Molecular Neurobiology (2019) 56:6460–6471
https://doi.org/10.1007/s12035-019-1533-2

Disease Modeling and Therapeutic Strategies in CMT2A: State of the Art

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Received: 13 November 2018 / Accepted: 19 February 2019 / Published online: 4 March 2019

Abstract

Mitofusin 2 (MFN2) is a protein of the mitochondrial outer membrane that belongs to a family of highly conserved dynamin-related GTPases. It is implicated in several intracellular pathways; however, its main role is the regulation of mitochondrial dynamics, in particular mitochondrial fusion. Mutations in *MFN2* are associated with Charcot–Marie–Tooth disease type 2A (CMT2A), a neurological disorder characterized by a wide spectrum of clinical features, primarily a motor sensory neuropathy. The cellular and molecular mechanisms by which *MFN2* mutations lead to neuronal degeneration are largely unknown, and there is currently no cure for patients. Here, we present the most recent in vitro and in vivo models of CMT2A and the more promising therapeutic approaches under development. These models and therapies may represent relevant tools for the study and recovery of defective mitochondrial dynamics that seem to play a significant role in the pathogenesis of other more common neurodegenerative diseases.

Keywords Mitofusin2 · Charcot–Marie–Tooth disease type 2 · Hereditary neuropathies · Mitochondrial diseases · Molecular therapy · Gene therapy · Mitofusin agonists

Abbreviation

CMT	Charcot–Marie–Tooth disease
MCNV	Motor nerve conduction velocity
MNs	Motor neurons
SNs	Sensory neurons
IPSCs	Induced pluripotent stem cells
ASO	Antisense oligonucleotide
RNAi	RNA interference
CRISPR	Clustered regularly interspersed short palindromic repeats
Cas9	Caspase 9
KO	Knockout

Introduction

Charcot–Marie–Tooth 2A (CMT2A) is an axonal peripheral neuropathy that belongs to the group of Charcot–Marie–Tooth diseases (CMT) [1]. CMT are part of a wide range of inherited sensory motor neuropathies with different clinical presentations and various genetic causes. The main clinical features include distal weakness, sensory loss, gait impairment, and foot deformities in the context of a very heterogeneous disease [2]. The first classification of CMT is based on the inheritance mode (autosomal dominant, autosomal recessive, and X-linked); however, the most used classification relies on the motor nerve conduction velocity (MNCV), thereby distinguishing a demyelinating form with a MNCV < 38 m/s (CMT1), an axonal form with a MNCV > 38 m/s (CMT2), and an intermediate form with a MNCV 25–45 m/s [3].

CMT disease is the most common inherited neuropathy and affects 1 in 2500 individuals [4]. CMT1A presents the highest prevalence and is mainly related to mutations in the gene of peripheral myelin protein 22 (*PMP22*) [5]. With respect to CMT2, the most frequent form is CMT2A (33% of CMT2 cases) [6], which is caused by mutations in the mitofusin 2 (*MFN2*) gene. Noteworthy, CMT2A accounts

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for the majority (91%) of severely affected CMT2 patients and only for a small amount (11%) of mild-to-moderate cases among CMT2 patients [7].

Züchner and colleagues first identified *MFN2*, located in the chromosomal region 1p36.2, as the causative gene of CMT2A [1]. Missense mutations are the most common *MFN2* mutations, followed by nonsense mutations and deletions [6]. The inheritance modality is almost always autosomal dominant (AD) [8], but autosomal recessive (AR) or semi-dominant cases due both to homozygous and to compound heterozygous *MFN2* mutations have been described as well [8–11]. *MFN2* mutations are not exclusive for CMT2A. They have been related to two other hereditary neuropathies, hereditary motor and sensory neuropathies (HMSN) V and VI, respectively, CMT2 with pyramidal signs and CMT2 with optic atrophy [12, 13].

MFN2 and its homolog mitofusin 1 (MFN1) are highly conserved transmembrane GTPase proteins of the outer mitochondrial membrane [14]. They share a common structure characterized by the following domains: N-terminal GTPase domain, first Heptad repeat coiled-coil region (HR 1), transmembrane domains, and second Heptad repeat coiled-coil region (HR 2). MFN2 has multiple functions. To date, its main well-known role is the regulation of the mitochondrial network with respect to mitochondrial fusion, mitochondrial transport along axons, and mitophagy [15]. Moreover, MFN2 participates in mitochondrial metabolism and intracellular signaling [15].

The complete pathogenesis of CMT2A remains unknown. A various range of alterations in MFN2 activity can equally drive to CMT2A development. Dominant mutations may lead either to a gain or to a loss of function according to the position of the mutation within MFN2 domains [16]. The same effects modulated by incomplete penetrance occur in case of recessive mutations [10]. To the best of our knowledge, no *MFN2* mutation ends in haploinsufficiency.

As concerns the clinical presentation, the typical features are length-dependent motor impairment and sensory loss. CMT2A patients present distal and slowly ascending weakness associated with hyporeflexia and muscle wasting in the same territories [17]. Gait impairment is typically related to foot-drop derived by paresis of the tibialis anterior [2]. A further clinical marker is represented by foot deformities, particularly *pes cavus* [2]. Other additional features with a lower incidence have been reported: *scapula alata*, bilateral facial weakness, vocal cord palsy, spasticity, deafness, and action tremor [17, 18].

Currently, there is no approved therapy for this disease; however, new approaches are now the focus of preclinical research. This review will provide an update on the in vitro and in vivo disease models developed to date and an overview of the therapeutic strategies that may be applied to CMT2A.

Models of CMT2A Disease

The lack of complete knowledge regarding CMT2A pathogenesis has clearly made it difficult to develop an effective curative treatment. The main obstacles are related to the heterogeneity of the disease due to the high number of mutations already identified and to the different clinical presentations among patients. Moreover, in CMT2A, the damage involves the axons of spinal motor neurons (MNs) and sensory neurons (SNs), which are hardly accessible in humans.

Due to the complexity of the disease, several CMT2A models have been developed, including gene knockout (KO) or gene-mutant in vitro models and in vivo models.

In Vitro Models

The generation of disease-specific in vitro models has recently been revolutionized by induced pluripotent stem cells (iPSCs) technology [19]. Through this approach, in vitro disease models can be obtained starting from easily accessible cells of patients and healthy subjects, such as blood cells and skin fibroblasts. These cells are induced to generate iPSCs, which notably share the same characteristics of embryonic stem cells (ESCs) and can be differentiated in the cell type of interest [19].

MNs differentiated from CMT2A-derived iPSCs were generated independently by two groups: Saporta et al., 2015, and Rizzo et al., 2016 [20, 21] (Table 1).

In particular, Saporta et al. worked on CMT2A MNs that carry the mutation arginine to tryptophan at position 364 (R364W) [20], whereas Rizzo et al. worked on CMT2A MNs that carry the mutation alanine to valine at position 383 (A383V) [21]. CMT2A MNs provide a cellular model closer to the disease than other previous models based on not-human cell types (e.g., MEFs and murine DRGs), based on not disease-relevant cell types (e.g., fibroblasts) or knockout samples [24–26]. In CMT2A MNs, one of the main processes affected was the transport of mitochondria along axons. Compared to controls, the anterograde and retrograde trafficking speeds were reduced and mitochondria tended to gather in perinuclear clusters [20, 21]. Furthermore, mitophagy was also impaired. Rizzo et al. found an enhanced rate of mitophagy (i.e., higher expressions of BECN1s, PINK1, PARK2, and BNIP3 than wild-type MNs), which resulted in mitochondrial depletion [21]. Rizzo et al. also investigated apoptosis and found decreased sensitivity of MFN2-mutants to apoptotic stimuli [21]. The latter anomaly described in CMT2A MNs regarded electrophysiological properties: Saporta et al. reported increased excitability likely related to voltage-dependent sodium and calcium channel abnormalities [20].

These impaired processes (mitochondrial trafficking, mitophagy, apoptosis, and ion channel dynamics) could be used as markers of disease when analyzing the potential

Table 1 Most relevant in vitro and in vivo models for CMT2A disease

	Substrate	Mutation	Remarks	Reference
In vitro models	iPSCs-derived MNs	R364W	Decrease of mitochondrial trafficking Impairment of electrophysiological properties, notably increased excitability, increased density of sodium currents, reduced inactivation of voltage-dependant sodium and calcium channels	Saporta et al. [20]
		A383V	Decrease of mitochondrial trafficking leading to perinuclear aggregation Increased mitophagy leading to mitochondrial depletion Decreased susceptibility to apoptosis	Rizzo et al. [21]
In vivo models	Drosophila	R94Q^{like} T105M^{like} R364W^{like} L76P^{like}	Motor impairment, mitochondrial loss at neuromuscular junctions and decreased oxidative metabolism Decreased rate of fusion leading to fragmentated and closely packed mitochondria Enhanced rate of fusion leading to giant round shape mitochondria and few tubular mitochondria throughout all the cytoplasm	El Fissi et al. [16]
	Mouse	R94Q restrained to neurons and with heterogenous (MitoCharc1) and homogeneous (MitoCharc2) expression	Age-related (from 5 months old on) motor impairment showed by MitoCharc1 and MitoCharc2 Postural and gait impairment showed specifically by MitoCharc2 Age-related (from 5 months old on) over-representation of axons smaller than 3.5 μm diameter Accumulation of mitochondria in the distal portions of sciatic nerve axons smaller than 3.5 μm diameter Increase in Aδ component ratio of MitoCharc1	Cartoni et al. [22]
		T105M restrained to neuroectoderm-derived cells	Gait impairment with shorter print length Reduction in mitochondrial content specifically in tibial axon Atrophy of muscle fibers of anterior tibialis Atrophy of muscle fibers, conversion of slow to mixed slow/fast fibers, decrease in sarcomeric actin expression, disruption of striatal mitochondrial organization and fusion of peripheral satellite cells to myofibers in the soleus	Bannerman et al. [23]
	Rat	R364W achieved in *Mfn2* through zinc finger nuclease-mediated genome editing in fertilized rat eggs	Motor impairment Age-related (from 40 weeks old on) active axonal degeneration with loss of myelinated axons in distal nerves Age-related (from 20 weeks old on) decrease of the amplitude of CMAP in the caudal nerve	Li et al. (http://www.psychogenics.com/abstract67.html)

therapeutic effects of novel therapies in vitro. Nevertheless, sensory neurons (SNs) generated from CMT2A-derived stem cells are missing. Their development would extend our possibilities of studying the disease and should be a major goal for future research.

In Vivo Models

Several in vivo models of CMT2A are available, as summarized in Table 1.

Drosophila and zebrafish transgenic models have been successfully generated [27–29]. Overall, they confirmed the central role of Mfn2 in mitochondrial dynamics and the strong link with the progressive loss of motor function [27–29].

The most recent Drosophila models have been provided by El Fissi et al. [16]. They generated flies that harbored four CMT2A-related mutations: R364W, arginine to glutamine at position 94 (R94Q), threonine to methionine at position 105 (T105M), and leucine to proline at position 76 (L76P). Mutations were inserted in the sequence of *marf*, the Drosophila gene homolog to human *MFN*. Although mutated flies showed several similar phenotypical and pathological features (Table 1), a relevant difference emerged when analyzing the mitochondrial morphology. Clusters of unfused mitochondria were reported in case of mutations located within the GTPase domain (R94Q and T105M). The decreased rate of fusion is likely to be caused by the loss of GTPase activity, whereas mitochondrial tethering leading to aggregate formation may be triggered by new properties acquired by MFN2-mutants, suggesting a loss of function by dominant negative effect rather than by haploinsufficiency [16]. This evidence confirmed in vivo the dominant negative effect of MFN2-mutants previously described in DRG cultures [25]. Opposing effects were induced by R364W and L76P mutations: large round-shaped mitochondria due to increased rate of fusion were detected, suggesting that some CMT2A-related mutations may exert a dominant positive effect on MFN2 activity [16]. Overall, this study shows that both *MFN2* loss and gain of function lead to phenotypical and pathological abnormalities in Drosophila models [16].

However, mammalian models represent a more relevant tool for human disease.

KO mice have been generated: knocking out *Mfn1* or *Mfn2* resulted in murine embryonal death, possibly due to a placental defect [24].

Transgenic mice that express specific mutations identified in CMT2A patients have been successfully created [22, 23]. In 2010, Cartoni and colleagues generated two lines of transgenic mice, MitoCharc1 and MitoCharc2, which expressed the mutation R94Q in the *MFN2* human gene in the heterozygous (MitoCharc1) and homozygous (MitoCharc2) genotypes. Transgene expression was induced only in neurons through a neuron-specific enolase promoter [22]. MitoCharc mice showed several phenotypical features, such as motor deficit and gait impairment, and pathological features, such as an increase in the mitochondria number in the distal part of sciatic nerve axons with diameters smaller than 3.5 μm, which were also overrepresented [22]. A strong limit of Cartoni's murine model is that the most relevant clinical data originated from MitoCharc2, which is the homozygote model: this contrasts with the typical dominant inheritance pattern and makes the model less reliable. Several years later, Bannerman and colleagues proposed another murine model with the mutation T105M in heterozygosis. The use of a nestin-cre-driven promoter resulted in the selective expression of $MFN2^{T105M}$ in cells of neuroectodermal origin [23]. The motor performance was first analyzed: the print length of mutant mice was decreased compared to wt mice, and researchers linked this result to one of the main typical clinical features of CMT2A patients, the *pes cavus* [23]. Pathological evidence concerned the mitochondrial content per tibial axon, which was reduced in mutant mice compared to wt mice, and several alterations attested in muscle fibers of the soleus and anterior tibialis [23].

A further step towards a better murine model would be the development of a mouse that expresses the *MFN2* mutant in all cell types and not selectively as exhibited in Cartoni's model (neuron-specific enolase promoter) and Bannerman's model (nestin-cre-driven promoter). Bannerman previously generated a mouse that expressed the *MFN2* mutant in neural and non-neural systems, and this attempt led to a severely diseased phenotype characterized by respiratory distress, ascites and the loss of mobility. The expression of $MFN2^{T105\ M}$ was then restrained to neuroectoderm-derived cells to obtain a less compromised murine model available for gait analysis [23].

Recently, a rat model of CMT2A, carrying the R364W mutation in the *Mfn2* gene, has been presented as an abstract (http://www.psychogenics.com/abstract67.html). This model is characterized by several motor deficits, active axonal degeneration in distal nerves, and gradual worsening of nerve conductions in the caudal nerve.

In general, all mammalian models present a milder phenotype than humans, particularly mice. An animal model of CMT2A that presents more accurate genetic features and develops a progressive, length-dependent axonal neuropathy will be a useful tool for investigating both the pathogenesis and new therapeutics for CMT2A.

Therapeutic Approaches

To date, no effective disease-modifying treatment for CMT has been achieved. Nevertheless, several studies on the development of potentially effective therapies are currently ongoing [30]. Besides classical pharmacological therapy, more

innovative fields of investigation include peptide therapy, molecular and gene therapy, and cellular therapy (Fig. 1).

Pharmacological Therapy

Several molecules have been investigated in the attempt to define novel pharmacological therapies.

To date, efforts have mainly focused on CMT1-related treatments. For example, ascorbic acid (AA) has been considered because it plays an important role in myelination [31]. However, evidence showed that AA does not ameliorate outcome parameters in CMT1A patients [32].

Progesterone has been a further target because it influences the expression of several myelin-related genes, such as *PMP22* and *MPZ*. In CMT1A transgenic mice, the

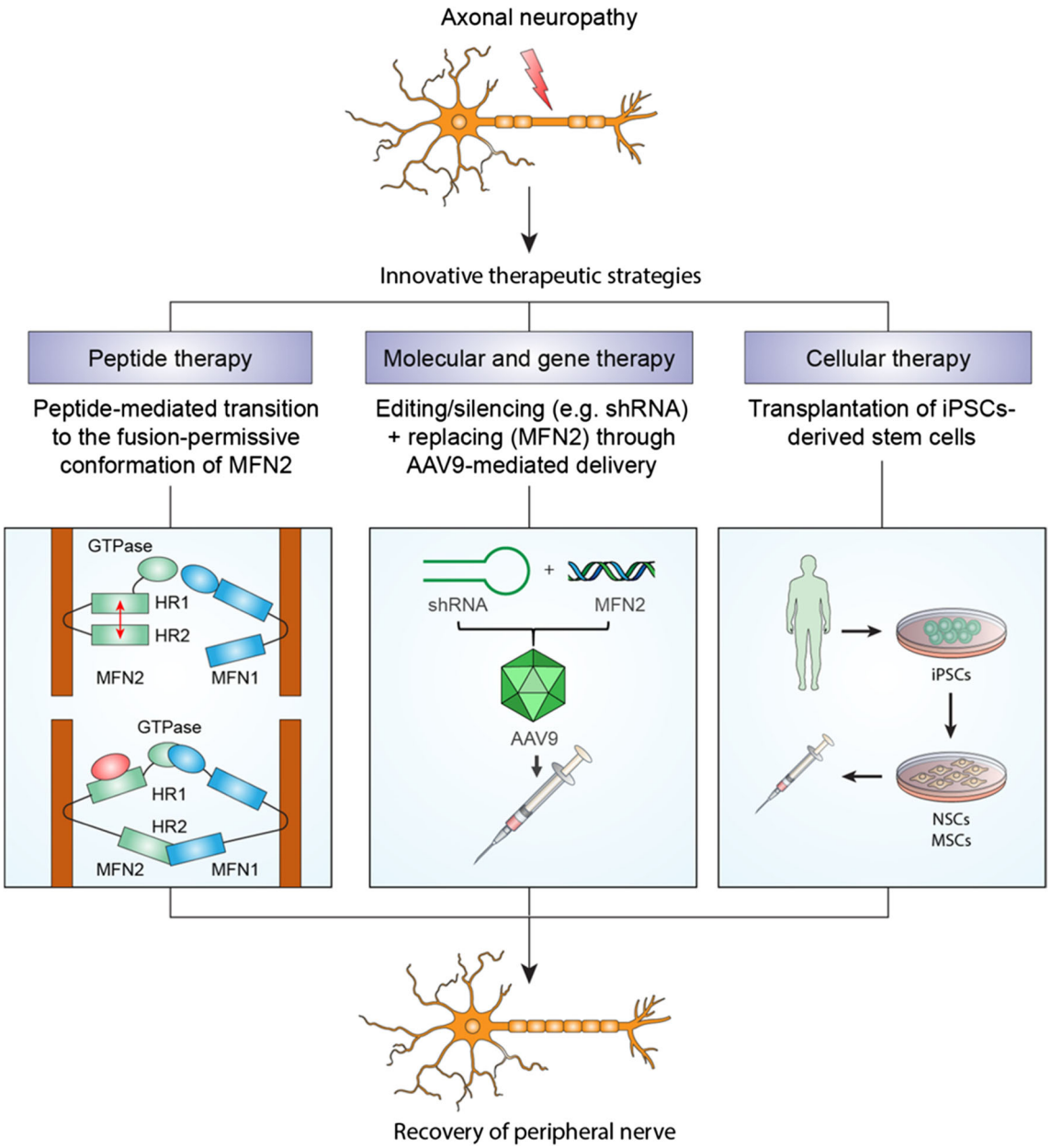

Fig. 1 CMT2A is a sensory motor neuropathy characterized by axonal damage. Given the natural history and the severity of the disease, CMT2A urgently requires a therapeutic solution, nowadays not available. The challenge of developing a successful treatment is even greater because of the lack of complete knowledge about the pathogenesis of the disease. Several innovative strategies may enhance the prospective of achieving a therapeutic solution. Peptide therapy aims to raise the HR2-HR2 interactions required for GTPase-dependent mitochondrial fusion. Molecular and gene therapy AAV9-delivered would restore the expression of wt *MFN2* while turning off the mutant-allele. Cellular therapy would substitute and sustain the affected neuronal population, possibly through NSCs or MSCs generated by iPSCs. Finally, the restoration of lost functions is expected thanks to peripheral nerves regeneration capacity

administration of progesterone worsened both pathological and clinical features, whereas the administration of progesterone antagonists led to an overall improvement [33]. A phase 2 clinical trial was carried out to investigate the effects of a progesterone antagonist (ulipristal acetate) in CMT1A patients; however, the data have not been provided to date (NCT02600286 at http://www.clinicaltrials.gov).

Melatonin has also been tested in CMT1A patients, resulting in the reduction of several markers of inflammation and oxidative stress, reasonably correlated with a possible pathological and clinical improvement [34]. In addition to these molecules more related to CMT1 treatment, other drugs could be more extensively addressed to all CMT patients.

Creatine administration and moderate home-based resistance training have been combined to improve the muscular performance of CMT1 patients, typically affected by muscle mass wasting [35]. The main effects were related to myosin heavy chain (MHC) contractile proteins: the data obtained showed that the combination of creatine administration and physical training modifies the MHC isoform expression (decrease in MHCI content and increase in MHCIIa content), which results in a muscle function improvement (reduced chair rise-times) in CMT1 patients [35]. Creatine could also potentially prove to be effective on CMT2 patients.

Coenzyme Q10 (CoQ10) effects on symptoms of weakness, fatigue, and pain have been investigated through a clinical trial extended to all CMT patients. CoQ10 is an electron acceptor in the mitochondrial electron transport chain. The results of the trial have not been published to date (NCT00541164 at http://www.clinicaltrials.gov).

Peptide Therapy

A new therapeutic approach aims to increase the rate of mitochondrial fusion through the administration of specifically designed peptides [36, 37]. Mitochondrial fusion is triggered by second heptad repeat (HR2)-HR2 intermolecular bindings between mitofusins on two opposed mitochondrial membranes. The HR2 domain can also be occupied by HR1-HR2 intramolecular binding. Therefore, according to the availability of the HR2 domain, mitofusins can transit from a fusion-constrained conformation (inactive) to a fusion-permissive conformation (active) [36]. This intrinsic structural plasticity was first exploited to generate a cell-permeant minipeptide (MP1) that competed with the HR1-HR2 interaction to promote the transition to the fusion-permissive status [36].

To allow permeability to cellular membranes, the minipeptide was associated with the TAT47–57 sequence (sequence of the HIV transactivator protein that allows transmembrane transfer). To obtain more flexibility, glycine residues were introduced in turn of leucine residues. The final molecule TAT-MP1Gly corrected the mitochondrial dysmorphology only in cultured rat neurons that expressed a wt allele of *MFN2* in addition to the mutated one (for example, $MFN2^{T105M}$). Notably, MFN-null cells were not rescued [36].

Recently, Rocha and colleagues discovered a new class of small-molecules, referred to as mitofusin agonists, which are able to allosterically overcome these HR1-HR2 interactions, leading to a definitive transition to the fusion-permissive conformation of MFN2 and an increase in fusion events [37]. Mitofusin agonists have shown promising results both in vitro and in vivo. They successfully reverted the formation of static mitochondrial clusters in cultured mouse neurons carrying $MFN2^{R94Q}$ or $MFN2^{T105M}$ and rescued the impairment of mitochondrial axonal transport in murine sciatic nerves carrying $MFN2^{T105M}$ [37]. Moreover, experiments performed on $MFN1^{-/-}$ and $MFN2^{-/-}$ cells showed that mitofusin agonists have no effects if wt MFNs are not available [37].

Additional studies are clearly needed to ensure the safety and efficacy. However, mitofusin agonists may represent a promising therapeutic strategy especially for dominant negative mutations. Rescue would be achieved through the stimulation of the wt fraction of the mitofusin pool in opposition to the dominant inhibition exerted by MFN2 mutants [37].

Molecular and Gene Therapy

Due to several innovative tools, one of the most promising fields of medicine is the treatment of genetic diseases. In general, in case of haploinsufficiency the therapy required is gene replacement [38], whereas in case of dominant effects (either positive or negative as seen in CMT2A [16]), the aim is to modulate the expression of the mutant-allele which interferes with the function of the wt-allele. This latter strategy could be suitable both for dominant CMT2A and for the fewer AR or semi-dominant cases related to incomplete penetrance [10, 16].

Mutant-allele modulation is the purpose of molecular therapy applied to CMT2A. Two main strategies are available: gene silencing and gene editing. Although molecular therapies are based on high specificity for the mutated allele, an unexpected reduction of the wt allele cannot be excluded. Therefore, the best solution would be to couple molecular and gene therapy, meaning that the treatment should address both silencing/editing (molecular therapy) and replacing (gene therapy). Notably, the DNA sequence administered as gene therapy should be manipulated through the insertion of silent mutations to resist molecular therapy while encoding the native amino acid sequence. This strategy, in which RNA interference (RNAi) inhibits both mutated and wt alleles while the native gene, ad hoc modified, is co-administered, has previously been used with good results in a model of Cu-Zn superoxide dismutase (SOD1)-related amyotrophic lateral sclerosis (ALS) [39]. These data raise hopes for the successful application of a similar strategy in CMT patients.

Strategies for Molecular Therapy

Different molecular systems can be used as therapeutic strategies in CMT2A, namely, antisense oligonucleotides (ASOs), RNAi, and clustered regularly interspersed short palindromic-Cas9 (CRISPR-Cas9) technology.

ASOs are synthetic short molecules of single-stranded RNA that modulate the target gene expression through several mechanisms, for example, by preventing mRNA translation through steric block of the ribosomes, covering the target sites of splicing, and triggering RNA-DNA hybrid degradation mediated by RNAse H [40]. These mechanisms are based on the specific complementary base pairing between the generated ASO and the mRNA carrying the information of the target protein [40].

During the years, the ASO structure was modified in several ways. Among others, great properties have been shown by morpholino phosphorodiamidate ASOs (PMOs). In PMOs, the main modifications achieved are related to the oligonucleotide backbone, in which the ribose was substituted with a morpholine ring, and the internucleotides linkages, represented by phosphorodiamidates in place of phosphodiesters [41]. Remarkably, morpholinos are substantially more resistant to cellular nucleases and proteases than previous ASOs [40]; thus, lower drug doses are necessary to achieve the same effect.

Currently, ASOs are among the most studied pharmacological tools, particularly in the neurological field. Recently, two new ASO-mediated therapies were approved for neurological diseases: nusinersen for spinal muscular atrophy (SMA) [42] and eteplirsen for Duchenne muscular dystrophy (DMD) [43].

Another promising technology is RNAi, a posttranscriptional regulatory pathway that results in target gene silencing. Small double-stranded RNA molecules are requested for target recognition: microRNA (miRNA), small interfering RNA (siRNA), and short hairpin RNA (shRNA) [44]. These sequences integrate in the RNA-induced silencing complex (RISC), where the two strands are divided so RISC can use the guide-strand to check the complementary or nearly complementary mRNA target sequence among the hundreds free in the cytosol. Once the target is recognized, the effector proteins of the RISC, the argonaute (AGO) proteins, conclude the silencing by blocking translation or degrading the mRNA [44].

Various classes of small RNAs are differently regulated, and they may originate endogenously (miRNA) or exogenously (siRNA, shRNA) [44]. Typically, siRNA- and shRNA-mediated recognition is based on a perfect match, whereas miRNAs may affect gene expression without a complete mRNA correspondence [45].

RNAi is a promising opportunity for gene-silencing for therapeutic purposes; however, several issues must be assessed to achieve the perfect RNAi-based therapy. The greatest limits include poor stability, as all RNAs are vulnerable to serum nucleases, and off-target effects, as inappropriate silencing of nontarget mRNA was quite frequent. Several chemical modifications (among others, ribose 2'-OH group modification and backbone modification through the introduction phosphorothioate linkage) can be introduced in the original dsRNA structure to obtain a higher success rate and less side effects [46].

CRISPR-Cas9 is a further powerful technique of gene editing. CRISPR-Cas9 is a site-specific engineered nuclease that can be properly programmed to recognize the gene site of interest, where it induces a DNA double-stranded break (DSB). The CRISPR are short repeated sequences of the bacterial genome. With CRISPR-associated proteins (Cas), CRISPR exerts an adaptive immune response against phagi via the cleavage of viral DNA. In particular, viral DNA sequences are properly recognized by two RNA constructs: the CRISPR targeting (crRNA) and the trans-activating RNA (tracrRNA). This machinery was improved by combining crRNA and tracrRNA into a single-guide RNA (sgRNA) and selecting type II Cas protein from *Streptococcus pyogenes* (SpCas9) [47, 48]. Thus, Cas9 is the gene editing effector, whereas sgRNA is specifically designed to recognize the target DNA sequence. SgRNA includes two portions: one portion (approximately 20 nt) is complementary to the target sequence, and the other portion is the photospacer adjacent motif (PAM), typically NGG (N could be any nucleotide) or NRG (R could be G or A). No recognition and no cleavage occur if a complementary PAM is not localized at the 3′ extremity of the targeted DNA sequence. This double recognition check reduces the risk of off-target effects; however, it cannot completely avoid them. Several strategies to reduce off-target effects have been proposed: among others, limitation of the Cas9-sgRNA concentration delivered, structural modifications of the complex and replacement by Cas9n (Cas9 nickase mutant) paired to two sgRNAs to attempt a single strand break [49].

Recently, CRISPR-Cas9 has also been tested for target gene activation (TGA) [50]. The purpose was to reduce off-target effects by avoiding DSBs. Therefore, with this new strategy, CRISPR-Cas9 was not used for gene editing; it was employed for a process defined by researchers as trans-epigenetic modulation: the CRISPR-Cas9 system was associated with a transcriptional activation complex to enhance the endogenous target gene expression. This innovated CRISPR-Cas9 TGA system was tested in vivo: murine models of muscular dystrophy, acute kidney disease, and diabetes showed phenotypical improvement after treatment [50]. Overall, CRISPR-Cas9 technology represents a relevant source for the development of future strategies for both gene therapy and epigenetic therapy.

Two phase 1 clinical trials focused on CRISPR-Cas9 technology are currently ongoing [51]. The first trial evaluates a

therapy based on ex-vivo CRISPR-edited autologous T cells for metastatic nonsmall cell lung carcinoma (NCT02793856 at http://www.clinicaltrials.gov). The second trial evaluates a therapy based on ex-vivo CRISPR-edited autologous T cells for multiple myeloma, melanoma, synovial sarcoma and myxoid/round cell liposarcoma (NCT03399448 at http://www.clinicaltrials.gov). The outcomes of these clinical trials will better indicate the future prospective for therapeutic strategies based on CRISPR-Cas9 editing.

The application of the CRISPR-Cas9 system to *MFN2* poses the problem of designing a specific guide in the presence of a single base difference in order to get the most specific therapeutic effect. Moreover, patients present different point mutations, which indicate it would be necessary to create several specific tools.

In theory, it is also possible to supply a repairing template vector; however, the efficiency of the proper correction is expected to be low in vivo, particularly in postmitotic cells, such as neurons.

Delivery Systems for Molecular and Gene Therapy

To enhance the clinical translatability of molecular and gene therapy, a high efficiency of intracellular delivery is required. For neurological diseases, an extremely relevant issue concerns the target site of therapy: the CNS is unique because of the low blood–brain barrier (BBB) permeability and the highly functional specialization of every brain and spinal cord area [52].

Specifically, ASOs must be injected directly beside the BBB, i.e., in the cerebrospinal fluid through lumbar puncture, without associated delivery systems [53]. In contrast, RNAi therapy, CRISPR-related therapy and gene therapy all require effective delivery systems into cells [52].

Given that the ideal properties for molecular transfer through the BBB are weak binding to serum protein, small dimensions and great lipophilicity, a solution is represented by delivery systems such as polymeric nanoparticles (NPs) and lipid complexes [52].

Viral vectors can also be used as delivery systems. Virus application enables exploitation of their ability to infect cells and induce viral genome transcription and translation through the host machinery. Retrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV) have all been tested as RNAi vectors [52].

For neurological diseases, the predominant solution is AAV. There are several advantages in their use: AAVs transfect both dividing cells (astrocytes) and nondividing cells (neurons), ensure a stable gene expression with each delivery, present low immunogenicity and no pathogenicity [54].

AAVs show different target-cell based on the capsid composition. Several serotypes have been identified according to this variability. The transfection of CNS cells is assured mainly by AAV2, AAV5, AAV8 and AAV9. Several studies have aimed to assess which AAV is more efficacious in CNS tissue transduction: in general, in murine samples, AAV8 shows a greater efficacy than AAV5 and AAV2 [54]; however, neurotoxic levels of green fluorescent protein (GFP) have been reported after AAV8 administration [55]. Extraneuronal transduction, also advisable in several neurological diseases, has been successfully obtained by AAV5 (in astrocytes) [56] and AAV1 (in oligodendrocytes and astrocytes) [57].

AAV9 is specifically studied for its uncommon ability to cross the BBB. Initial studies were performed on murine models through AAV9-GFP intravenous administration. A widespread distribution of GFP to all cell types, including dorsal root ganglia (DRGs), MNs and neurons in the brain, was identified in neonatal mice. In contrast, in adult mice, GFP preferentially localized to astrocytes in the entire CNS, and the neuronal distribution was very limited [58]. The intrathecal route has also been evaluated. Overall, both lumbar and cisternal intrathecal administrations of AAV9-GFP resulted in higher rates of GFP expression [59, 60]; however, the following specific pattern of expression was detected in the spinal cord: positive and intense expression in superficial cellular lawyers, with no expression in the deeper layers [61]. Thus, Miyanohara and colleagues suggested that a further barrier could oppose free AAV9-GFP distribution in addition to the BBB. They addressed the pia mater for this role and evaluated their hypothesis by testing a subpial (SP) delivery method for AAV9-GFP or AAV9-RFP (red fluorescent protein) in adult rats and pigs. Positive results were obtained as SP delivery resulted in a wider distribution of the transgene in the spinal parenchyma and descending and ascending axons [61].

Thus, if an SP delivery system is adequately implemented, it may replace intrathecal administration. Moreover, SP administration requires a local laminectomy; thus, the procedure is more invasive, although the greater transgene expression achieved through the SP route would lead to a reduced frequency of administrations [61].

The first AAV gene therapy was recently approved: Voretigene Neparvovec is a therapy for inherited retinal diseases associated with a RPE65 mutation (2% of AR retinitis pigmentosa and 16% of Leber's congenital amaurosis). The viral vector used for Voretigene is AAV2: it vehicles the human RPE65 cDNA into retinal pigment epithelial cells. The therapy is administered through an injection in the subretinal space [62].

In the neurological field, relevant results have been achieved in both preclinical and clinical research [38, 63–65].

AAV9 intrathecally administered could be exploited for the delivery of CMT2A-specific treatments. For the purpose, a combined approach of mutant gene silencing and wild-type gene replacement could be considered.

Cellular Therapy

Stem cell transplantation could be a potential new therapeutic strategy for CMT2A disease.

Regarding neuropathies, potential cellular sources for transplantation are represented by neural stem cells (NSCs) and mesenchymal stem cells (MSCs), which are expected to improve these diseases through several mechanisms. NSCs could replace specific neuronal cells and produce beneficial neuroprotective factors [66], substitute non-neuronal cells [67], and reduce toxic molecules in the microenvironment [68]. Specifically, neuroprotective factors produced by NSCs act as inhibitors of the neuronal damage-related GSK-3 and HGK kinase pathways [69, 70].

NSC transplantation has already been performed on murine models with significant results [68, 70].

MSCs may play a neuroprotective role in differentiating Schwann cells, producing antiapoptotic factors and inhibiting inflammation [71]. MSCs are more indicated for neurodegenerative demyelinating diseases, such as CMT1.

Stem cells for cellular therapy could be obtained from iPSCs and ESCs. The use of iPSCs technology avoids the ethical concerns strongly related to ESCs use for research and therapeutic purposes.

The first transplantation of iPSC-derived tissue in patients was performed in 2014 and consisted of retinal pigment epithelium (RPE) transplantation as a therapy for age-related macular degeneration [72]. It was temporarily suspended because genetic mutations were found in the iPSCs of a patient [72]. Other trials in cardiac and neurodegenerative diseases are ongoing [73].

The cell transplantation approach has not been explored in MFN2 preclinical models to date. Moreover, the mild phenotype of the currently available rodent models would pose challenges in evaluating the efficacy of this approach.

Outcome Measures and Prognosis

A useful instrument for the assessment of the disease, as well as in future clinical trials, is the Charcot-Marie-Tooth neuropathy score second version (CMTNS2) [74]. There are three items related to symptoms (sensory symptoms, motor symptoms at legs, and motor symptoms at arms), four items related to signs (pinprick sensibility, vibratory sensibility, leg strength, and arm strength) and two items related to neurophysiology (ulnar CMAP and radial SAP amplitude). Each item is scored on a 0–4-point scale (where 4 indicates the worst score). Patients are classified as mild (final score ≤ 10), moderate (final score 11–20), or severe (final score > 20) [74]. A further variant of the score, the CMTPedS, is provided for the pediatric age [75].

Although CMTNS2 shows high reliability for the assessment of disease severity, it should be further improved for the analysis of the on years-period [74]. Therefore, researchers are implementing new instruments in addition to the CMTNS2 to assess the prognosis and verify the response within clinical trials.

Among instrumental evaluations, quantitative muscle MRI can provide feasible outcome measures through the analysis of muscle atrophy and fatty substitution. The correlation of these parameters with both the clinical severity and the rate of progression is sufficiently strong to suggest MRI as a promising assessment tool [30].

Currently, new possibilities are emerging only for CMT1A. Several biomarkers available through skin biopsies have been described [76]. The cutaneous gene expressions of selected genes resulted to be linked with a high sensitivity (90%) to disease severity as assessed by the CMTNS, while a lower sensitivity was detected when used for the progression of the disease. However, given their maximum specificity of 100%, their reliability when used to assess the response to therapies should be good. Overall, five of these genes (*CDA*, *CTSA*, *ENPP1*, *GSTT2*, and *PPARG*) were suggested by the authors as a valid set of biomarkers for CMT1A severity and progression [76].

As similar solutions are not available for CMT2A disease, classic scores may be used to obtain useful information. Recently, Cornett and colleagues performed a longitudinal study ($n = 206$) concerning the natural history of CMT disease in the pediatric age [75]. The study was performed over a 2-year period and compared the CMTPedS at the baseline and the end of the observation period. Overall, the progression rate was assessed to 2.4 CMTPedS points (14%). This finding should be considered an important deterioration rate of the disease, expected by the clinical observation that an early-onset disease has a worst progression. The genetic subtype also affects the natural history of the disease: for example, one of the faster progression rates was detected in the CMT2A population (particularly during childhood rather than adolescence). In contrast, CMT1A patients showed the most favorable natural history of the disease. No gender differences were detected in any CMT form [75].

Conclusion

CMT2A is a severe neurodegenerative disease that causes progressive disability over time. It leads to wheelchair dependency in approximately 30% of cases [6]. In general, CMT2A-related disability severely impacts patient quality of life. No effective curative therapy is available, and researchers are looking for an effective solution.

Innovative molecular strategies seem more promising than traditional pharmacological treatments. We could potentially

work on several steps of the pathogenetic chain that lead from the mutation to the disease.

First, we could work at the origin of the disease: the gene itself. Treatments for genetic disease are currently feasible, and the same goal may be achieved for CMT2A through the coupling of mutant gene silencing and wild-type gene replacement.

Second, we could work on the protein (MFN2) through peptide therapy to improve the activity of the wt pool of mitofusins and overcome the pathogenetic mechanisms related to the abnormal activity of MFN2 mutants.

Third, we could work on the affected neuronal population, enriching it with substitutive cell elements by stem cell transplantation and providing neuroprotective factors secreted by the engrafted cells.

Hopefully, the progress of our knowledge concerning CMT2A will continue and ultimately will lead to phase 1 clinical trials in patients.

Acknowledgements We thank Associazione Progetto Mitofusina 2 Onlus and Associazione Amici del Centro Dino Ferrari for their support.

Funding This study is supported by grant "Ricerca Corrente 2019: Analisi dei pathway molecolari condivisi coinvolti nella patogenesi delle malattie neurodegenerative mediante modelli in vitro basati su cellule staminali" from the Italian Ministry of Health.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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