r.1354_1467del	p.Phe452_Gln489del	Inherited (*de novo*, in mother)	Severe	Turkey
20938	c.1509_1510del	Exon 14	Out-of-frame deletion	r.(?)	p.(Asn503Lysfs*2)	Unknown	Severe	United Kingdom
20952	c.1519_1522del	Exon 14	Out-of-frame deletion	r.(?)	p.(Glu507Asnfs*28)	Inherited (MC)	Severe	Germany
24705	c.1600T>C	Exon 14	Missense	r.(?)	p.(Trp534Arg)[b]	Inherited (MGC)	Severe (at birth)	Germany

Abbreviations: ID, patient identification in database; MC, mother carrier; MGC, maternal grandmother carrier.
Variants are described according to the reference sequence NM_000252.2, using HGVS nomenclature guidelines.
[a]Predicted to affect splicing by bioinformatic analysis.
[b]Pathogenicity assessment of missense variants in Supplementary Information (S3).
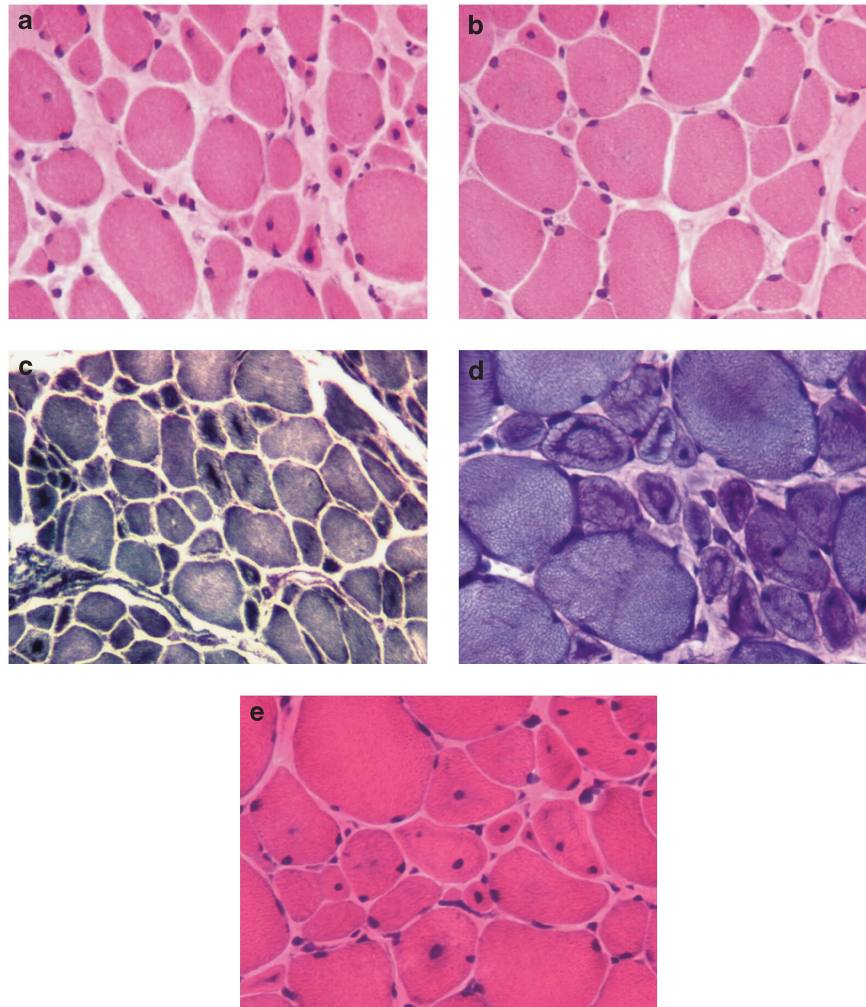
Figure 2 Histological features of patient 1. Histological analysis of the left deltoid muscle biopsy performed using staining with hematoxylin and eosin (**a**, **b** and **e**), histochemical reactions for NADH-TR (**c**), and PAS (**d**). Abnormal fiber size variability is seen on all stainings. The key feature of the pathology is the central nuclei (more evident on H&E image **a** and **e**). The dark central staining areas accompanying the central nuclei are another striking feature (**c** and **d**). The 'necklace fibers', recently described as a histopathological marker of *MTM1*, can be best appreciated on PAS staining (**d**) and faintly on H&E (**e**).

loss of ~87% of primary protein sequence including all of the functionally relevant myotubularin domains.

DISCUSSION
MTM1-LOVD
Providing evidence for pathogenicity of variants is costly and time-consuming, so diagnostic laboratories rely on previous reports of variants detected in patients. For *MTM1* however, such data has been dispersed in the literature, in various formats. These difficulties prompted us to develop an LSDB for the *MTM1* gene. We also engaged in this task because the scientific community has expressed the importance and the need for establishing a dedicated XLMTM database (mutational and clinical).[23] The implementation and curation of this LSDB followed guidelines reported elsewhere,[32,33] and is registered with the HGVS (LSDB list, http://www.hgvs.org/dblist/glsdb.html).

The main objective for this freely accessible database is the description of *MTM1* sequence variants with phenotypic impact, collected from different sources. These data ultimately will allow further insights with respect to the mutational spectrum of the *MTM1* gene and epidemiology, abetting the molecular diagnosis and research in this field. Currently > 11% of the entries included in this database have not been previously described in the literature. This means that this database contributes to the visibility of new *MTM1* mutations within the public domain that would otherwise have a limited possibility to be released as single or individual case reports in the literature.

Database content analysis
Although large genomic deletions contributed to narrowing down the XLMTM candidate region and ultimately the identification of the *MTM1* gene,[34] large intragenic deletions appear to be a relatively rare cause of myotubular myopathy (6.3% of all cases) compared with point mutations. In fact, these deletions are generally identified in single case reports, with the exception of exon 1 and full *MTM1* gene deletions. The majority are associated with a severe disease outcome, even in deletions that are predictably in-frame. However, it should be noted that besides the large deletion reported in this work (P2) only a few mutations, namely the deletion of exon 9 and the deletion of
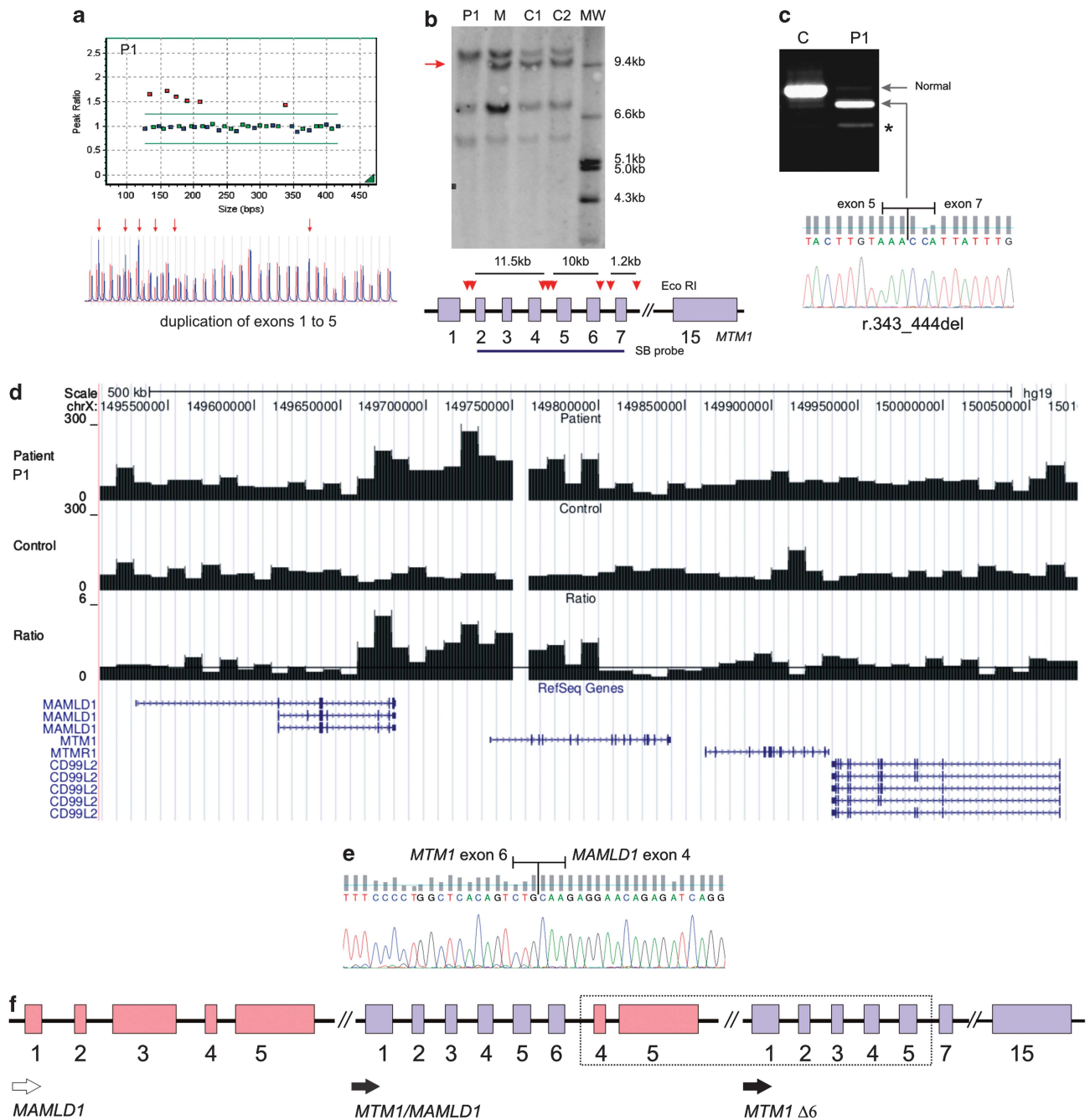
Figure 3 Large genomic duplication detected in patient 1. (**a**) MLPA analysis in patient 1 (P1) shows duplication of exons 1–5 of the *MTM1* gene. Red arrows indicate the duplicated probes. (**b**) Southern blot technique using a cDNA probe spanning exons 2–7 (SB probe); red arrow-heads represent *EcoR*I restriction sites; red arrow highlight the absence of a band in the patient, corresponding to exons 5 and 6. (**c**) RT-PCR analysis of mRNA obtained from muscle, revealing a mutated isoform lacking exon 6; (*) – smaller faint band corresponding to an alternative splicing product (Δ exon 5 and 6, ENSEMBL transcript ID: ENST00000424519). (**d**) Low coverage NGS analysis of a 500 kb interval within the X chromosome. Histograms correspond to the total number of reads aligned per 10 kb interval for the patient (top), an unrelated normal control sample (middle) and ratio between the patient and control (bottom) where the line corresponds to ratio value of 1. (**e**) Partial sequence of the fusion transcript, showing *MTM1* exon 6 adjacent to *MAMLD1* exon 4. (**f**) Schematic representation of duplicated region (dashed rectangle); resulting transcripts are indicated by black arrows – observed (filled) and predicted (unfilled). P1, patient; C/C1/C2, controls; M, patient's mother; MW, molecular weight marker.

exon 13, have been studied in enough detail to allow a more accurate prediction of their impact at the protein level.[10,35]

The largest proportion (93.3%) of pathogenic sequence variants described to date in *MTM1* is that comprising small mutations. Since the last published *MTM1* mutation update,[23] there has been a disproportional increase of missense and splicing mutations reported in *MTM1*. This might be attributed to the recent identification of additional XLMTM cases with a milder phenotype, often associated with these types of sequence variants.[36–39] Analysis of the distribution of point mutations reveals a higher incidence in exons

Table 3 Frequent mutations in MTM1-LOVD

Number of DB entries (n)	Gene region	Mutation (DNA)	Mutation (protein)	Sequence context	Possible cause
12	Exon 3	c.109C>T	p.Arg37*	TCCTCGACT	CpG
13	Exon 4	c.141_144delAGAA	p.Glu48Leufs*24	CAAAGAAGT	Slippage
10	Exon 4	c.205C>T	p.Arg69Cys	TTATCGTCT	CpG
9	Exon 8	c.614C>T	p.Pro205Leu	GTTCCGTAT	CpG
13	Exon 9	c.721C>T	p.Arg241Cys	TGTGCGTTG	CpG
28	Intron 11	c.1261-10A>G	p.Ser420_Arg421insPheIleGln	ATCAATTTA	Unknown
9	Exon 12	c.1261C>T	p.Arg421*	TCAGCGAAT	CpG
18	Exon 12	c.1262G>A	p.Arg421Gln	CAGCGAATA	CpG

Abbreviations: DB, database; CpG, dinucleotide mutational hotspot.
Description of mutations with nine or more independent entries. Nucleotides affected by the mutation are underlined. Variants are described according to the reference sequence NM_000252.2, using HGVS nomenclature guidelines.

Table 4 Analysis of MTM1-LOVD missense mutations

			Missense mutations				Nonsense mutations
	Residues involved			Phenotype reported[a]			
Protein domain	(% of total protein)	n (% of total)	Severe	Mild/Moderate	Inconsistent	Unknown	n (% of total)
GRAM	aa 34–149 (19.1%)	9 (12.3%)	2 (22.2%)	5 (55.6%)	0	2 (22.2%)	5 (15.6%)
RID[b]	aa 162–265 (17.1%)	28 (38.4%)	15 (53.6%)	7 (25.0%)	3 (10.7%)	3 (10.7%)	6 (18.8%)
PTP	aa 274–434 (26.5%)	28 (38.4%)	18 (64.3%)	5 (17.9%)	1 (3.5%)	4 (14.3%)	3 (9.4%)
SID	aa 435–486 (8.5%)	5 (6.8%)	3 (60.0%)	1 (20.0%)	0	1 (20.0%)	5 (15.6%)
Other	(28.8%)	3 (4.1%)	1 (33.3%)	2 (66.7%)	0	0	13 (40.6%)
	Total	73	39	21	4	9	32

Abbreviations: aa, amino acid position within myotubularin protein (reference sequence NP_000243.1); GRAM, glucosyltransferase, Rab-like GTPase activators and myotubularins; PTP, protein tyrosine phosphatase; RID, Rac-induced recruitment domain; SID, SET-interacting domain.
[a]Only phenotypes reported in male patients were considered for this analysis; the mild and moderate phenotypes were combined as several reports do not distinguish the two clinical classifications.
[b]This domain partially overlaps desmin-binding region.

8, 11, 9, 4 and 12 (in decreasing order of frequency), and this should be taken into consideration when conducting *MTM1* gene mutation screening. Altogether, mutations in these exons account for almost half of all reported XLMTM cases.

A significant number of *MTM1* point mutations coincide with the hypermutable CpG dinucleotides that are amenable to methylation-mediated deamination. This mechanism could explain the recurrence, hence higher frequency, of some variants (Table 3). In fact six mutations (c.109C>T, c.141_144delAGAA, c.205C>T, c.1261-10A>G, c.1261C>T and c.1262G>A) were each detected in nine or more patients, accounting for ∼24% of total cases in the MTM1-LOVD database. The most frequent variant in the MTM1-LOVD is the splicing mutation c.1261-10A>G that activates a cryptic splice site, promoting the inclusion of 9 bp in the open reading frame. This cryptic splice site is preferentially used in *MTM1* transcripts detected in skeletal muscle.[40,41] The change is predicted to include three amino acids in the core of the PTP (active) site. It was identified in several patients from different ethnic origins (US, Japan and Europe) and associated with a severe disease outcome in male patients. The cause of this recurrent mutational event is still unknown.

In a previous study,[12] the analysis of a large number of patients revealed a statistically significant association between non-truncating mutations and the mild phenotype, as opposed to the intermediate/severe phenotype associated with mutations that give rise to PTC. We performed a similar analysis of all data available in MTM1-LOVD with the aim of obtaining further correlation between genotype and phenotype. In line with previous reports, it was evident that

truncating mutations are predominantly associated with a severe phenotype. A total of 188 male patients were reported to have mutations that originate PTC. Clinical classification was available in 146 of these (77.7%). The majority (*n* = 139, 95.2%) were reported as severe. Only four patients were reported as mild or mild/moderate, and the PTC inducing mutations were c.836delC, c.1558C>T (*n* = 2) and c.1792delC. Besides these, there are three patients with a moderate phenotype, one associated with a splicing mutation (c.137-7T>G) and the other two with nonsense mutations which had also been found in patients with a severe disease outcome. Overall, the registries show a clear bias towards the severest end of the disease, which may reflect either a specific biological pattern related to defects in myotubularin or the patient selection criteria used to conduct *MTM1* gene analysis.

As the majority of non-truncating mutations are of the missense type (∼80%) we centered our analysis on these variants and their localization on the protein (Table 4). Nonsense mutations were also included for comparative purposes. Most missense mutations (96%) are located in myotubularin regions with known function (representing 71% of the total protein sequence), whereas only 59% of nonsense mutations are located in these domains. This suggests that missense changes are not randomly scattered in the protein. Moreover, 85% of mutations associated with a severe phenotype are located in the PTP (catalytic) domain and the RID/desmin-binding region. In terms of its relative proportion, mild/moderate missense mutations are more frequent in the GRAM domain and in the remaining regions of myotubularin. However, it should be noted that there is some degree

of phenotypic variability; four missense mutations were classified as 'inconsistent' in terms of the reported clinical severity. The presence of more than one sequence variant in a patient can hamper attempts at genotype/phenotype correlations. In fact two independent publications reported male patients with double *MTM1* mutations. In the first case a patient classified as having a severe phenotype (died at 6 weeks of age) two missense changes were identified (p.Asp431Asn and p.Asp433Asn).[42] The second patient was reported with severe neonatal hypotonia requiring assisted ventilation due to respiratory failure. Mutational analysis revealed the presence of two *MTM1* changes that create PTC: c.109C>T (p.Arg37*) and c.386_387insA (p.Ser129Argfs*7).[43]

Novel multi-exonic duplication. We describe two patients with large rearrangements in the *MTM1* gene, one of which (P1) carries the first multi-exonic duplication described to date. The clinical presentation of this patient is milder than the classical form of XLMTM – although he had delayed motor development skills and a progressive tetraparesis, at age 7 he remains ambulant and there is no report of respiratory impairment. Analysis of the patient's muscle biopsy revealed variation of fiber size and typical central nuclei, which lead to the diagnosis of CNM. Additionally, 'necklace' fibers were identified. These structures, characterized by a basophilic ring deposit following the contour of the cell in which the nuclei are aligned, were initially described as a particular feature of older CNM cases.[1] It is now evident that 'necklace' fibers can also be found in early onset cases with a milder phenotype. Using a variety of experimental methods, we have shown that the duplication encompassing exons 1–5 in *MTM1* has an unexpected effect at the mRNA level, resulting in an in-frame deletion of the sequence corresponding to exon 6 (r.343_444del). At the gDNA level, using a genome wide low coverage analysis of the patient's genome we were able to confirm the amplification within the *MTM1* gene and obtained evidence that it extended into the *MAMLD1* gene, known to be associated with hypospadias.[44] However, unlike intragenic duplications, it is foreseeable that because this duplication affects only the 3′-end of the *MAMLD1* gene, a normal copy would be left intact, hence the duplication should have little or no phenotypic consequence, thereby explaining why no genital abnormalities were found in the patient.

MLPA analysis had shown that the average ratio of duplicated *MTM1* probes (1.6) was significantly lower than expected for a male (~2.0). This, together with the presence of a residual amount of normal transcript and the finding that the patient's mother was not a carrier, provides strong evidence for somatic mosaicism in the patient – the first such case described to date – which might explain the patient's relatively mild phenotype in relation to what could be predicted from the genotype.

Standard routine methods for XLMTM molecular diagnosis, such as genomic analysis of *MTM1* by Sanger sequencing, do not allow the identification of duplications and may also fail to detect mosaics. The only other description of a duplication involving a complex *MTM1* rearrangement concerns a male infant with characteristic clinical and histopathologic findings of XLMTM.[24] The patient had severe neonatal hypotonia and respiratory insufficiency. Other XLMTM compatible findings included the absence of deep tendon reflexes, cryptorchidism and elongated fingers and toes. There was a progressive respiratory deterioration culminating in death at 1 month of age, due to respiratory failure. It is conceivable that there may be a considerable number of CNM cases with *MTM1* duplications, and it is therefore advisable to perform MLPA analysis in all CNM cases, after excluding point mutations.

Future perspectives

It would be important to gather more *MTM1* mutational data that are presently dispersed among other non-public databases (such as UMD-MTM1 and the Cardiff database for XLMTM),[45] as well as in private registries of diagnostic laboratories around the world. According to the meeting report of the 6th ENMC workshop on centronuclear (myotubular) myopathies, efforts are currently being made to develop a patient registry for CNM with the support of TREAT-NMD and the Myotubular Trust.[45] Until now the harmonized clinical items to be included in this registry are not yet in the public domain. After full release of LOVD software v3.0 the clinical items to be included will follow these international recommendations for the *MTM1* registry, ultimately allowing the data integration, which at this point in time is a recognized difficulty.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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