Centronuclear myopathies under attack: A plethora of therapeutic targets

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Abstract

Centronuclear myopathies are a group of congenital myopathies characterized by severe muscle weakness, genetic heterogeneity, and defects in the structural organization of muscle fibers. Their names are derived from the central position of nuclei on biopsies, while they are at the fiber periphery under normal conditions. No specific therapy exists yet for these debilitating diseases. Mutations in the myotubularin phosphoinositides phosphatase, the GTPase dynamin 2, or amphiphysin 2 have been identified to cause respectively X-linked centronuclear myopathies (also called myotubular myopathy) or autosomal dominant and recessive forms. Mutations in additional genes, as RYR1, TTN, SPEG or CACNA1S, were linked to phenotypes that can overlap with centronuclear myopathies. Numerous animal models of centronuclear myopathies have been studied over the last 15 years, ranging from invertebrate to large mammalian models. Their characterization led to a partial understanding of the pathomechanisms of these diseases and allowed the recent validation of therapeutic proof-of-concepts. Here, we review the different therapeutic strategies that have been tested so far for centronuclear myopathies, some of which may be translated to patients.

Keywords: Centronuclear myopathy, myotubular myopathy, myotubularin, amphiphysin, dynamin, gene therapy, autophagy, phosphoinositides, oligonucleotide, neuropathy

BACKGROUND

Centronuclear myopathies (CNM) are usually defined as congenital myopathies with abnormally positioned nuclei in the center of myofibers, in the absence of increased muscle regeneration commonly observed in dystrophies [1]. Of note, CNM are linked with a general disorganization of myofibers, with abnormal positioning of different organelles, internal membrane alterations and often myofibrillar misalignment and fiber shape defects.

Since the first clinical and histopathological description of a 12-year-old affected boy with myotubular myopathy in 1966 by Spiro et al. [2], remarkable advances have been made in understanding the physiopathology of CNM. A major step forward was the identification of the causative genetic
anomalies. MTMI was the first identified gene mutated in the most severe form of CNM: X-linked centronuclear or myotubular myopathy (XLTM, CNMX, OMIM#310400) [3, 4]. This form is characterized clinically by a severe hypotonia and a generalized muscle weakness often leading to respiratory failure and swallowing difficulties, and usually correlated with a poor prognosis [5–7]. In the vast majority of cases, loss-of-function mutations cause a strong decrease of the MTM1 protein [8, 9], a phosphoinositides phosphatase implicated in multiple cellular processes such as endosomal trafficking [10–12], excitation-contraction coupling [13, 14], intermediate filament organization [15], neuromuscular junction (NMJ) structure [16, 17], satellite cell proliferation [18–20], and autophagy [21, 22]. Female carriers can display a wide spectrum of clinical and pathological involvement [23].

The autosomal dominant (ADCNM) form is mainly caused by mutations in the dynamin 2 (DNM2) gene (ADCNM; OMIM#160150) [24]. The severity ranges from severely affected infant to mildly affected adults [25]. In severe early onset cases, infants present with generalized muscle weakness, hypotonia with facial weakness in addition to ophthalmoplegia [26], while in the late-onset milder cases, young adult patients present with moderate muscle weakness leading to motor defects [27]. DNM2 is a GTPase mechanoenzyme involved in endocytosis [28], exocytosis [29, 30], intracellular membrane trafficking [31–33] and cytoskeletal organization [34–36]. In vitro studies showed that CNM mutations in DNM2 increase the GTPase activity and promote higher stability of polymers, suggesting a gain-of-function mechanism in ADCNM [37, 38]. This is supported by the appearance of a CNM-like phenotype in mice over-expressing wildtype DNM2 [39, 40].

Mutations in the BIN1 gene encoding amphiphysin 2 have been reported in CNM patients linked to two modes of inheritance: autosomal recessive [41, 42] and autosomal dominant [43] (OMIM#255200). A large variability in severity is observed in affected patients, however the main consistent presentation is muscular atrophy with diffuse muscle weakness [1, 44, 45]. BIN1 generates and maintains membrane curvature and tubulation through the BAR domain [46], while the SH3 domain is involved in recruitment of endocytotic proteins such as DNM2 [41, 47, 48]. Recessive mutations decrease the membrane tubulation properties of BIN1 [41]. Of note, BIN1 also binds phosphoinositides and MTM1 [46, 47, 49, 50], suggesting a common pathway linking MTM1, BIN1 and DNM2 [51].

The incidence of CNM is about 1-2 per 100,000 births, comprised of mutations in MTM1 (~50%), DNM2 (~15%) or BIN1 (~3%), other implicated genes (~12%), or CNM with unknown genetic basis (~20%) [52]. Mutations in Ryanodine receptor 1 (RYR1) [53, 54], Titin (TTN) [55], SPEG [56], DHPR (CACNA1S) [57], ZAK [58], and potentially myotubularin-related protein 14 (MTMR14) [59] and CCDC78 [60], have been reported to cause congenital myopathies with a CNM-like phenotype. We concentrate here on CNM linked to MTMI, BIN1 or DNM2, as these forms present with highly similar pathogenesis and most translational research has focused on these forms in the recent years. We provide a summary of the different preclinical therapeutic developments that have been explored for XLTM and autosomal forms of CNM.

INCREASING OUR UNDERSTANDING OF CNM THROUGH PHENOTYPING OF ANIMAL MODELS

Development of different animal models and their characterization greatly accelerated the understanding of the clinical, physiopathological and molecular aspects of CNM (Table 1). CNM, or their molecular defects, have been modelled and studied in cultured cells, yeast, drosophila, zebrafish, mice and dogs [51]. Moreover, they represent precious tools to test and validate the different therapeutic approaches preclinically before embarking on clinical trials.

| Table 1 |

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6218136/
Comparison between the different therapeutic approaches tested on animal models of centronuclear myopathies

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prevention</th>
<th>mTOR inhibitors</th>
<th>Mtm1KO (oral)</th>
<th>Lifespan</th>
<th>Body weight</th>
<th>Muscle mass and/or force</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophagy activation</td>
<td>Prevention</td>
<td>RAD001 (AZD8055)</td>
<td>Systemic (oral)</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myostatin inhibition</td>
<td>Prevention</td>
<td>ActRIIB-mFC</td>
<td>Mtm1KO mouse (intraperitoneal)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Prevention</td>
<td>AAV-PropD76A</td>
<td>Mtm1KO mouse (intraperitoneal)</td>
<td>ND</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Other potential approaches</td>
<td>Prevention</td>
<td>edrophonium</td>
<td>mtl1 morphant</td>
<td>Systemic (oral)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acetylcholinesterase inhibition</td>
<td>Reversion</td>
<td>pyridostigmine</td>
<td>dnm2 S619L zebrafish</td>
<td>Systemic (oral)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Reversion</td>
<td>XLMTM patient</td>
<td>Systemic (oral)</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reversion</td>
<td>DNM2-CNM patients</td>
<td>Systemic (oral)</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Cell transplantation</td>
<td>Prevention</td>
<td>syngeneic WT myoblasts</td>
<td>Mtm1KI R69C mouse (intramuscular)</td>
<td>Local</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Notes: ND: no data; NA: not applicable. Most animal models are presented in Cowling et al. 2012. Extent of positive outcomes in animal models do not necessarily translate in human. In several set-up, only disease prevention was tested.
Three different mouse lines were developed to mimic XLMTM. The Mtm1 knockout (KO) mice with a complete knockdown of the MTM1 protein present a progressive myopathy starting from around week 3 with a shortened lifespan to around 7-8 weeks of age [13, 61]. They exhibit similar features to XLMTM patients, including muscle atrophy and severe weakness, and muscle biopsies feature small rounded fibers with a high frequency of abnormal localization of nuclei and disorganized mitochondrial distribution. Ultrastructural analysis revealed dramatic decrease of normal triads [13], membrane invagination implicated in excitation coupling machinery, as well as sarcomere disorganization and enlargement of neuromuscular junctions [17, 62]. Additional studies have shown defects in mitochondrial dynamics, desmin aggregation and alteration in autophagy and ubiquitin-proteasome pathways [15, 63]. A knock-in (KI) mouse carrying the R69C mutation was generated to mimic a mutation identified in XLMTM patients with a mild phenotype [64]. The R69C mutation results in exon 4 skipping leading to premature termination of myotubularin translation. However, some full-length MTM1 protein was still expressed in this model, resulting in a milder phenotype compared to Mtm1KO mice, with a median lifespan of 66 weeks. This observation suggests that expression of a small amount of MTM1 may have significant clinical benefits in XLMTM. A third mouse model, the Mtm1gt/gt, was generated using a gene trap strategy, and these mice survive for at least 41 weeks [22]. Mtm1gt/gt mice present a milder phenotype than Mtm1KO mice with a similar histopathology including hypotrophic fibers with a mild increase in mislocalized organelles and internal nuclei, and autophagy defects. Overall these mouse models present with CNM phenotypes mimicking those observed in patients, making these models very attractive, and are therefore not surprisingly frequently used in preclinical research.

In addition to murine models, a zebrafish model of XLMTM was generated using morpholinos [14]. Injection of antisense oligonucleotides into embryos resulted in reduction of myotubularin expression resulting in a CNM phenotype resulting in myofiber hypotrophy, centralization of nuclei, as well as mislocalization of organelles and NMJ defects [14, 16]. Triad defects were also noted in this model. These defects caused abnormal dorsal curvature with impairment in spontaneous movements and swimming capacities. More recently, a novel zebrafish model for XLMTM was created using zinc finger nucleases to delete 8-bp in exon 5 of the mtm1 gene causing a frameshift and premature stop codon [65]. In addition to a shortened survival of 7 days, mutants present a severe muscular phenotype similar to the morphant model, including reduced motor abilities, and morphological and muscle structural defects.

Finally, two naturally occurring canine models were identified for XLMTM: a Labrador Retriever dog and a Rottweiler dog with progressive myotubular myopathy harboring the N155K and Q384P mutations, respectively, in the MTMI gene [66, 67]. The affected dogs develop a progressive myopathy and exhibit CNM features such as gait abnormalities with muscle weakness, and hypotrophic fibers with an increase in mis-positioned nuclei and organelles.

A third naturally occurring canine model was identified with a splicing mutation in BIN1 leading to a strong decrease in protein level and a rapidly progressive muscle atrophy coupled with exercise intolerance and a typical CNM histology [42]. This latter line represents a model for the recessive form of BIN1-CNM.

Concerning ADCNM due to DNM2 mutations, a K1 mouse model harboring the most frequent ADCNM mutation R465W was generated [68]. While the homozygous K1 mice present a neonatal lethality due to metabolic defect including hypoglycemia, increased liver glycogen content and hepatomegaly [69], the heterozygous K1 mice are viable and have a normal lifespan, and develop a progressive myopathy with impairment of muscle contractile properties, atrophy and defects in mitochondria and reticular networks [68]. Similarly to Mtm1KO mice, alterations of autophagy were reported, in addition to defective clathrin-dependent endocytosis and calcium homeostasis [68–71]. As an alternative to germline mouse mutagenesis, adenov-associated virus (AAV) containing mutant
DNM2-R465W were intramuscularly injected into WT mice and resulted in development of several CNM features such as fiber hypotrophy, histological abnormalities and muscle contraction defects, despite the endogenous expression of DNM2 [39]. In addition, overexpression of WT DNM2 in mouse, either with the same AAV transduction strategy or through transgenesis, also resulted in a CNM phenotype [39, 40]. These results suggest DNM2-CNMs is not due to haploinsufficiency of DNM2, and support other in vitro observations suggesting that DNM2 mutations leading to CNM may be gain-of-function [37, 38]. Lastly, a more severe DNM2-CNMs mutation, S619L, was modeled in zebrafish, resulting in severe weakness and motor defects with histopathological and NMJ abnormalities [72].

Overall, these animal models recapitulate many CNM features and have contributed to the identification of several pathomechanisms. They all have strengths and limitations; the small size and high offspring number of drosophila and zebrafish make these models well adapted for preclinical work including drug screening, whereas the murine models allow in-depth analysis of the pathomechanisms in mammal. The canine models have similar body weight to affected children and are thus better suited for pharmacokinetic/pharmacodynamic modeling and validation of delivery methods. Based on their various strengths these animal models were used in several preclinical studies as discussed below.

**GENE, RNA AND ENZYME REPLACEMENT STRATEGIES**

**MTM1 (Myotubularin) gene therapy**

Gene therapy is a promising therapeutic approach for many monogenic disorders, especially with loss-of-function mechanism, which relies on the knowledge of the implicated genes but not on an in-depth understanding of the pathomechanisms. It was thus the first approach tested in CNM models [73]. Moreover, recent progress has greatly increased the prospects for successful gene transfer [74–76]. Among the different options, AAV vectors have emerged as effective and potentially safe candidates for certain indications such as neuromuscular or neurodegenerative diseases, hematologic disorders or heart defects [77, 78]. This is reflected by the increasing number of successful clinical trials that have driven the approval and marketing of different products based on AAV-gene delivery, such as alipogene tiparvovec (AAV1-LPLS447X) (commercial name: Glybera) for lipoprotein lipase deficiency (LPLD) [79, 80]. MTM1 gene replacement was first tested through intramuscular injection of Mtm1KO mice with AAV expressing the Mtm1 cDNA under the control of a CMV promoter, which resulted in the recovery of muscle mass and force and a normal histology [73]. Subsequently, AAV-Mtm1 with a desmin promoter was administered through loco-regional or systemic injections in both mice and dogs lacking MTM1 [81–83]. Significant improvements of survival and whole-body rescue were noted after a single injection, with increase of muscle force and mass as well as correction of histopathological hallmarks of CNM. An absence of obvious toxicity or immune response was noted, except histological alteration of cardiac tissue specifically in mice [81]. In treated dogs, amelioration of the phenotype was seen from 4 weeks post injection, and both survival and health status were greatly improved. This exciting proof-of-concept demonstrates the efficacy of gene therapy-based approaches in both small and large animals and indicates that postnatal gene replacement can reverse the XLMTM phenotype.

The selection of the best AAV serotype and promoter that controls the MTM1 expression is essential for effective and safe gene delivery. Although XLMTM patients exhibit mainly a muscular phenotype, MTM1 is ubiquitously expressed and might have other key roles in other tissues and organs. Tissue-tropism of AAV-Mtm1 should respect the natural tissue-distribution of MTM1 with similar expression level specific to each organ. The desmin promoter is highly active in skeletal and cardiac muscles after systemic delivery in mouse, but not in dog, resulting in massive overexpression of MTM1 in heart (about 700 fold) with an impact on cardiac histology [81]. The MTM1 promoter may be an ideal regulator of myotubularin expression in the different tissues. Furthermore, since MTM1 is barely expressed in XLMTM patient cells [8, 9], it may be necessary to ensure that delivering an “exogenous”
protein will not trigger an immune response against the transgene. Also, as AAVs are present within cells mostly as non-integrated episomes, normal muscle turnover will likely lead to loss of transgene expression and reduce the efficacy; however this may be less the case for CNM than for the tested dystrophies as there are no signs of increased muscle regeneration in CNM [1]. Whether re-administration of these viral vectors several years after the initial infusion may be necessary, without eliciting immunoresponse, is unclear. A recent study in dog showed longterm expression of MTM1 four years after a single AAV-MTM1 isolated limb perfusion despite decrease in AAV vector copy number, correlating with near-normal respiratory and motor functions [82]. Finally, production of an effective dose of AAV vectors for human administration presents a manufacturing challenge and requires high titer solutions ranging from 10^{13} to 10^{14} vector genome per kg of body weight for effective gene delivery to at least vital affected muscles as shown in several ongoing clinical trials (NCT03199469, NCT03368742, NCT03375164). Production of these highly concentrated solutions relies on the use of costly methods that guarantee high-quality and reliable lots [84]. Importantly, a clinical trial delivering the AAV-MTM1 gene therapy to XLMTM patient was started by Audentes Therapeutics (www.audentestx.com, NCT03199469) and exciting positive preliminary results were very recently reported (www.audentestx.com). Significant improvements of neuromuscular and respiratory functions were detected, and the earliest treated patient has become ventilator independent 16 weeks post-injection.

**Myotubularin replacement**

Enzymotherapy or enzyme replacement is another approach that was tested to deliver the MTM1 protein to myofibers [85]. Preclinical research suggests a small amount of fully functional myotubularin is enough to significantly prevent or improve the disease. Indeed several female carriers are asymptomatic, and there is a tendency for a milder disease in patients with a residual protein level if the mutation is not in the catalytic site [8]. This is supported by animal models, such as the Mtm1KI R69C mouse that produces a small amount of myotubularin and is less affected than the Mtm1KO mouse [64]. For enzyme replacement, MTM1 was fused with a single chain fragment derived from a mouse monoclonal antibody (3E10Fv) [85]. 3E10Fv fusion proteins penetrate cells through interactions with the equilibrative nucleoside transporter 2 (ENT2), a plasma membrane receptor widely distributed in mammalian tissues with high abundance in skeletal muscle [86]. Interestingly, the phosphatase activity of the fused myotubularin-3E10Fv was similar to myotubularin alone suggesting that this fusion does not interfere with the enzymatic activity [85]. Intramuscular injection of 3E10Fv-MTM1 into Mtm1KO mice significantly improved the muscle function with an increase in muscle contraction that may be correlated to the increase in correctly positioned triads and T-tubules observed by electronic microscopy. However, no obvious improvement was seen in general muscle histology. Assessment of the beneficial effect of 3E10Fv-MTM1 was limited to intramuscular injections to date. Due to the generalized muscle defect in XLMTM disease, systemic delivery will be required and the efficacy of systemic delivery of 3E10Fv-MTM1 remains to be elucidated. In addition, the manufacturing of this active MTM1 protein for human delivery represents a challenge. The major advantage of this approach is the flexibility in dosing; however the expected short effect duration will necessitate a repeated re-administration, which may induce an immunoresponse against the 3E10Fv-MTM1 complex. Similar therapeutic strategies have been successfully employed especially in multisystemic lysosomal storage disorders including alglucosidase alfa (Myozyme) for Pompe disease, Agalsidase alfa (Replagal) or Agalsidase beta (Fabrazyme®) for Fabry disease, and Imiglucerase (Cerezyme®) for Gaucher disease [87–90]. These marketed enzymotherapies showed the feasibility of such approaches and may be used as a roadmap for a therapeutic development initiated by Valerion Therapeutics (www.valerion.com) based on myotubularin enzyme replacement for myotubular myopathy.
RNA Trans-splicing for DNM2-related myopathy

DNM2-related CNM is a dominant form of CNM potentially due to gain-of-function mutations, and may not be a good target for conventional gene replacement. Reprogramming the Dnm2 RNA to replace the mutated part with a normal sequence was tested in the mouse through spliceosome-mediated RNA trans-splicing [91, 92]. In this specific example, the classical 3’ trans-splicing strategy led to toxic intermediate. However, the alternative usage of 5’ trans-splicing successfully allowed the detection of trans-splicing events at the mRNA and protein levels, both in vitro in fibroblasts and in vivo in WT murine tibialis muscle [91].

RNA trans-splicing was successful for different diseases including several myopathies in a pre-clinical setting [92–96]. This promising strategy appears still in its infancy and additional studies will have to improve the in vivo efficiency of the trans-splicing and the phenotypic rescue, while carefully investigating potential toxicity linked to the used pre-trans-splicing molecules. Also, these molecules have to be vectorized to be efficiently provided [97].

Allele-specific RNA silencing for DNM2-related myopathy

Another approach aiming to decrease the amount of the mutated DNM2 transcript consisted in silencing specifically the mutated RNA through the siRNA technology [98]. Allele-specific siRNA sequences were developed in order to specifically knock down the human or murine DNM2-mRNA harboring the p.R465W mutation without affecting the wild-type allele. Scanning the sequence encompassing the mutated site was necessary to identify the best siRNA molecules efficient in heterozygous Dnm2KI R465W mouse embryonic fibroblasts. The in vivo proof-of-concept was obtained in the Dnm2KI R465W mouse after intramuscular injection of AAV expressing the selected shRNA molecules. After 3 months, most CNM histological signs were partially or fully rescued, including muscle mass and specific force, fiber size and central accumulation of oxidative staining. A similar approach was validated in patient fibroblasts expressing the R465W mutation. The R465W RNA was strongly reduced while the WT transcript appeared unaffected. This was correlated with a normalization of endocytosis measured by transferrin uptake, while this is decreased in scramble-transfected cells. Overall, this allele-specific targeting approach appears very specific and bears promise for future pre-clinical development.

NORMALIZING THE DISEASE PATHWAY

MTMR2 expression

The MTM1 gene has many close homologs, which may provide an alternative therapeutic option. Indeed, 14 myotubularins have been identified in human [99, 100]. They express common protein domains including the PH-GRAM and coiled-coil that are implicated in phosphoinositide binding and in their oligomerization, respectively [4]. Of note, 6 myotubularins do not have an enzymatic activity and are named dead-phosphatases, while 8 display a phosphatase activity [4]. The myotubularin dead-phosphatases do however regulate their cataclystically active homologs, either by modifying their subcellular localization or by potentially changing the allosteric activity [101]. MTM1 and MTMR2 share 65% of amino acid sequence identity and similar phosphatase activity [100, 102]. Of note, while MTM1 is mutated in XLMTM, MTMR2 mutations are linked to demyelinating Charcot-Marie-Tooth neuropathy (CMT4B1; OMIM 601382) [103]. Raess et al. studied the molecular basis for the functional difference between MTM1 and MTMR2 [104]. Two MTMR2 isoforms were previously identified [105]. The most studied and longest isoform has an additional N-terminal extension prior to the PH-GRAM domain, which is absent in the short MTMR2 isoform. Importantly, unlike the long isoform, the short MTMR2 isoform displays an MTM1-like activity in a yeast complementation system and was found to be downregulated in Mtm1KO muscle [104]. Upon intramuscular injection of AAV
expressing the long MTMR2 isoform in the Mtm/KO mouse, a significant rescue of muscle phenotype was seen, however a better amelioration in muscle mass, force and histology were observed with the short isoform \[104\]. In addition, the elevated level of the MTM1 substrate phosphoinositol-3-phosphate (PtdIns3P) in Mtm/KO muscles was corrected upon this treatment. These observations suggest that the additional N-terminal extension in MTMR2 is responsible for the functional difference between MTM1 and MTMR2. More recently, intravenous administration of a single dose of the AAV9-Mtmr2 vector in the Mtm/KO mice was shown to improve the motor activity and muscle strength and prolonged survival throughout a 3-month study \[106\].

Thus, exogenous overexpression of short MTMR2 isoform or alternative splicing modulation of endogenous MTMR2 transcripts may be potential strategies to compensate for the loss of MTM1. This strategy could be applied as an approach to normalize the phosphoinositides level and treat XLMTM. While the targeting of an MTM1 homolog may avoid potential immune response compared to exogenous expression of the missing MTM1 protein, the rescue may be less efficient than using MTM1 itself, and the challenges for the delivery (e.g. AAV) remain as discussed above.

Rebalancing 3-phosphoinositides level

MTM1 is a PI 3-phosphatase that dephosphorylates PtdIns3P and PtdIns(3,5)P2 into PtdIns and PtdIns5P respectively \[107–109\]. Different XLMTM animal models have shown an increase in PtdIns3P as a direct consequence of loss of MTM1 \[14, 64, 65, 110\]. These low-abundant phospholipids are key regulators of different cellular processes like membrane trafficking and intracellular signaling \[111, 112\]. Concerning PtdIns3P, several kinases were implicated in its generation including class II (PI3K) and class III (PIK3C or VPS34) PI 3-kinases. Reducing the elevated PtdIns3P level was investigated through downregulation or inhibition of these kinases. Based on the observation made in Drosophila melanogaster showing depletion of the class II PI 3-kinase PI3K68D rescued the defects caused by mtm (ortholog of both MTM1 and MTMR2) deficiency \[113\], depletion of the class II PI3K2C was achieved in Mtm/KO mice through the production of a double knockout Mtm/KO/muscle-specific Pike2BKO \[65\]. PIK3C2B was potentially the best candidate to target as its total loss in mice is viable whereas loss of other PI 3-kinases including PIK3C3 leads to strong defects \[114\]. Genetic reduction of PIK3C2B before or after disease onset prevented or reverted the myopathy and extended the life expectancy, improved body weight and restored muscle force and histology. Surprisingly, genetic reduction of PIK3C3 in Mtm/KO mice (Mtm/KO/Pik3c3 heterozygous) worsened the CNM phenotype, suggesting that MTM1 and PIK2B may control the same sub-pool of PtdIns3P and that blocking the phosphorylation activity of PIK3C2B, but not PIK3C3, would be a therapeutic strategy for XLMTM. To develop this approach further, the identification of drugs that can specifically target PIK3C2B is required. In the mtml zebrafish model, inhibitors of PI 3-kinase class I or III, GDC-0941 or VPS34-IN1 respectively, did not improve the motor function and morphological defects. In the absence of specific class II inhibitors, pan PI 3-kinase inhibitors were tested, including PI-103, LYS294002 and wortmannin, and significant improvements of the survival, motor function and morphology were observed in the treated larvae \[65\]. In addition, treatment of Mtm/KO mice with wortmannin prolonged the survival, ameliorated the CNM phenotype and histology, and greatly improved Ca\(^{2+}\) release defects \[65, 115\].

A great advantage of this therapeutic strategy is that the target is an enzymatic activity and thus druggable. The therapeutic window, i.e. the range of doses which optimize between efficacy and toxicity, has to be defined and genetic studies suggested full depletion of PIK3C2B achieved a better rescue than partial depletion \[65\]. This successful strategy focused on targeting one function of MTM1, PtdIns3P dephosphorylation, but not PtdIns5P production or phosphoinositides-independent functions, suggesting increased in PtdIns3P is a main cause of XLMTM. Interestingly, a significant prevention of CNM phenotypes was also achieved by AAV-mediated expression of several phosphatase-dead MTM1
mutants in the MtmIKO mouse [116] and confirmed in an independent study [63]. Thus targeting phosphoinositide-independent functions of MTM1 also appears promising as normalization of PtdIns3P level was not needed for the rescue of the intracellular organization of myofibers, including positioning of nuclei, mitochondria and triads, nor for muscle force, but seems important for triad shape and fiber size [116].

In conclusion, inhibiting specifically PIK3C2B in muscle represents a promising therapeutic approach in XLMTM; however development of specific PIK3C2B inhibitors is required, as alteration or inhibition of other PI 3-kinases is highly detrimental [117].

### Normalizing or reducing DN2M in several forms of CNM

As mutations in either MTM1 or DN2M lead to CNM, it is probable that these proteins act in the same pathway for normal muscle maturation and/or maintenance [51]. In vitro experiments showed several CNM mutations increase the oligomerization and GTPase activity of DN2M [37, 38], and in vivo overexpression of wildtype DN2M in mice either through transgenesis or AAV injection produced a CNM-like phenotype [39, 40]. Moreover, it was realized that DN2M protein level is increased in XLMTM patient muscles and in muscles from the MtmIKO mouse [118]. Based on these results, and the rationale that DN2M mutations in CNM are gain-of-function, the potential therapeutic effect of DN2M downregulation was tested in MtmIKO mice.

To test a negative epistasis between MTM1 and DN2M, a genetic reduction of DN2M in MtmIKO mice was first investigated in MtmIKO/Dnm2 heterozygous KO animals. Unlike MtmIKO mice, the double transgenic mice showed normal lifespan and whole-body weight, and greatly improved muscle histology, mass and force [118]. These results were confirmed recently using antisense oligonucleotides (ASO) targeting specifically Dnm2 transcript [119]. Systemic ASO delivery in pre-symptomatic MtmIKO mice efficiently decreased DN2M expression at RNA and protein levels and prevented the development of CNM. More importantly, post-symptomatic ASO systemic treatment stopped disease progression after a single injection and reverted CNM phenotypes within only 2 weeks. In this later experiment, the disease correction correlated with a normalized DN2M expression, suggesting that decreasing DN2M level to normal range is enough to restore muscle function in severely affected mice thereby reducing side effects related to DN2M reduction below normal level.

These observations make the reduction/normalization of DN2M by ASO injection an attractive therapeutic approach. Indeed, injection and dosing regime can be easily adapted during the treatment. In addition, many clinical trials using ASO have been made with some ASO-based drugs approved for use in clinic, such as fomivirsen upon local administration in Cytomegalovirus retinitis, or systemic delivery of mipomersen for familial hypercholesterolemia, and more recently nusinersen and eteplirsen for spinal muscular atrophy and Duchenne muscular dystrophy, respectively [120]. Nevertheless, repeated injections are needed and certain ASO chemistries have been reported to preferentially distribute into liver and kidney potentially causing toxicity [121–124]. Thus, translation of this approach into XLMTM patients will require the development of efficient and safe human ASO candidates that allow a preferential targeting of skeletal muscle. Also, DN2M has important roles in cellular organization in different tissues; therefore determining the benefit:risk ratio and to which extent DN2M can and needs to be downregulated is a main point being investigated by Dynacure ([www.dynacure.fr](http://www.dynacure.fr)) which is developing clinical candidates for DN2M targeting.

The concept of “cross therapy” in CNM, i.e. targeting one CNM gene (DN2M) to rescue the myopathy due to mutations in another CNM gene (MTM1 as above), has been recently further developed by showing that DN2M downregulation can also rescue BIN1-related CNM [125]. While Bin1KO mice die at birth from a skeletal muscle defect, Bin1KO mice with reduced level of DN2M (Bin1KO/Dnm2 heterozygous KO) survive at least 18 months, and have normal muscle force and intracellular
organization of muscle fibers. Modulating DNM2 level thus represents a first therapeutic concept for patients with BIN1 defects.

Taken together, these results validate the epistasis between MTM1, BIN1 and DNM2. Of note, DNM2 targeting appears to be the first therapeutic strategy targeting several forms of CNM, suggesting a single ASO therapy may thus be sufficient to target a wide range of CNM mutations.

**TREATING THE GENERAL MUSCLE IMBALANCE**

XLMTM is characterized by severe muscle atrophy with small muscle fibers in Human and animal models [1, 14, 61, 66]. Moreover, a correlation between myofiber size, MTM1 mutation and clinical outcome has been documented by comparing histology of XLMTM infant muscle biopsies and prognosis [126]. Other data on murine and canine models suggest that a very useful histological indicator of disease improvement is normalization of myofiber size together with organelle distribution. These observations support that therapeutic strategies increasing fiber size and/or muscle bulk together with myofibers intracellular organization may be beneficial. The regulation of muscle mass and fiber size is reflected by the balance between protein synthesis and degradation, which are controlled by several intracellular signaling pathways. Major pathways implicated to date are the IGF1-AKT-mTOR pathway leading to muscle hypertrophy mainly through increased protein synthesis, the myostatin-Smad2/3 pathway which is a negative regulator of protein synthesis, and the protein degradation pathways, autophagy and the ubiquitin-proteasome system [127]. These pathways represent interesting targets that were investigated mainly for XLMTM.

**Targeting cellular homeostasis: Autophagy and ubiquitin-proteasome system**

The ubiquitin-proteasome and autophagy-lysosomal pathways play a vital role in cellular homeostasis by degrading damaged organelles and unnecessary or dysfunctional components such as proteins, and were shown essential for normal muscle growth and maintenance [128]. Alterations of these cellular processes have been reported in different Mtmt1-deficient animals [15, 21, 22, 63]. Analysis of muscles from Mtm1 KO mice revealed an accumulation of ubiquitinated proteins and defective mitochondria as well as a low conversion rate of LC3I to LC3II suggesting a blockade of the late stage of autophagy [15, 22]. In addition, misfolding and aggregation of intermediate filaments, such as desmin, were also noted [15]. Loss of MTM1 was associated with hyperactivation of the IGF1R/AKT/mTOR pathway reflected by an hyperphosphorylation of S6K1Thr389, S6Ser240/244 and 4EBP1Thr37/46 and an increase of the autophagy induction marker phospho-ULK1Ser757 [19, 22]. On the other hand, hypophosphorylation of FoxO3aSer318/321, the transcription factor implicated in muscle protein degradation, was noted in MTM1-deficient muscles resulting in increased transcription of atrogens such as Atrogin1, Murf1, Nedd4 and ZNF2016 [21]. Moreover, a molecular interaction was characterized between MTM1 and UBQLN2, an ubiquitin protein regulating the degradation of ubiquitinated proteins by the proteasome [63]. Taken together, these results indicate that MTM1-deficiency results in defects in cellular clearance pathways and suggest that activating these degradation processes may normalize homeostasis of the muscle fibers.

Pharmacological inhibition of mTOR, a major negative regulator of autophagy, using the allosteric inhibitor RAD001 or the ATP-competitive inhibitor AZD8055 remarkably normalized autophagy markers by lowering p62 and increasing LC3I/LC3II conversion [22]. Moreover, AZD8055 treatment in MTM1-deficient mice had positive effect on muscle mass and reduced the abnormal accumulation of desmin intermediate filament. These encouraging results highlight the implication of MTM1 in autophagy-lysosome and ubiquitin-proteasome pathways and propose autophagy normalization as a therapeutic target for XLMTM. As drugs affecting these pathways are in clinical development for different diseases [129], it may benefit the field of centronuclear myopathies.
It is worth noting that DNM2 was also reported to be implicated in autophagy [68, 69]. Liver, skeletal muscle and fibroblasts of Dnm2 R465W KI mice presented a defect in autophagosome maturation due to a decrease in the autophagic flux [69]. As these data were mainly obtained on cells and mice homozygous for this specific DNM2 mutation, while DNM2-CNM is linked to different heterozygous mutations, the importance of autophagy in the DNM2-CNM forms remains to be confirmed. To this end it was shown that fasting in heterozygous animals induced an increase in LC3-II, p62 and autophagosomes in muscle [69]. These findings suggest a common molecular defect between XLMTM and ADCNM, and broaden the therapeutic interest of the autophagy normalization approach.

Targeting muscle growth pathways with Activin Receptor type IIB inhibition

Myostatin, a member of the TGFβ (transforming growth factor beta) family, is a highly conserved negative regulator of skeletal muscle bulk [130]. Furthermore, natural deficiency or pharmacological inhibition of the myostatin pathway has been reported to stimulate muscle growth by increasing myofiber size and muscle mass [131–135], which makes anti-myostatin based approaches very attractive to different muscle wasting conditions including Duchenne muscular dystrophy [136], amyotrophic lateral sclerosis [137], sarcopenia [138] and cancer cachexia [139, 140]. Indeed, various myostatin inhibitor approaches have been identified and tested in preclinical or clinical trials with varying efficacies, e.g. gene delivery of follistatin and homologues [135], myostatin propeptide [141, 142], myostatin antibodies [136] and more recently a soluble form of myostatin receptors [143, 144]. In Mtm1KO mice, administration of a soluble form of the extracellular region of the myostatin receptor (ActRIIB) fused to the Fc portion of human IgG resulted in a transient increase in muscle mass and force and myofiber size, that was reflected clinically by a significant albeit modest extension of lifespan [145], whereas no clinical improvements were noted following ActRIIB injection in the less severely affected Mtm1KI R69C mice apart from hypertrophy of gastrocnemius muscle [19]. Histology analysis of skeletal muscles of both treated Mtm1KO or Mtm1KI R69C mice showed that the hypertrophy was restricted to myofiber type 2b, suggesting that inhibiting myostatin has an effect mainly on fibers with low oxidative capacities. However, in XLMTM cases, muscle biopsies are characterized by predominance of highly oxidative type 1 fibers [1], which may compromise the efficacy of this strategy in XLMTM patients. An explanation for the modest benefit of downregulating the myostatin pathway in the XLMTM mice models is that, in the Mtm1KO at least, there is a strong decrease of myostatin and a corresponding increase of the myostatin antagonist follistatin, supporting that the myostatin pathway is already turn down in the disease and thus is not available for further inhibition [146]. Moreover, despite the substantial increase in myofiber type 2b size, no improvement of the excitation-contraction coupling related defects was noted, supporting myostatin inhibition as a potential additive strategy for increasing muscle bulk in combination with another strategy improving excitation-contraction coupling. In addition to its skeletal muscle expression, myostatin is also expressed at much lower level in the heart and adipose tissue. Myostatin null mice exhibit cardiac hypertrophy with changes in structure and contractile parameters [147]. Consequently, the benefit: risk balance for an anti-myostatin approach in XLMTM should be considered before application on patients.

**OTHER POTENTIAL THERAPEUTIC APPROACHES**

Acetylcholinesterase inhibition

Defects of NMJ have been documented in XLMTM patients and animal models of myotubular myopathy [16, 17, 148]. This chemical synapse is where a motoneuron activates muscle contraction upon the arrival of an action potential at the presynaptic neuron leading to a release of neurotransmitters. Its function is defective in congenital myasthenic syndromes (CMS) that share some clinical features with CNM. Previous studies described structural and functional defects of NMJ in
some patients with CNM without genetic diagnosis at that time [149, 150]. Using electron microscopy, muscle biopsies from CNM affected neonates showed shallow primary synaptic clefts with few large synaptic vesicles indicating a poor NMJ transmission. Furthermore, similar defects have been observed in a zebrafish model of XLMTM (mtmi hypomorphic morpholino model) or ADCNM transiently overexpressing the most severe DNM2 S619L mutation [16, 151]. In fact, these zebrafish models exhibit an abnormal distribution of acetylcholine receptors that was associated with a decrease in swimming capacities, while treatment with edrophonium, an acetylcholinesterase inhibitor, dramatically enhanced both spontaneous and touch evoked movement. Finally, additional studies confirmed the NMJ defect in two mouse models of XLMTM, Mtm1/KO and Mtm1/KI R69C [17, 148]. In Mtm1/KO, through either intravitral imaging or examination of muscle sections, NMJs appear larger and ultrastructural examination revealed simplification of post-junctional folds with presence of many vacuolar structures, resembling NMJ ultrastructure described in XLMTM patients. In all, these findings suggest a contribution of NMJ defects in the muscle weakness of CNM patients and a role of two CNM-causing genes (MTM1 and DNM2) in the regulation of NMJ. Whether NMJ defects are a primary defect of the nerve or secondary to muscle alteration is not yet known. Importantly, a significant clinical improvement was noted in a confirmed case with XLMTM following treatment with pyridostigmine, an acetylcholinesterase inhibitor which increases acetylcholine half-life within the synapse and is commonly used to treat myasthenia gravis and CMS [16]. Thus, acetylcholinesterase inhibitors may represent an effective therapy to treat symptoms for patients with CNM.

Cell transplantation or cell therapy

Cell transplantation has been tested in the clinic for many diseases with variable positive outcomes [152]. At present, the most successful trials have been achieved in hematological diseases using bone marrow cells which are relatively easy to harvest, manipulate and replace, and have good safety profiles and obvious selective advantages [153, 154]. In contrast, due to the wide tissue distribution and slow cellular turnover of muscle fibers, stem cell transplantation showed very limited beneficial effects when used in muscle disorders [155]. In this field, the majority of trials focused on Duchenne muscular dystrophy which is characterized by myofiber degeneration/regeneration and satellite cell pool exhaustion [156–159]. Albeit XLMTM is not a dystrophy, the number of satellite cells in three different muscles of XLMTM patients is significantly lower than controls [20]. The Mtm1/KO mice also present a decrease in satellite cells [18, 19]. In addition, Mtm1/KO muscle cells displayed decreased proliferation, increased apoptosis and poor cell engraftment indicating an inability of satellite cells to “rebuild” damaged skeletal muscle [18] and suggesting cell replacement as a potential therapeutic option. In the Mtm1KI R69C mice, a model of mild XLMTM, injection of syngeneic skeletal muscle-derived WT myoblasts into the gastrocnemius resulted in moderate improvements in skeletal muscle mass and force [160]. This encouraging, albeit limited, positive effect demonstrated the potency of this approach in XLMTM disease.

Furthermore, tremendous progress has been achieved in the isolation of muscle satellite cells and understanding the molecular and cellular mechanisms that control their activity [156, 161–164]. Combination of autologous cell therapy with other approaches like ex vivo genome editing or ex vivo gene replacement may present a safe and efficient way to achieve a successful cell therapy-based approach, while avoiding immunoreaction to exogenous cells in case of allotransplantation.

Interestingly, the myostatin inhibition-based approach described above showed a significant increase of satellite cells activation in XLMTM animals [19]. Importantly, myostatin inhibition increased the efficacy of myoblast transplantation in other myopathy models [165], indicating that inhibition of myostatin could potentiate myoblast transfer. Nonetheless, systemic delivery of stem cells to repopulate most affected muscles represents the major limitation of this approach due to the wide distribution required to target this tissue. Overall, this approach has a promising future and encouraging perspectives in XLMTM disease assuming methodological bottlenecks are solved.
CONCLUSIONS

Substantial advances have been achieved in the recent years in our understanding of the molecular pathomechanisms causing CNM, and have allowed the emergence of therapeutic strategies targeting different cellular defects (Fig. 1) and the creation of several companies dedicated to developing these approaches to clinic.

![Diagram](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6218136/)

**Fig. 1**

Conceptual figure depicting the different cellular defects reported in X-linked and/or autosomal CNM (in red) and indicating the various therapeutic strategies tested (in green). CNM fibers can be rounder and smaller, especially for the X-linked form. NMJ: neuromuscular junction.

However, while the overlap observed in clinical features, histology and several cellular defects between different CNM forms suggests a common pathway where the different mutated proteins may be involved, the functional interplay between these proteins and their links with the different cellular abnormalities are not well understood. Moreover, a significant number of individuals with CNM-like phenotype remains without genetic diagnosis, suggesting the implication of other genes. Identification of novel CNM genes and pathomechanisms will certainly contribute to the characterization of additional defective cellular pathways that represent new therapeutic targets.

Each of the therapeutic approaches has its own pros and cons (summarized in Table 2). Current bottlenecks for translation of therapeutic concepts achieved in cellular and animal models need to be overcome, such as increasing efficacy, decreasing on-target toxicity and off-target effects, benefit sustainability, tissue specific targeting, and vector/drug production and delivery. A first clinical trial has been started in 2017 for *MTMI* gene therapy, underlining that myotubular and centronuclear
myopathies are now under attack. The plethora of therapeutic targets recently identified should pave the way to future combinatorial therapies for the benefit of patients.

Table 2
Pros and Cons of the potential therapeutic approaches for centronuclear myopathies

<table>
<thead>
<tr>
<th>Approach</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTMR2 expression AAV-MTMR2</td>
<td>Single injection; longterm expression; partial organ-specific delivery</td>
<td>Not fine-tunable; no treatment interruption; immunity to AAV</td>
</tr>
<tr>
<td>PI3K inhibition Wortmannin; LY294002; PI-103</td>
<td>Fine-tunable; possible treatment interruption</td>
<td>Re-administration needed; poor organ specificity; on-target toxicity (lack of specific PIK3C2B inhibitor)</td>
</tr>
<tr>
<td>DNM2 reduction or normalization antisense oligonucleotides</td>
<td>Fine-tunable; possible treatment interruption; sequence substitution</td>
<td>Re-administration needed; poor organ specificity; on-target toxicity</td>
</tr>
<tr>
<td></td>
<td>AAV-shRNA</td>
<td>Not fine-tunable; no treatment interruption; immunity to AAV; on-target toxicity</td>
</tr>
</tbody>
</table>

Treating the general muscle unbalance

<table>
<thead>
<tr>
<th>Approach</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophagy activation mTOR inhibitors (RAD001, AZD8055)</td>
<td>Fine-tunable; possible treatment interruption</td>
<td>Re-administration needed; poor organ specificity; on-target toxicity</td>
</tr>
</tbody>
</table>

Myostatin inhibition ActRIIB-mFc | Fine-tunable; possible treatment interruption; in clinical use | Re-administration needed; poor organ specificity; on-target toxicity; good histological but poor functional improvements in XLMTM model |

Other potential approaches

<table>
<thead>
<tr>
<th>Approach</th>
<th>Pros</th>
<th>Cons</th>
</tr>
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<tbody>
<tr>
<td>Acetylcholinesterase inhibition pyridostigmine; edrophonium</td>
<td>Fine-tunable; possible treatment interruption; in clinical use for myopathies (FDA approved)</td>
<td>Symptomatic treatment</td>
</tr>
<tr>
<td>Cell transplantation syngeneic WT myoblasts</td>
<td>Ex vivo cell manipulation possible</td>
<td>Re-administration needed; systemic delivery difficult; longterm effect unknown</td>
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</tbody>
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Open in a separate window
Notes: Extent of positive outcomes in animal models do not necessarily translate in human. Pros and Cons and mode of delivery may change depending on results in pre-clinical development and methodological updates.

LIST OF ABBREVIATIONS

XLMTM: X-linked myotubular or centronuclear myopathy
CNM: centronuclear myopathy
ADCNM: autosomal dominant centronuclear myopathy
MTM1: myotubularin
BIN1: amphiphysin 2
DNM2: dynamin 2
NMJ: neuromuscular junction
PtdIns3P: phosphoinositide 3-monophosphate
PH-GRAM: pleckstrin homology- glucosyltransferases, Rab-like GTPase activators and myotubularins
ASO: antisense oligonucleotide
CMS: congenital myasthenic syndrome

DECLARATIONS

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and material
Not applicable

Competing interests
H.T., J.L. and B.S.C. are inventors of a patent on targeting DNM2 for the treatment of centronuclear myopathies. J.L. and B.S.C. are scientific advisors for Dynacure.

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Authors’ contributions
H.T. wrote the manuscript draft with editions by B.S.C and J.L.

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