Received: 28 December 2022 | Revised: 16 February 2023 | Accepted: 24 February 2023

DOI: 10.1111/jns.12539

REVIEW

WILEY

Recent advances in the treatment of Charcot-Marie-Tooth neuropathies

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Funding information
AFM-Telethon, Grant/Award Number: 24110; Charcot-Marie-Tooth Association USA; Charcot-Marie-Tooth Research Foundation USA (CMTRF); Fondazione Telethon, Grant/Award Numbers: GGP19099, GGP20063, GSA21E002; Italian Ministry of Health, Grant/Award Numbers: RF-2016-02361246, RF-2019- 12369320; Muscular Dystrophy Association, Grant/Award Number: MDA 957250

Abstract

Charcot-Marie-Tooth (CMT) neuropathies are one of the most common neuromuscular disorders. However, despite the identification of more than 100 causative genes, therapeutic options are still missing. The generation of authentic animal models and the increasing insights into the understanding of disease mechanisms, in addition to extraordinary developments in gene and molecular therapies, are quickly changing this scenario, and several strategies are currently being translated, or are getting close to, clinical trials. Here, we provide an overview of the most recent advances for the therapy of CMT at both the preclinical and clinical levels. For clarity, we have grouped the approaches in three different categories: gene therapy based on viral-mediated delivery, molecular therapies based on alternative delivery systems, and pharmacological therapies.

KEYWORDS
Charcot-Marie-Tooth, therapy

1 | INTRODUCTION

Inherited peripheral neuropathies represent a vast group of very heterogenous disorders characterized by a complex phenotype where the neuropathy is one of many different clinical manifestations.[1] When the neuropathy is instead the predominant aspect of disease, it is usually referred to as Charcot-Marie-Tooth (CMT) disease.[2,3] CMT is also clinically and genetically very heterogenous, with more than 100 causative genes identified so far.[4] In CMT, usually both the motor and sensory components are affected, but it can also present with a predominantly motor or predominantly sensory neuropathy. On the basis of clinical and neurophysiological criteria, three main categories have been identified in CMT, namely demyelinating CMT1 with MNCV (Motor Nerve Conduction Velocity) at median nerves lower than 38 m/s; axonal CMT2 with MNCV higher than 38 m/s, and intermediate CMT-I, with MNCV ranging from 25 to 45 m/s. Independently of the primary cause of the disease either in the myelin or in the axon, the neuropathy progresses and impairs axonal physiology, leading to axonal degeneration and loss, which correlate with clinical disability.

Many progresses have been made in the last three decades in the identification of genes and mutations and in the elucidation of the disease pathogenesis, through the generation of animal and cellular models, that have been instrumental to study disease physiology.[5,6] However, therapies are not yet available for any CMT form. This can be due to several issues: CMTs are caused by more than 1500 mutations in at least 100 genes; the therapy should be delivered to the PNS (Peripheral Nervous System), that, as the CNS (Central Nervous System), is protected by the blood–nerve barrier; CMT models not always reproduce the severity of disease, particularly for axonal

CMT2, and, finally, there is the need of natural history studies for many CMT forms with the identification of biomarkers and of informative outcome measures to adequately monitor disease progression that is often very slow.[7–9] Nevertheless, many therapeutic strategies have been designed on the basis of recent advances in the understanding of disease pathogenesis, which have been or are in the process of being validated at the preclinical level. In some cases, promising data from preclinical studies have been already translated into clinical trials, for example, the PXT3003 drug repurposing strategy in demyelinating CMT1A; ACE-083, an inhibitor of negative regulators of skeletal muscle growth, in CMT1 patients; aldose reductase inhibition in the axonal neuropathy due to mutations in *SORD* (Sorbitol dehydrogenase); and the gene therapy for GAN, the giant axonal neuropathy (ClinicalTrials.gov Identifiers: NCT04762758; NCT03124459; NCT05397665; and NCT02362438).

In this review, we will describe these approaches, for clarity, we grouped into three main categories: gene therapy (viral-mediated delivery), molecular therapy (gene editing strategies) not based on viral delivery, and drug-based therapeutic approaches (Table 1). We will also provide the reader with a summary of the therapeutic attempts for the demyelinating CMT1A neuropathy, the most frequent form of CMT, which accounts for up to 50%–60% of cases (Table 2).

2 | GENE THERAPY FOR CMT

Viral vector-mediated gene therapy is based on the use of lentiviral vectors (LVs) and adeno-associated adenoviral vectors (AAVs). LVs have the advantage of a bigger capacity as compared to AAVs (up to 10 Kb) and of lower but stable expression levels of the transgene. However, LVs integrate into the genome, thus raising concerns for translatability into the clinics. LVs are used in cell-mediated ex vivo gene therapy approaches, using autologous cell transplantation.[64] This is the case, for example, of metachromatic leukodystrophy, where LV-transduced hematopoietic stem cells are used to replace defective arylsulfatase A (ARSA) lysosomal enzyme. ARSA is secreted by the donor hematopoietic LV-transduced cells and up-taken by recipient cells that express mannose-6-phosphate receptors, which are then trafficked from plasma membrane to lysosomes.[65]

On the other hand, AAVs, have a lower capacity (up to 4.7 Kb), do not integrate into the genome, can diffuse from the site of injection, and are characterized by low immunogenicity.[66,67] AAV's genome is single-stranded and must be converted in second-strand to be functional, which can limit transduction rate and expression. Self-complementary AAVs (scAAV) carry instead a double-stranded genome with two complementary transgenic sequences, which limit the capacity up to 2.6 Kb but have the advantage of a faster onset and sustained level of expression. Different AAV serotypes have been developed based on distinct sequences of the capsid protein to minimize immunogenicity and maximize the ability to cross the BBB (brain–blood barrier). The tropism of AAVs, which is the cell population that the viruses efficiently transduce, depends on the serotype of the virus itself and on the route of administration. Both have a great impact on transduction efficiency. These aspects have been recently reviewed by Hudry and Vandenberghe.[66] Concerning the PNS, in particular, the AAV9 serotype efficiently targets motor and sensory neurons and axons, but much less Schwann cells at least when administered systemically or in the cerebrospinal fluid (CSF) following intrathecal lumbar injection.

Proof-of-principle of AAV9-mediated gene therapy at the preclinical level has been provided in animal models of both demyelinating and axonal CMT neuropathies.[67,68] For the demyelinating forms, intrathecal route of administration has been used in CMT1A and CMT1X to target Schwann cells.[11,12,68] Of note, intrathecal and intravenous routes of administration have been recently compared in adult mice using AAV9 carrying an *Mpz* (myelin protein zero) Schwann cell-specific promoter and similar efficiency of Schwann cell transduction has been observed.[69] However, intraneural injection has been found to be the most efficient route of administration to specifically target Schwann cells in the demyelinating CMT1A.[10] For axonal forms, AAV9 vectors have been delivered by intracerebroventricular (i.c.v.) injection (CMT4J and CMT2D) or intrathecal injection through a lumbar puncture (CMT2D and GAN, Giant Axonal Neuropathy).[13–15]

2.1 | Gene therapy for demyelinating CMT

CMT1A is a demyelinating generally slowly progressive neuropathy caused by duplication of the *PMP22* gene, which encodes the peripheral myelin protein 22 (PMP22), specifically expressed in Schwann cells in the PNS as well as in other cells.[70] A 1.4 Mb duplication on chromosome 17p11.2 encompassing the *PMP22* gene represents the most frequent mutation responsible for approximately 50%–60% of all CMT neuropathies, depending on the population.[5,71] Thus, it is intuitive that most of the research efforts since the discovery of this gene in 1992 have been focused on CMT1A.[72–74] A gene dosage effect has been proposed at the basis of the CMT1A Schwann cell pathogenesis. Approximately 80% of the PMP22 protein is recognized as misfolded and physiologically eliminated through the proteasome, which in CMT1A Schwann cells, that carry three copies of *PMP22*, is overwhelmed and blocked resulting in the activation of cell stress mechanisms.[48,75,76] This has been nicely recapitulated in animal models, expressing extra copies of the *PMP22* gene.[5] These models have been particularly useful to assess that, in addition to the proteostatic stress due to PMP22 cytosolic accumulation, PMP22 overexpression impairs Schwann cell differentiation.[39] However, in human, depending on the nerve, three *PMP22* copies do not necessarily correspond to the same extra level of *PMP22* mRNA, thus highlighting the fact that the regulation of *PMP22* expression might be nerve and tissue-dependent and may account for the variability observed among different patients carrying the same 1.4 Mb duplication.[71] This of course implies that therapeutic strategies aimed at lowering PMP22 levels could benefit CMT1A in principle, but with variable outcomes depending on tissues/nerves and stage of disease.

TABLE 1 Summary of the therapeutic strategies for CMT neuropathies (in vivo).

Category	CMT type	Compound	Action	Delivery	References
Gene therapy	CMT1A	AAV2/9 shRNA	To decrease PMP22 expression	Intraneural	10
Gene therapy	CMT1A	scAAV9 miRNA	To decrease PMP22 expression	Intrathecal	11
Gene therapy	CMT1X	AAV9, CX32	To replace CX32	Intrathecal	12
Gene therapy	GAN	scAAV9/JeT-GAN	To replace GAN1	Intrathecal	13
Gene therapy	CMT2D	scAAV9 RNAi	To target allele specific mutations in *GARS*	Intracerebroventricular and intrathecal	14
Gene therapy	CMT4J	AAV9 FIG4	To replace FIG4	Intracerebroventricular and intrathecal	15
Gene therapy	CMT1E, CMT1X, CMT2D	AAV1.tMCK.NT-3	To deliver NT3	Intramuscular	16–18
Molecular therapy	CMT1A	ASO	To decrease PMP22 expression	Sub-cutaneous	19
Molecular therapy	CMT1A	siRNA squalene nanoparticle	To decrease PMP22 expression	Intravenous	20
Molecular therapy	CMT1E	siRNA	To target mutated allele	Intraperitoneal	21
Molecular therapy	CMT1A	CRISPR/Cas9 RNP	To decrease PMP22 expression	Intraneural	22
Molecular therapy	CMT2A	SARM1 KO	To ablate SARM1 NADase activity at the basis of axonal degeneration	Transgenic	23
Molecular therapy	CMT2A	MFN1	To increase MFN1 expression	Transgenic	24
Drug therapy	CMT1A	PXT3003	To decrease PMP22 expression	Oral	25–28
Drug therapy	CMT1A	Ascorbic acid	To decrease PMP22 expression	Intraperitoneal and oral	29–33
Drug therapy	CMT1A	Onapristone	To decrease PMP22 expression	Intraperitoneal	34
Drug therapy	CMT1A, CMT1E	Fasting diet	To activate autophagy	Oral	35
Drug therapy	CMT1E	Rapamycin	To activate autophagy	Intraperitoneal	36
Drug therapy	CMT1A	AUY922	HSP90 inhibitor	Intraperitoneal	37
Drug therapy	CMT1A	A438079	P2X7 channels inhibitor	Intraperitoneal	38
Drug therapy	CMT1A	hrNRG1	To improve differentiation and myelination	Intraperitoneal	39
Drug therapy	CMT4B1 HNPP	Niacin	TACE secretase activator	Intraperitoneal	40
Drug therapy	CMT1A	Lipids	To increase lipid expression	Oral	41
Drug therapy	CMT4B1	PIKfyve inhibitors	To decrease $PI3,5P_2$ levels	Oral	42
Drug therapy	CMT1E, CMT1B, CMT1A	Curcumin	To decrease UPR and cell stress	Oral	43–45
Drug therapy	CMT1A, CMT1B	Sephin1/IFB-088	To decrease UPR and cell stress	Oral	46–48
Drug therapy	CMT1B	Sildenafil	To increase proteasome activity	Intraperitoneal	49
Drug therapy	CMT1A, CMT1B, CMT1X	PLX5622	To decrease inflammation-CSF1R inhibitor	Oral	50–52
Drug therapy	CMT2F, CMT2A, CMT2D	Tubastatin-A	HADC6 inhibitor. To increase tubulin acetylation	Intraperitoneal	53–56
Drug therapy	CMT2F	ACY-738, ACY-775, ACY-1215	HADC6 inhibitor. To increase tubulin acetylation	Intraperitoneal	57
Drug therapy	CMT1A	CKD-504	HADC6 inhibitor. To increase HSP90/70 acetylation	Oral	58
Drug therapy	CMT1A	RGFP966	HDAC3 inhibitor. To improve Schwann cell differentiation	Sub-cutaneous and intraperitoneal	59

(Continues)

TABLE 1 (Continued)

Category	CMT type	Compound	Action	Delivery	References
Drug therapy	CMT2A	MFN2 agonists, MiM111	To increase function of MFN2 and MFN1	Intramuscular	60,61
Drug therapy	CMT2, SORD	Aldolase reductase inhibitor	To reduce sorbitol accumulation	Oral	62,63

TABLE 2 Summary of the therapeutic strategies for CMT1A/CMT1E neuropathies (in vivo).

Category	CMT type	Compound	Action	Delivery	References
Gene therapy	CMT1A	AAV2/9 shRNA	To decrease PMP22 expression	Intraneural	10
Gene therapy	CMT1A	scAAV9 miRNA	To decrease PMP22 expression	Intrathecal	11
Molecular therapy	CMT1A	ASO	To decrease PMP22 expression	Sub-cutaneous	19
Molecular therapy	CMT1A	siRNA squalene nanoparticle	To decrease PMP22 expression	Intravenous	20
Molecular therapy	CMT1E	siRNA	To target mutated allele	Intraperitoneal	21
Molecular therapy	CMT1A	CRISPR/Cas9 RNP	To decrease PMP22 expression	Intraneural	22
Drug therapy	CMT1A	Ascorbic acid	To decrease PMP22 expression	Intraperitoneal and oral	29–33
Drug therapy	CMT1A	Onapristone	To decrease PMP22 expression	Intraperitoneal	34
Drug therapy	CMT1A	PXT3003	To decrease PMP22 expression	Oral	25–28
Drug therapy	CMT1A CMT1E	Fasting diet	To activate autophagy	Oral	35
Drug therapy	CMT1E	Rapamycin	To activate autophagy	Intraperitoneal	36
Drug therapy	CMT1A	AUY922	HSP90 inhibitor	Intraperitoneal	37
Drug therapy	CMT1A	A438079	P2X7 channels inhibitor	Intraperitoneal	38
Drug therapy	CMT1A	hrNRG1	To improve differentiation and myelination	Intraperitoneal	39
Drug therapy	CMT1A	Lipids	To increase lipid expression	Oral	41
Drug therapy	CMT1E CMT1B	Curcumin	To decrease UPR and cell stress	Oral	43–45
Drug therapy	CMT1A CMT1B	Sephin1/IFB-088	To decrease UPR and cell stress	Oral	46–48
Drug therapy	CMT1A CMT1B CMT1X	PLX5622	To decrease inflammation-CSF1R inhibitor	Oral	50–52
Drug therapy	CMT1A	CKD-504	HADC6 inhibitor. To increase HSP90/70 acetylation	Oral	58
Drug therapy	CMT1A	RGFP966	HDAC3 inhibitor. To improve Schwann cell differentiation	Sub-cutaneous and intraperitoneal	59

Two very promising preclinical studies have recently reported proof-of-principle data of the efficacy of gene therapy-based strategies to lower PMP22 levels.[10,11] RNA interference (RNAi) is a fundamental process of gene silencing mediated by endogenous miRNA (microRNAs), small non-coding molecules that negatively regulate gene expression at post-transcriptional level.[77,78] Physiological microRNAs can be artificially modified in the form of siRNA, shRNA and synthetic miRNA and targeted to specific gene regions to downregulate expression. The first study by Gautier et al., is based on intraneural delivery in sciatic nerves of AAV2/9-shRNA targeting the mouse *Pmp22* gene, which is overexpressed in the transgenic CMT1A rat, a bona fide model of the CMT1A neuropathy.[10,79] Of note, the injected vector efficiently spread into the nerve and remained localized in the tissue, thus also avoiding a humoral immune response. A single injection was performed in rat pups at P6/P7 and the observed beneficial effects lasted up to 1 year. In AAV2/9-shRNA transduced CMT1A rat nerves, PMP22 overexpression was normalized to wild-type levels and morphological and neurophysiological analyses showed a significant amelioration of the demyelinating phenotype. Finally, transcriptomic analysis of biomarkers from forepaw skin biopsies showed a nice correlation with the treatment outcome at 12 months of age.[10] The local intraneural delivery of AAV9 has the

advantage to use a lower viral dose, vectors are confined in the tissue of injection, and that myelin-forming Schwann cells are very efficiently transduced. However, local delivery into the nerve has the limitation of being restricted to sciatic nerves and to be hardly translatable to human.

A second very interesting study by Stavrou et al. recently reported beneficial effects of scAAV9-miRNA, targeting both mouse and human *Pmp22*/*PMP22* gene, delivered in the C61 mouse model of CMT1A, that carries four copies of the human *PMP22* gene.[11] Viral vectors were administered intrathecally at P60 by performing lumbar injection and outcome measures were scored after 4 and 8 months. Also, delivery at 6 months, at a more advanced stage of disease, was tested. Several outcome measures, including mRNA and protein expression levels, neurophysiology, morphology, and biomarker analyses, showed significant amelioration of the phenotype, with a better improvement when mice were treated earlier rather than later at 6 months.[11]

Another demyelinating CMT neuropathy for which AAV9-mediated gene therapy approach has been explored is CMT1X.[12,68] This X-linked type of CMT represents the second most common form and is the consequence of mutations in the *GJB1* gene, which encodes the CX32 (connexin 32) protein.[5] CX32 is expressed by Schwann cells in the nerve as in other cells. In non-compact myelin such as Schmidt–Lanterman incisures and paranodal regions, CX32 forms gap junction channels that allow fast communications of small molecules such as metabolites, ions and others across multiple layers of the Schwann cell plasma membrane. Both loss-of-function and gain-of-function mutations have been reported for *GJB1*. Gain-of-function mutations cause the retention of the mutant protein in the ER and/or Golgi compartments and impaired trafficking of the wild-type protein. Other mutations result in the correct delivery of mutated CX32 to the plasma membrane, but with altered biophysical properties. Thus, even if males have one copy of the mutant allele, if the mutation has a dominant negative effect, the overexpression of a wild-type transgene may not be sufficient to fully revert the phenotype as the endogenous mutant protein may interfere with it.[68] However, the majority are loss-of-function mutations which are nicely modeled by the *Cx32* KO mouse. Proof-of-principle of AAV9-mediated gene replacement therapy has been obtained using this model.[12] Intrathecal injection at 2 and 6 months of age of AAV9 expressing CX32 under the rat *Mpz* Schwann cell-specific promoter, of 1.2 Kb in size significantly ameliorated the demyelinating phenotype. Kagiava et al., reported an improvement of muscle force and of MNCV, which was more pronounced with an early rather than late treatment. From the histological point of view, CX32 AAV9-mediated transduction reduced aberrant myelination and inflammation of mutant nerves.[12]

2.2 | Gene therapy for axonal CMT

Proof-of-principle of AAV9-mediated gene therapy approaches has been provided also for axonal neuropathies. Giant axonal neuropathy (GAN) is a very rare form of autosomal recessive neuropathy caused by loss-of-function mutations in *GAN1*, which encodes an E3 ubiquitin ligase. Hallmark of GAN is the presence of enlarged axons which are abnormally packed with microtubules and intermediate filaments.[6,13,67,80] Human and mouse models heterozygous for a loss-of-function allele are normal, suggesting that the increase of wild-type protein by exogenous delivery may be beneficial. Indeed, preclinical studies demonstrated that AAV-mediated GAN1 delivery reverted the enlarged axon hallmark phenotype.[13] Of note, a scAAV9/JeT-GAN safety and efficacy clinical trial is ongoing for GAN with first patients already treated intrathecally (ClinicalTrials.gov Identifier: NCT02362438).

A second type of axonal CMT for which a gene therapy approach has been validated at the preclinical level is the autosomal dominant CMT2D neuropathy, caused by heterozygous mutations in *GARS*, which encodes the glycyl-tRNA synthase.[14,67] Mutated GARS in vitro displays reduced enzymatic activity, but null alleles in human and mouse do not cause neuropathy. Moreover, overexpression of the wild-type protein does not ameliorate the neuropathy phenotype in mouse, suggesting a gain-of-function mechanism rather than haploinsufficiency and that the specific deletion of the mutant allele would be beneficial. Morelli et al., provided proof-of-principle of this strategy using two mouse models, one carrying a mutant $Gars^{P278KY}$ allele that does not correspond to a human mutation but causes neuropathy in mouse, and a second exhibiting a four amino acid GARS deletion (p.Glu299_Gln302del) found in a patient with a severe early onset CMT2D phenotype.[14] In both cases, RNAi specifically targeting the two alleles were packaged into scAAV9 and delivered either by i.c.v. (intracerebroventricular) injection at birth or by intrathecal injection at 5 and 9 weeks of age, postdisease onset. Mice treated at birth displayed a significant improvement of the neuropathy phenotype, according to behavioral, neurophysiological, and morphological analyses of nerves and of neuromuscular junctions in both mutant models. On the contrary, the postonset treatment only partially ameliorated the phenotype. If this is due to decreased transduction and/or allele targeting efficiency or to the inability of the strategy to act on axonal/neuronal degeneration when delivered postonset remains to be clarified.

Another example of AAV9-mediated gene therapy is autosomal recessive CMT4J, due to loss-of-function mutations in *FIG4*, encoding a phosphatase acting on PtdIns(3,5)P_2 phosphoinositide.[15] Mutations in *FIG4* in human are associated with different phenotypes ranging from CMT4J, ALS (Amyotrophic Lateral Sclerosis), Yunis-Varon syndrome, and epilepsy.[81] Phenotypes may correlate with the type of mutation and the residual FIG4 enzymatic activity. Studies in yeast and mammalian cells showed that FIG4 is also an activator of PIKfyve, the kinase which generates PtdIns(3,5)P_2 and that cells lacking FIG4 displayed reduced levels of this lipid.[82] Decreased PtdIns(3,5)P_2 levels are associated with a distinctive cellular phenotype, the vacuolization of the late endosomal-lysosomal compartment due to the impaired activation of a lysosomal calcium channel on the organelle and the consequent influx of water causing enlarged size and vacuolization.[81] The *plt* (Pale tremor) mouse is a spontaneous mutant due to loss-of-function of FIG4 and characterized by a severe CNS phenotype consisting of spongiform degeneration due to extensive vacuolization of

neurons and peripheral neuropathy, thus modeling the more severe Yunis-Varon disease rather than CMT4J.[82,83] AAV9-mediated replacement of FIG4 in the *plt* mouse by i.c.v. injection at P1 and P4 significantly ameliorated lifespan and growth over 12 months.[15] Intrathecal injection at P7 and P11 was also performed but the effect on survival and growth were different, suggesting that treatment is effective if FIG4 is provided earlier rather than later, as expected by this severe phenotype. Almost a full rescue was observed in *plt* mutants treated at P1 or P4 based on behavioral, neurophysiological, and morphological analyses. Of note, vacuolization of brain, spinal cord, DRG (Dorsal Root Ganglia), and sciatic nerves was almost completely prevented by the therapy, whereas a minor but still significative effect was observed following intrathecal injection at P7, particularly in spinal cord and nerve, and at a lesser extent in the brain. However, even if overexpression of FIG4 in a wild-type background did not result in toxic effect in neurons, hepatotoxicity and HCCs (Hepatocellular carcinomas) were observed in some treated mice at 12-month postinjection, likely due to the high viral titer used and the combination of strong ubiquitous promoters such as CMV and CBA.[15] Another consideration is that demyelination is also present in CMT4J nerves, which is not only the consequence of axonal degeneration. Consistent with this, conditional ablation of *FIG4* in mice showed a Schwann cell autonomous role for FIG4.[84] Thus, whether also Schwann cell-specific targeting might be necessary to treat CMT4J in human represents an unsolved question.

A more transversal gene therapy approach which can be beneficial to different CMTs independently on the specific etiology is based on the intramuscular delivery of NT3 (Neurotrophin 3).[85] NT3 is one of the autocrine factors that Schwann cells secrete to survive and proliferate in the immature stage of development, which is a prerequisite for myelination.[86] Sahenk et al., hypothesized that supplementation of exogenous NT3 could maintain terminal Schwann cells in a growth-promoting state, thus overcoming the loss of regeneration capacity following the chronic progression of the neuropathy.[85] Independently on the primary cause of the neuropathy, demyelinating or axonal, length-dependent axonal degeneration and fiber loss is the common consequence, which correlate with clinical disability. Heterozygous and homozygous null mice for NT3 display axonal degeneration and motor neuropathy, with loss of nerve terminals, thus corroborating the evidence for NT3 as a key factor for terminal Schwann cells. Sahenk et al., validated a gene therapy approach based on scAAV1.tMCK.NT-3 delivery by intramuscular injection in the *Tr-J* (Trembler-J), a model of CMT1E (point mutations in the *PMP22* gene), in CX32 KO (CMT1X) and in CMT2D (*GARS*) models.[16–18] A phase I/IIa trial evaluating the safety scAAV1.tMCK.NT-3 has been then proposed on the basis of results from preclinical studies (ClinicalTrials.gov Identifier: NCT03520751).

3 | MOLECULAR THERAPY IN CMT

As previously mentioned, one of the biggest challenges for the treatment of peripheral neuropathies is the correct delivery of the desired therapeutic tool to Schwann cells. Although AAV9 has shown great promise in the treatment of preclinical models of CMT, concerns about their long-term safety still remain.[87] The identification of alternative delivery approaches is therefore highly desirable, and synthetic nucleic acids such as antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), two of the most widely used strategies for silencing gene expression, represent ideal candidate tools to test different delivery methods.

As already discussed in the AAV-mediated gene therapy section, the decrease of *PMP22* expression represents a candidate therapeutic strategy in CMT1A. Interestingly, several approaches using ASO or siRNAs aimed at lowering *PMP22* mRNA expression have been proposed. ASOs are synthetic single-strand oligonucleotides that recognize a complementary sequence on the mRNA target in a Watson and Crick base pairing, usually leading to RNase–H activation and subsequent mRNA degradation.[88] Subcutaneous injection of ASO effectively suppressed *PMP22* mRNA in affected nerves in two murine models of CMT1A, the C22 mouse and the CMT1A rat.[19] In particular, weekly subcutaneous injection in symptomatic mice of an ASO targeting the 3′ UTR of the human *PMP22* gene (which is overexpressed in the C22 mouse), restored MNCV and CMAP (Compound Motor Action Potential) almost to the levels seen in wild-type animals, and corrected several other endpoints including motor capacity, pathology, and transcriptomic changes. Importantly, at the highest doses of ASO, neuropathy endpoints where not only arrested but reversed, suggesting that therapy in patients may not only arrest disease progression but result in some functional improvements.[19] Independent studies on the CMT1A rat also showed that ASO-mediated reduction of *Pmp22* mRNA partially restored myelination and electrophysiology.[19] Of note, in this work, the authors also identified several disease biomarkers that appeared to be modulated by ASO treatment and could be potentially used to monitor disease progression and efficacy of treatments.[19] Despite these exciting results, important challenges remain for the translation of this approach. Specifically, the need for recurrent injection, the mode of administration, the ability to reach all Schwann cells in the body as well as the level of silencing of *PMP22* need to be further explored. In fact, while PMP22 overexpression is the cause of CMT1A, PMP22 haploinsufficiency results in a different neuropathy, namely Hereditary Neuropathy with Liability to Pressure Palsies (HNPP). As such, the levels of PMP22 need to be carefully controlled. Finally, the possibility of off-target effect and the fact that some ASO can have mild to moderate side effect, including thrombocytopenia[89] need to be taken into consideration.

The use of small-interfering RNA (siRNA) represents another approach that has shown promising results in pre-clinical studies of CMT1A (see also above). Differently from ASO which are single-stranded synthetic nucleic acids, siRNAs are small double-stranded RNAs that can selectively silence the expression of a targeted gene by degrading its mRNA. Boutary et al. recently used intravenous (i.v.) injection of *PMP22* siRNA to treat two mouse models of CMT1A, the JP18 and JP18/JY13 mice, that carry one and two extra copies of *PMP22*, respectively.[20] Since hydrophilicity and short plasmatic half-life are important limitations of siRNA, the authors stabilized the

siRNA by conjugating them to squalene nanoparticles (SQ-NP). Treatment of mice with siRNA PMP22-SQ NP normalized PMP22 expression, improved motor function and limb strength, and restored CMAP and sensory conduction velocities. Importantly, the positive effects of the treatment lasted for at least 3 weeks after the last injection.[20]

The use of siRNA has also been explored for the selective knockdown of the disease-causing allele, sparing the wild-type allele, in the Trembler-J (*Tr-J*) mouse, a naturally occurring authentic model of CMT1E due to an L16P mutation in *PMP22*.[90] Allele-specific siRNA was intraperitoneally injected in postnatal Day 6 (P6) *Tr-J* pups and resulted in reduction of mutant PMP22 expression, amelioration in motor function and increase in NCV and CMAP, accompanied in some ameliorations in myelin pathology.[21]

Finally, a recent work showed that *PMP22* downregulation can also be achieved by targeting the TATA-box of the *PMP22* P1 promoter. *PMP22* in fact has two promoters, P1 and P2, with P1 being Schwann cell-specific. Lee et al. used intraneural injection of CRISPR/Cas9 ribonucleoprotein (RNP) complexes in sciatic nerves of C22 mice at P6 and P21. The P6 treatment resulted in improvements in NCV and CMAP, and in amelioration of myelin pathology, with the P21 treatment also able to provide some benefits.[22] While this would have the advantage of being a one-off treatment, the translatability of intraneural injection in humans remains to be explored.

4 | PHARMACOLOGICAL APPROACHES IN DEMYELINATING CMT

4.1 | Demyelinating CMT1A: to decrease *PMP22* expression

In addition to gene and molecular therapies aimed at silencing *PMP22* as described above, other approaches have been tested to decrease *PMP22* expression and improve CMT1A Schwann cell differentiation. One of the first attempts made was based on the use of ascorbic acid (AA), which was shown to inhibit adenylate cyclase and *PMP22* gene expression, whose promoter is responsive to cAMP levels.[19] However, despite promising data at the preclinical level supporting the rational for this strategy, clinical trials based on different amount of AA in different patient cohorts failed to demonstrate efficacy.[30–33] Importantly, these studies revealed the need for clinical trial development of alternative outcome measures and biomarkers informative enough to detect small changes in this slowly progressive clinical form.[9]

Interestingly, the *PMP22* promoter is also responding to progesterone, which can be inhibited by onapristone. This compound was shown to be effective at preclinical level in CMT1A rat.[34] However, due to safety concerns, onapristone has not been tested in clinical trials in CMT1A patients.

A system biology approach screened for repurposed drugs promoting differentiation and decreasing *PMP22* expression. A combination of compounds including baclofen, a GABA receptor agonist which reduces *PMP22* expression; naltrexone, believed to potentiate baclofen activity, and D-sorbitol, a metabolite involved in the polyol pathway thought to stabilize misfolded protein, was selected under the name of PXT3003.[25–27] Following proof-of-principle at preclinical level, a phase 2 study was conducted that confirmed safety and tolerability of PXT3003 and showed improvement of Charcot-Marie-Tooth Neuropathy Score (CMTNS) and the Overall Neuropathy Limitations Scale (ONLS) using the highest dose for longer treatment period.[27,28] Clinical trials phase III are now ongoing to assess the efficacy of this treatment in CMT1A patients (ClinicalTrials.gov Identifier: NCT04762758).

4.2 | Demyelinating CMT1A: to improve clearance of PMP22 aggregates

As previously discussed in the gene therapy section, overexpressed PMP22 in CMT1A overwhelms the proteasome, which is blocked resulting in PMP22 aggregates and cell stress. Activation of autophagy has been proposed as a strategy to clear PMP22 aggregates in CMT1A Schwann cells to release cell stress and improve differentiation and myelination.[75] In Schwann cells, a cross talk between proteasome and autophagy has been suggested so that when proteasome is blocked autophagy is activated. However, in CMT1A Schwann cells, the physiological attempt of inducing autophagy is inefficient to clear aggregates, whereas boosting autophagy, for example, using Rapamycin, a known mTORC1 inhibitor, was shown to decrease PMP22 and improve myelination.[91,92] Proof-of-principle of this strategy was provided in vitro using myelin-forming Schwann cell/DRG neuron co-cultures established from both C22 (with extra copies of PMP22) and *Tr-J* mouse models.[93] Consistent with this, the intermitting fasting diet, which is known to activate autophagy to supply to decreased nutrient metabolism, had a beneficial effect in the locomotor capacity and myelination in the *Tr-J* mutant,[35] whereas Rapamycin treatment of *Tr-J* mutants increased myelinated fibers and myelin thickness but had no effect on neuromuscular function.[36] Of note, PMP22 cellular aggregates colocalize with heath shock proteins (HSP), chaperones, and with proteasome and autophagic markers. Synthetic small molecules inhibitors of HSP90 enhanced the expression of cytosolic chaperones and improved myelin formation and PMP22 processing in DRG from CMT1A mice.[94] Moreover, in vivo treatment of C22-PMP22 (CMT1A) and *Tr-J* mice with the HSP90 inhibitor AUY922 maintained myelinated fibers and limited the decline in rotarod performance in both models,[37] indicating that also the HSP90 pathway is a potential target for inherited neuropathies where the mutant protein is incorrectly trafficked. However, while *Tr-J* is an authentic model of CMT1E carrying a mutation also found in humans, the C22 mouse carries up to seven copies of the human *PMP22* gene resulting in a much higher PMP22 overexpression as compared to CMT1A patients. Indeed, the phenotype of the C22 mice reflects more the severe congenital hypomyelination neuropathy rather than CMT1A. As such, whether the formation of protein aggregates is really a relevant feature of CMT1A remains questionable.

4.3 | Demyelinating CMT1A: to improve Schwann cell differentiation

Additional therapeutic strategies for CMT1A have been tested at the preclinical level, which are not based on the decrease of *PMP22* gene expression or clearance of PMP22 protein aggregates. In CMT1A, the P2X7 channel is overactivated resulting in increased calcium influx and demyelination. Nobbio et al. found that antagonizing P2X7 using the A438079 pharmacological inhibitor may improve the myelination of CMT1A Schwann cells.[95] Sociali et al. showed that A438079 was well tolerated in CMT1A rat when administered daily by i.p. (intraperitoneal injection) for 4 weeks and it was able to improve myelination and muscle strength but not CMAP amplitudes.[38] Clinical trials were not envisaged due to safety concerns of this compound in human.

Another strategy tested at the preclinical level is the treatment of CMT1A rat with soluble NRG 1 (Neuregulin).[39] Among different isoforms of Neuregulin 1, NRG1 type III is expressed on the axonal surface and signals with ErbB2/B3 receptors in Schwann cells, whereas NRG1 type I is mainly expressed by Schwann cells.[96–98] NRG1 type III has been demonstrated to dictate the fate of Schwann cells as pro-myelinating or non-myelinating and represents an instructive signal for the amount of myelin to be produced. Above a certain threshold, Schwann cells myelinate and the more NRG1 type III is expressed by axons, the thicker is the myelin.[99] This function is reflected by the constant ratio between axon diameter and myelin thickness. Fledrich et al. demonstrated that CMT1A rat Schwann cells have a differentiation defect, with enhanced ERK1/2 signaling and decreased AKT phosphorylation.[39] The group suggested that by treating CMT1A rat with soluble NRG1, myelination is improved and the correct ERK/AKT phosphorylation ratio is restored. However, the treatment should be administered very early during postnatal development from P6 to P18, at the peak of myelination. Later treatment had minor effect on the CMT1A phenotype. Notably, NRG1 type III overexpression was found to improve the phenotype of two CMT1B models, the $Mpz^{Q215x/+}$ and the Mpz^{S63del} mice characterized by congenital hypomyelination and demyelination, respectively.[100,101] In both models, crossbreeding the neuropathic mice with mice overexpressing NRG1 type III,[99] resulted in increased myelin thickness and improved neurophysiological parameters. Surprisingly, in both models, the mechanism appeared to be independent of the upregulation of Egr2/Krox20 (Early growth response 2, the master regulator of the essential myelin genes) but to rely on the rebalancing of myelin lipid:protein stoichiometry and on the expression of other myelin proteins such as myelin protein 2.[100,101]

The opposite strategy aimed at decreasing myelination signaling has been tested in demyelinating neuropathies with myelin outfoldings and aberrant myelin. NRG1 type III is cleaved and activated by the BASE secretase, whereas its activity is downregulated by TACE secretase.[102] TACE in turn is activated by Niacin-vitamin B3, a precursor of NAD^+ metabolism. *Mtmr2* KO mice, which model the CMT4B1 neuropathy with myelin outfoldings (see following paragraph) and *Pmp22* haploinsufficient mice with tomacula, a model of the HNPP neuropathy was treated using niacin daily administered by i.p. injection.[40] Morphological analysis showed decreased number of fibers with myelin outfoldings in *Mtmr2* KO-treated animals and reduced tomacula with improved myelin thickness in $Pmp22^{+/-}$ treated mutants.

In another study, Fledrich et al. found that the transcriptomic profile of CMT1A rat nerves correlated with the severity of the phenotype.[41] In particular, lipid-related genes were differentially expressed and, among this category, those genes coding proteins involved in phospholipid, cholesterol, and glycosphingolipid biosynthesis were significantly downregulated in CMT1A nerves as compared to controls. Among phospholipids, the metabolism of phosphatidylcholine was most affected. Thus, the authors hypothesized that supplementation with this lipid may ameliorate Schwann cell differentiation and myelination, ultimately leading to axonal preservation and decreased loss of fibers. CMT1A rats were treated from P2 to P112 with different lipid concentrations supplemented in their food. At the end point, muscle force, mass, and neurophysiology (MNCV and CMAP) showed a modest but significant improvement. Morphological analysis showed increased myelinated axons and improved myelin ultrastructure in lipid-treated CMT1A rat sciatic nerves. A milder but significant amelioration of the phenotype was also observed when the treatment started at P21, after the peak of myelination.[41] Of note, even if the lipid treatment did not solve the problem of impaired differentiation and low PI3K-AKT signal in CMT1A Schwann cells, exogenous phosphatidylcholine up-taken by diseased Schwann cells stimulated the synthesis of cholesterol and of myelin biosynthesis in general.

4.4 | Demyelinating CMT4B1

The Myotubularin-related (MTMR) protein family is a well-conserved family of proteins sharing homology with protein tyrosine phosphatase/dual specificity phosphatase proteins. Loss-of-function mutations in *MTMR2*, *MTMR5*, and *MTMR13* are associated with CMT4B1, B3, and B2, respectively.[103] MTMR2 is a catalytically active enzyme, whereas MTMR5 and MTMR13 are catalytically inactive. The interaction of MTMR2 with either MTMR5 or MTMR13 is thought to potentiate enzymatic activity and regulate subcellular localization of the complex. Active phosphatases downregulate levels of PtdIns3*P* and PtdIns(3,5)P_2 phosphoinositides, key signaling molecules that participate to complex signaling platforms on subcellular membranes together with kinases, phosphatases, Rab GTPases, their regulators, and effector proteins.[104] While loss of MTMR2 and MTMR13 cause a similar demyelinating phenotype, whose hallmark is the presence of myelin outfoldings-redundant myelin in the nerve that degenerates causing axonal loss, MTMR5 mutations are mainly associated with a complex and very severe CNS phenotype.[103] Thus, the function of these complexes is not redundant but rather they control cell-specific functions. Vaccari et al. suggested that the preferential substrate of MTMR2 is PtdIns(3,5)P_2 so that MTMR2 loss is associated with increased level of this lipid in CMT4B1 mouse and patient cells.[105] However, why dysregulation of this lipid causes aberrant myelin growth and degeneration in this model is not clear. Guerrero-Valero

et al. suggested that this lipid may regulate the mTORC1 and RhoA pathways, known to be relevant for myelin biology.[42] PIKfyve is the kinase that produces the PtdIns(3,5)P_2 lipid[81] and thus the decrease of PIKfyve expression both in vivo and in vitro or of PIKfyve activity in vitro was shown to ameliorate the myelin outfoldings phenotype.[42,105] Inhibitors of PIKfyve are being developed particularly because these drugs are relevant for the treatment of cancers and COVID-19.[106] PIKfyve inhibitors and decrease of PtdIns(3,5)P_2 may block SARS-CoV-2 entry via loss of positive regulation of TPC2 (Two-Pore Channel 2), a downstream target of PIKfyve that resides on lysosomes. If these inhibitors are effective in vivo to treat CMT4B neuropathies with altered levels of PtdIns(3,5)P_2 remains to be further elucidated.

4.5 | Targeting protein misfolding in demyelinating CMT

As described above for CMT1A, the accumulation of misfolded proteins and the activation of stress pathways is believed to play a role in other forms of CMT.[107,108] Misfolded mutant PMP22 proteins (CMT1E phenotype) can be retained in the ER/Golgi compartment,[109–111] where they activate the unfolded protein response (UPR). This is also the case of many mutations in the myelin protein zero (*MPZ*) gene that cause CMT1B.[112,113] Administration of curcumin, a low-affinity SERCA inhibitor derived from the spice turmeric, has been shown to improve trafficking, reduce UPR marker expression and alleviate disease severity of in vitro and in vivo models of CMT1E and CMT1B.[43,44] More recently, curcumin has been shown to alleviate disease manifestation in a CMT1A rat model, probably acting on oxidative stress.[45] However, due to poor pharmacokinetic[114] and rather unclear mechanism of action, it is unlikely that curcumin will be considered for clinical trials.

In models of CMT1B, it has been shown that modulation of the UPR represents an appealing therapeutic option for those neuropathies with ER-retention of the mutant protein.[112] In mammalian cells, the UPR consists in the activation of inositol requiring kinase 1 (IRE1), activating transcription factor 6 (ATF6) and pancreatic ER kinase (PERK), three ER sensors that activate a transcriptional and translational program aimed at reducing the load of unfolded proteins through upregulation of chaperones, attenuation of protein synthesis and increased protein degradation.[115,116] In particular, in condition of ER-stress, the ER-resident kinase PERK phosphorylates eIF2α, to attenuate protein translation.[117] Genetic ablation of PPP1R15A/Gadd34, which is part of the phosphatase complex that dephosphorylates eIF2α to reactivate translation,[118] or its pharmacologic inhibition with the small molecule Sephin1/IFB-088 ameliorated motor, neurophysiological and morphological parameters in the Mpz^{S63del} mouse,[46,47] suggesting that phosphorylation of eIF2α, the crucial step in the integrated stress response (ISR), is a fundamental checkpoint in Schwann cell viability.[119] More recently, it has been shown that oral treatment with IFB-088 is capable to ameliorate disease parameters in a second model of CMT1B, the $Mpz^{R98C/+}$ mouse, and also in C3-PMP22 CMT1A mice, where PMP22 overexpression causes activation of the UPR.[48] In both models, IFB-088 improved motor capacity, neurophysiology, myelin pathology, and partially readjusted Schwann cell proteostasis.[48] These data suggest that IFB-088 could be a viable therapy for multiple forms of CMT with UPR activation. A phase I clinical trial of oral IFB-088 has been successfully completed (ClinicalTrials.gov Identifier: NCT03610334).

It is proposed that by its mode of action, IFB-088 limits the accumulation of mutant protein in the ER, allowing the cellular protein quality control (PQC) system to better dispose of the mutant protein. Indeed, it has been shown that mutant P0 (myelin protein zero) proteins accumulate in the ER and are degraded via the ER-associated degradation (ERAD)/proteasome system.[120] Molecules such as GlucNac, which stimulate PQC mechanisms have been shown to improve myelination in DRG explants from CMT1B models.[120] In line with this, a recent work has shown that in vivo treatment of Mpz^{S63del} mice with agents like sildenafil, which raises cyclic guanosine monophosphate (cGMP) and activates proteasome activities, protein ubiquitination and degradation of misfolded proteins, improved myelination, and nerve conduction velocities,[49] suggesting that compounds capable of increasing cGMP could be useful therapies for CMT1B and proteotoxic neuropathies in general.

4.6 | Immunomodulation in CMT1 forms

Mounting evidence suggests a role for low-grade inflammation as a disease amplifier in many forms of demyelinating CMT.[121] In *Mpz* heterozygous null mice (P0het) that can be considered a model for the mild forms of CMT1B due to loss of function mutation in P0,[122] demyelination in aged mice is accompanied by increase in infiltrating lymphocytes and macrophages.[123] Crossbreeding of P0het mice with mice deficient in macrophage colony-stimulating factor (M-CSF), resulted in reduced demyelination.[123,124] Breeding with the M-CSF KO mice ameliorated demyelination and overall axonopathy also in CX32 KO mice, a model of X-linked CMT (CMT1X),[125] indicating a role for immune cells in different neuropathies. In the absence of the endoneurial fibroblast expressed CSF-1, CX32-deficient mice not only showed amelioration in myelination, but also failed to upregulate the Schwann cell dedifferentiation markers NCAM and L1.[126] Long-term treatment (9 months treatment, starting at 3 months) with an orally available CSF-1 receptor (CSF1R) inhibitor (PLX5622) resulted in a robust decline in macrophages in P0het and CX32 deficient mice, and improved neuropathological and clinical features in both mouse models.[50] While the same treatment protocol did not significantly improve the phenotype of CMT1A mice,[50] targeting macrophages with PLX5622 at an earlier time point, starting by feeding lactating mothers with chow containing PLX5622 and then continuing the treatment up to 6 months, substantially ameliorated the peripheral neuropathy also in CMT1A mice, leading to preserved motor function and modulated the abnormal differentiation phenotype of CMT1A Schwann cells.[51] Along the same lines, treatment of P0het mice with PLX 5622 starting at 6 months led to macrophage depletion but only to modest improvements in histopathological and clinical features,

whereas early and continuous treatment (from 1 to 12 months) recapitulated the beneficial effects previously seen with the treatment starting at 3 months.[52] Importantly, Ostertag et al. also showed that early treatment lasting only 6 months, while it did not reduce the demyelinating profiles, resulted in the improved axonal phenotype of P0het mice at 12 months, suggesting that continuous treatment may not be always necessary for disease improvement, at least at the axonal level.[52] How macrophages mediate axonal damage in peripheral nerve remains an open question. However, these data indicate that targeting macrophages can represent a general approach for different subtypes of demyelinating neuropathies, as long as the proper therapeutic window is identified. Whether this approach would be effective also in other forms of CMT, including primarily axonal ones, remains to be elucidated.

5 | PHARMACOLOGICAL APPROACHES FOR AXONAL CMT2

5.1 | SARM 1 inactivation to prevent axonal degeneration in CMT2

Although hereditary neuropathies can be divided into demyelinating (CMT1) and axonal (CMT2), ultimate progression of neuropathy in patients reflects axonal degeneration in either type. Limiting axonal degeneration would therefore provide significant benefits for most forms of CMT. Recent advances in the understanding of the axonal degeneration program are leading to the identification of therapeutic targets that could be broadly applicable. In this respect, Sterile alpha and toll/interleukin 1 receptor motif-containing 1 (SARM1) represent one of the most appealing candidates.[127,128] SARM1 is an NAD^+ hydrolase,[129] whose action results in NAD^+ depletion followed by a wave of events in which ATP is lost, mitochondria stall and depolarize, extracellular calcium enters the axon and membrane integrity is disrupted, ultimately leading to axonal degeneration.[130] In *Sarm1*$^{-/-}$ mice, severed axons survived for several weeks both in vitro and in vivo, indicating SARM1 as the central executioner of the axonal degeneration program,[127] and suggesting that inhibiting SARM1 could be a promising strategy for pathological axonal degeneration. Indeed, deletion of SARM1 was shown to be protective in models of metabolic or chemotherapy-induced neuropathy.[131,132] Currently, no small molecules are available to inhibit SARM1 in vivo, but a gene therapy approach using adeno-associated virus (AAV)-mediated expression of dominant negative (DN) SARM1 mutants has been devised.[133] Intrathecal injection of the most potent SARM1 DN protected from axonal degeneration after sciatic nerve cut similarly to SARM1 genetic ablation, suggesting that AAV-mediated expression of these dominant-negatives could represent a widespread approach for the treatment of multiple models of pathological axonal degeneration. Whether SARM1 inhibition could also apply to CMT is still under investigation. In this respect, it has been recently shown that SARM1 deletion does not rescue the behavioral, neurophysiological, or morphological phenotype of the C3-PMP22 mice, a model of CMT1A.[134] However, contrary to humans affected by CMT1A that experience significant axonal loss as disease progresses, the C3 mouse model is mostly affected by severe dysmyelination and shows little to no axonal degeneration,[134] at least at the relatively early adults time points analyzed in the study. This finding renders these results hard to interpret and indicates the need to analyze the mice at later time points and possibly the necessity for improved CMT1A models. On the other hand, it is also possible that the SARM1-driven axon degeneration pathway is not involved in the secondary axonal degeneration seen in CMT1A patients. Indeed, several papers suggest that alternative degeneration pathways also exist.[135,136] Conversely, SARM1 ablation appears to be highly protective in a CMT2A rat model.[23] CMT2A is a primary axonal neuropathy caused by mutations in the mitofusin2 gene (*MFN2*) and represents the most common form of CMT2[137] (see below). MFN2 is a GTPase that resides on the outer mitochondrial membrane, and mutations in *MFN2* result in profound mitochondria abnormalities,[138,139] but how these abnormalities lead to axonal loss is unknown. In the *Mfn2*^{H361Y} rat, a model that recapitulates several hallmarks of the human disease, such as progressive dying back axonal degeneration, muscle atrophy, neuromuscular junction (NMJ) abnormalities and mitochondrial defects, deletion of *Sarm1* rescued the axonal, synaptic, muscle and functional phenotypes, indicating that SARM1 is directly responsible for most of the pathological findings.[23] Interestingly, also some of the mitochondrial abnormalities were rescued in these mice after *Sarm1* deletion, despite the presence of the mutant MFN2. This suggests that the initial mitochondrial defects due to the MFN2 mutation lead to SARM1 activation that in turn feeds back on the mitochondria to worsen their pathology.[23] This exciting work identifies SARM1 as a potent target for CMT2A but also for other neurodegenerative conditions with significant mitochondrial involvement and warrants further investigations on the role of SARM1 in other forms of CMT2 but also in models of CMT1 with more extensive secondary axonal degeneration. Currently, great efforts are devoted to developing SARM1 inhibitors and recently a potent and selective small molecule isoquinoline inhibitor of SARM1 NADase activity has been identified.[140] This compound, named DSRM-3716, strongly prevented axonal degeneration in axotomized mouse DRG neurons, and maintained viable functional mitochondria. Importantly, these protective effects were also observed in human iPSC-derived motor neurons after axotomy.[140] Although these compounds have not been tested in vivo yet, they hold great promise for the treatments of human diseases characterized by axonal loss.

5.2 | HDAC6 deacetylase inhibition

Disturbances in axonal mitochondrial transport are often associated with peripheral neuropathies.[141] As such, axonal transport and mitochondrial distribution may also represent attractive downstream targets for therapy shared by several CMT subtypes. One of the key players in axonal transport is α-tubulin acetylation which is believed to increase microtubules stability and facilitate in particular the anterograde transport of cargoes along microtubules by promoting their

interaction with, amongst other, KIF5 as well as dynein.[142,143] The dynamic between α-tubulin acetylation and deacetylation is therefore essential for axonal transport modulation. Histone deacetylase 6 (HDAC6) is the enzyme responsible for α-tubulin deacetylation. HDACs are mainly known to be enzymes involved in transcriptional regulation by epigenetically modifying histones. HDAC6, contrary of conventional HDACs, is mostly located in the cytosol, where it has been shown to modulate a variety of non-histone substrates, including α-tubulin.[53]

The first indication of a decrease in the amount of acetylated α-tubulin, that correlated with a reduction of axonal transport rate, came from work on a mouse model carrying *HSPB1* mutations, that in humans cause CMT2F. Cultured DRG from these mice showed defects in axonal transport of mitochondria, and peripheral nerves presented a marked reduction of acetylated α-tubulin, which appeared instead normal in the spinal cord. Treatment of DRGs and mice with HDAC6 inhibitors such as tubastatin restored axonal transport and reversed the CMT2 phenotype.[54] Subsequently, deficiencies of acetylated tubulin and axonal transport have been shown in mouse models of CMT2D (*GARS* mutation) and of CMT2A (*MFN2* mutations),[55,56,144] indicating that reduction in tubulin acetylation within axons may represent a common defect across genetically diverse forms of axonal neuropathy. In the $Gars^{C201/+}$ model of CMT2D selective HDAC6 inhibition with intraperitoneal injection of tubastatin-A improved motor and sensory defects, which correlated with an increased innervation and a higher α-tubulin acetylation.[8,55] Similar results were obtained using a second CMT2D model, the $Gars^{P234KY/+}$ mouse.[56] Finally, in the $Mfn2^{R94Q}$ model of CMT2A, pharmacologic and genetic inhibition of HDAC6 were able to ameliorate or prevent the sensory and motor defects, respectively.[144]

Overall, these data suggest that inhibition of HDAC6 has the potential to be a unifying treatment approach for many axonal neuropathies. Whether this would apply for all CMT2 types is still unknown, but HDAC6 inhibitors are surely worthy of further exploration. In this respect, Benoy et al. tested three small molecules (ACY-738, ACY-775, and ACY-1215) that demonstrated selective and potent inhibition of HDAC6.[57] All three molecules were able to improve motor and sensory defects in CMT2F mice. Importantly, ACY-1215, also known as Ricolinostat, is being tested in a phase II clinical trial for multiple myeloma.[57] Similarly, two recently developed HDAC6 inhibitors CHEMICAL X4 and CHEMICAL X9 where able to improve mitochondrial mobility in CMT2F patients derived motor neurons.[145]

In addition to its role in mitochondrial dynamics and axonal transport, HDAC6 has been also involved in proteotoxic stress and protein clearance.[146] Amongst its targets are in fact HSP90 and HSP70, which are important players in the folding and clearance of proteins (see above). It is well established that PMP22 is a highly metastable protein, and that overexpression of PMP22, which is the cause of CMT1A, causes the activation of proteotoxic stress, as previously discussed. Ha et al. showed that CKD-504, a specific HDAC6 inhibitor, is capable of modestly improve behavior, neurophysiology, and myelin pathology in the C22 mouse, a model of CMT1A.[58] Nerves from treated mice showed increased HSP90 acetylation, augmented expression of HSP70, and reduced PMP22 protein aggregation, suggesting that HDAC6 inhibition could ameliorate cellular protein quality control mechanisms. Whether HDAC6 inhibition may improve additional CMT caused by proteotoxic proteins remains to be established.

A number of other histone deacetylases have been implicated in Schwann cell development myelination, and remyelination after injury.[147] In particular, HDAC3 has been shown to act as a potent inhibitor of Schwann cell myelination by negatively regulating the NRG1-PI3K-AKT and the MAPK/ERK1/2 signaling pathways[148]; this restrain on NRG1 ensures proper myelin growth and the entering of Schwann cells in a homeostatic state in adult nerves.[149] In the C3-PMP22 model, the PI3K-AKT and ERK1/2 activity as well as myelin protein expression are significantly downregulated.[59] Treatment of C3-PMP22 mice with the selective HDAC3 inhibitor RGFP966 resulted in increased myelin protein synthesis, mildly improved neurophysiological parameters and in an amelioration in myelination.[59] However, HDAC3 inhibition, in particular at the highest doses used, also resulted in reduced motor performance and in increased number of macrophages in C3 nerves, highlighting the crucial importance of proper dosing of HDAC3 inhibitors should they reach the clinical phase.[59]

5.3 | Restoring MFN2 in CMT2A

Mutations in mitofusin2 (*MFN2*) cause CMT2A, the most common form of CMT2, accounting for 20%–30% of all CMT2 cases. MFN2, an outer mitochondrial membrane GTPase, is a key mediator of mitochondrial fusion. MFN2 and its close homolog MFN1 are characterized by a large cytosolic N-terminal GTPase domain, two transmembrane domains and two coiled-coil heptad repeat (HR) domains, named HR1 and HR2. It has been shown that interactions of HR2 domains from MFN2 proteins on adjacent mitochondria are critical for the initial tethering, while the GTPase domain is involved in the completion of mitochondrial fusion.[150] In turn, fusion affects mitochondrial dynamics, quality control, distribution, and functions. Moreover, through interactions with Miro1 and Milton, MFN2 has also been suggested to be essential to mitochondrial transport along the axons.[151] Due to their unique morphology characterized by long axon projections, which likely entails higher reliance on mitochondrial energetic, peripheral neurons are particularly sensitive to mutations in MFN2, and are the most affected in CMT2A. More than 100 dominant mutations in *MFN2* have been reported in CMT2A patients and many of them reside close to the GTPase domain. Most mutations result in mitochondrial fusion defects, which may lead to mitochondrial aggregation and defective quality control,[152,153] but mutations affecting transport have also been identified.[154,155] While the HR2-HR2 interactions between MFN2 on opposing membrane are necessary for mitochondria tethering and fusion, it has also been shown that intramolecular HR1-HR2 interactions maintain MFN2 in a tethering-non-permissive state.[156] Small TAT-peptide targeting and destabilizing the HR1-HR2 conformation can act as MFN2 agonist, promoting the tethering permissive HR2-HR2 interaction, and can reverse mitochondrial abnormalities in cultured fibroblast and neurons from mice carrying the CMT2A $Mfn2^{T105M}$ mutation.[156]

Similarly, small molecules mimetics of MFN2 HR1 amino acid side chains that interact with HR2 can act as potent mitofusin agonists.[60] These mitofusin agonists corrected mitochondrial dysmorphology and fragmentation in cultured mouse neurons expressing, in addition to the endogenous MFN1 and MFN2, the CMT2A mutants *MFN2*^{R94Q} or *MFN2*^{T105M}. Moreover, mitofusin agonists restored mitochondria motility in cultured neurons and sciatic nerves from *Mfn2*^{T105M} mice,[60] mechanistically linking CMT2A abnormal mitochondrial trafficking to MFN2 dysfunction. Importantly, these studies showed that these agonists did not act by restoring the function on the dominant negative mutant MFN2 but, rather, by stabilizing the fusion permissive HR2-HR2 conformation of the endogenous normal MFN1 and MFN2.[60] Indeed, recent work showed that pharmacologic activation on endogenous MFN1 and MFN2 reversed mitochondrial abnormalities in diverse CMT2A patients motor neurons and that intermittent mitofusin activation with daily intramuscular administration of the small molecule MiM111, reversed neuromuscular dysfunction in CMT2A mice expressing the human *MFN2*^{T105M} mutation, as measured by ex-vivo mitochondrial motility and in vivo motor and neurophysiological parameters.[61] Moreover, treatment with MiM111 also reversed histopathological findings in nerve and muscle of *Mfn2*^{T105M} mice.[61] These improvements were linked to enhanced mitochondrial function and axon outgrowth in DRG neurons from CMT2A mice. Remarkably, mitofusin activation also stimulated postaxotomy regrowth in CMT2A cortical neurons in vitro.[61]

Several studies have shown that MFN1 is highly homologous to and can interact with MFN2, and that MFN1 can compensate for MFN2 deficiency.[24] The low levels of expression of MFN1 in axons as compared to non-neuronal tissues, may explain the peculiar sensitivity of motor and sensory axons of the PNS to MFN2 mutations. This led to the hypothesis that increased expression of MFN1 or the WT form of MFN2 could be a viable therapeutic option for CMT2A. Indeed, it has been recently shown that increased expression of MFN1 in the nervous system rescued the phenotype of *Mfn2*^{R94Q} mice.[157] These mice express the human *MFN2*^{R94Q} mutation under the neuronal Thy1.2 promoter, and display classic features of CMT2A, including severe early sensorimotor deficits, vision loss, altered mitochondrial dynamics, and widespread axonal loss. Crossing of the *Mfn2*^{R94Q} mice with mice overexpressing human MFN1 under the prion promoter resulted in an almost complete rescue of the defects in locomotor activity, sensorimotor coordination, and visual loss.[157] Of note, also the crossing of *Mfn2*^{R94Q} with *Mfn2*^{WT} mice largely rescued the CMT2A phenotype, strongly supporting the notion that CMT2A mutations act via a dominant negative effect that can be countered by increasing the amount of fusion-competent MFN2 or MFN1 molecules, opening to the possibility of future gene therapy approaches in humans.

5.4 | Reducing sorbitol accumulation in CMT2 due to SORD deficiency

Recently, biallelic mutations in the *SORD* (sorbitol dehydrogenase) gene have been identified in autosomal recessive predominantly motor axonal CMT2.[62] SORD encodes sorbitol dehydrogenase, which catalyzes the conversion of glucose to fructose in the polyol pathway. When SORD is mutated and lost, sorbitol accumulates and this is thought to be the basis of the neurotoxicity even if the mechanism has not been completely elucidated. Of note, aldose reductase is the upstream enzyme that in the reaction generates sorbitol from glucose. Aldose reductase activity can be inhibited by already FDA-approved compounds, thus reducing sorbitol accumulation in SORD-deficient cells and potentially toxicity. Clinical trials as well as natural history studies for SORD neuropathy are in progress.[63]

6 | CONCLUDING REMARKS

Since the discovery in 1992 of the first causative gene for CMT, enormous progresses have been made in the understanding of the disease pathomechanisms. This in turn has led to several therapeutic approaches, ranging from pharmacologic to gene therapies, being tested in preclinical models and, more recently, to the development of the first clinical trials in patients. While many challenges still lay ahead, including natural history studies, more effective clinical evaluation scores, and the identification of treatment responsive biomarkers, there is optimism that the first effective treatments for several forms of CMT will be available in the near future.

ACKNOWLEDGMENTS

The authors apologize for the work that we did not have the possibility to mention for space concerns. The authors thank all members of the Bolino and D'Antonio laboratories. Research in the Bolino laboratory is currently supported by the Muscular Dystrophy Association (#MDA 957250), Fondazione Telethon (#GGP20063), the Charcot-Marie-Tooth Association USA (CMTA), the Charcot-Marie-Tooth Research Foundation USA (CMTRF), and the Italian Ministry of Health (#RF-2016-02361246). Research in the D'Antonio laboratory is currently supported by Fondazione Telethon (#GGP19099 and #GSA21E002), the Charcot-Marie-Tooth Association USA (CMTA), AFM-Telethon (#24110), and the Italian Ministry of Health (#RF-2019-12369320). Open access funding provided by BIBLIOSAN.

CONFLICT OF INTEREST STATEMENT

M. D'Antonio acts as a scientific advisory board member for Inflectis Bioscience. Other author declares no conflict of interest.

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How to cite this article: Bolino A, D'Antonio M. Recent advances in the treatment of Charcot-Marie-Tooth neuropathies. *J Peripher Nerv Syst*. 2023;1-16. doi:10.1111/jns.12539