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A multicentre retrospective study of Charcot-Marie-Tooth disease type 4B (CMT4B) due to mutations in Myotubularin-related proteins (MTMRs)

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Potential Conflicts of Interest

None to report.

Data availability

The data that support the findings of this study are available from the corresponding authors, upon request.

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Abstract

Objective.—Charcot-Marie-Tooth disease 4B1 and 4B2 (CMT4B1/B2) are characterized by recessive inheritance, early onset, severe course, slowed nerve conduction, and myelin outfoldings. CMT4B3 shows a more heterogeneous phenotype. All are associated with Myotubularin-related proteins (MTMRs) mutations. We conducted a multicentre retrospective study to better characterize CMT4B.

Methods.—We collected clinical and genetic data from CMT4B subjects in 18 centres using a predefined minimal dataset including MRC scores of nine muscle pairs and CMT Neuropathy Score.

Results.—There were 50 patients, 21 of whom never reported before, carrying 44 mutations, of which 21 novel and six representing novel disease associations of known rare variants. CMT4B1 patients had significantly more severe disease than CMT4B2, with earlier onset, more frequent motor milestones delay, wheelchair use and respiratory involvement as well as worse MRC scores and motor CMT Examination Score components despite younger age at examination. Vocal cord involvement was common in both subtypes, whereas glaucoma occurred in CMT4B2 only. Nerve conduction velocities were similarly slowed in both subtypes. Regression analyses showed that disease severity is significantly associated with age in CMT4B1. Slopes are steeper for CMT4B1, indicating faster disease progression. Almost none of the mutations in the *MTMR2* and *MTMR13* genes, responsible for CMT4B1 and B2, respectively, influence the correlation between disease severity and age, in agreement with the hypothesis of a complete loss of function of MTMR2/13 proteins for such mutations.

Interpretation.—This is the largest CMT4B series ever reported, demonstrating that CMT4B1 is significantly more severe than CMT4B2, and allowing an estimate of prognosis.

Introduction

Charcot-Marie-Tooth disease (CMT) encompasses a highly heterogeneous group of hereditary neuropathies including some rare subtypes[1–4]. It is commonly subdivided into two main forms, demyelinating and axonal, according to the motor nerve conduction values, with a recognized group of intermediate subtypes laying in between. All Mendelian inheritance patterns have been described for CMT, and recessive demyelinating types are conventionally classified as CMT4. Further subdivision is based mainly on involved genes and loci[5].

Among demyelinating recessive forms, CMT4B is characterized pathologically by the presence of focal hypermyelination on nerve biopsy, with myelin outfoldings constituted by redundant loops of myelin lamellae involving the vast majority of fibers[6]. CMT4B includes three clinical and genetically distinct subtypes. CMT4B1 and CMT4B2 are associated with mutations in the *MTMR2* and *MTMR1*3 (myotubularin-related protein 2 and 13, which is also named SET binding factor 2, *SBF2*) genes, respectively[7–10]. Both CMT4B1 and CMT4B2 are characterized by early onset, demyelinating neuropathy, disabling progression,

and may be complicated by vocal cord palsy and respiratory involvement[6, 11]. CMT4B2 patients may have congenital or juvenile glaucoma[12]. CMT4B3 has been more recently associated with mutations in the *MTMR5/SBF1* gene but is characterized by different phenotypes with either a pure demyelinating neuropathy with myelin outfoldings or an axonal polyneuropathy complicated by multiple cranial involvement, intellectual disability, microcephaly and dysmorphic features. All three subtypes are extremely rare, with about 38 CMT4B1, 37 CMT4B2, and 14 CMT4B3 patients described so far, mostly from countries with high frequency of consanguineous marriages[7–9, 11–36].

MTMR2, MTMR5 and MTMR13 belong to the MTMR protein family, with homology to protein tyrosine phosphatase/dual specificity phosphatase family (PTP/DSP). Among 14 members, eight are catalytically active phosphatases, whereas six represent catalytically inactive proteins, also called “dead” phosphatases[10, 37]. MTMR2 dephosphorylates PtdIns3*P* and PtdIns(3,5)P_2 phosphoinositides, which are potent signalling molecules regulating membrane trafficking within the cell. On the contrary, MTMR5 and MTMR13, which are highly homologous, are not able to dephosphorylate phospholipids and are thought to assist catalytically active enzymes to properly localize at sub-cellular membranes and to increase their catalytic activity[37]. MTMR2 can heterodimerize with MTMR13 and MTMR5 through coiled-coil domains[38–40].

There are promising potential treatments under investigation for CMT4B, such as the FDA approved drug Niaspan[41] or PIKfyve kinase inhibitors[42] which stimulated us to perform a retrospective study to pave the way for clinical trial readiness. We aimed at defining the clinical and electrophysiological phenotype, evaluating genotype-phenotype correlations, and identifying patients for future studies on natural history and treatment efficacy for these diseases.

Material and Methods

Patients

This is a retrospective study on CMT4B patients with data collected from experienced neurologists working at several sites between July 2016 and January 2019 according to a specifically designed protocol. This was derived from a minimal dataset of clinical, genetic, electrophysiological information used within the Inherited Neuropathy Consortium[43] and expanded to include data on specific features of CMT4B. We also collected MRC scores from nine muscle pairs (first dorsal interosseous, abductor pollicis brevis, wrist extensors, biceps, deltoid, foot dorsiflexors, foot plantar flexors, knee extensors, hip flexors) as well as the Charcot-Marie-Tooth Examination Score and Neuropathy Score (CMTES/CMTNS)[44]; we also calculated the motor sub-score of the CMTES (motor CMTES), i.e., the sum of the four items concerning motor symptoms and signs in upper and lower limbs, and the sensory CMTES, the sum of the three items concerning sensory symptoms and loss of pinprick and vibration sense in lower limbs. We weren’t able to collect data about the CMTPedS, the specific scale for children[45], since it was not available at many sites either because it had not yet been published when the patients were evaluated or was not available at the site where the patient was seen; patients ageing 10 years and more were evaluated with the CMTES/

CMTNS. For each patient, we obtained data at least at baseline and, whenever possible, also from follow up visits.

Several centres across five continents were invited to participate in the study if they were following or had followed patients with a genetic diagnosis of CMT4B. Eighteen centres answered the invitation and adhered to the retrospective study, filled the minimal dataset of information forms which were collected and analysed centrally at the Fondazione IRCCS Istituto Neurologico Carlo Besta in Milan, Italy. Patients gave informed consent to clinical and genetic studies according to respective national regulations and in agreement with the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013). The study was notified to the Ethics Committee of the Fondazione IRCCS Istituto Neurologico Carlo Besta. All data were collected in an anonymised way with a sequential consecutive code for patients from each centre.

Genetic analyses

Variants were identified using whole exome sequencing (WES), or next generation sequencing (NGS) panel analyses followed by Sanger sequencing analysis, or directly by Sanger sequencing of the *MTMR2* and *MTMR13* genes when clinical diagnosis was compatible with mutations in these two genes.

The Genome Aggregation Database (GnomAD) was used to annotate each novel variant for its frequency in the general population and specifically in the population of European ancestry. Polyphen2 and Provean softwares were used to predict *in silico* the impact of missense variants on the corresponding protein sequences. “Novel variants” are those changes not reported in variant databases (GnomAD, 1000 Genome, ExAc, etc.) and thus for which the allele frequency is not available (AF=0). “Novel disease associations of known rare variants” are instead those changes reported in variant databases but with a very low allele frequency (AF<0.01), which have not previously associated with a disease, thus representing novel disease associations. The following criteria suggest pathogenicity of missense variants according to ACMG (The American College of Medical Genetics and Genomics) guidelines: i) very low (AF<0.01) or not reported (AF=0) allele frequencies, PM2 (pathogenic moderate) category classification; ii) Provean software scores less than −2,5 (deleteriousness prediction) and Polyphen-2 software prediction scores equal or near 1 (probably damaging), PP3 (pathogenic supporting) category classification; and iii) detected in *trans* with a pathogenic variant, PM3 (pathogenic moderate).

Statistics

Data are expressed as mean ± standard deviation, min and max values; t-test for unpaired data and Fisher’s exact test were used for comparison between CMT4B1 and CMT4B2 characteristics, as appropriate; univariate and multivariate regression analyses were performed in order to assess significant associations between the disease severity scores (MRC, CMTES, motor CMTES, and sensory CMTES scores) and the age at examination for CMT4B1 and CMT4B2 separately, significant differences between CMT4B1 and CMT4B2 age-adjusted severity, and mutation influence on age-adjusted severity for CMT4B1 and CMT4B2 separately; statistical tests were two-tailed and significance was set at $p<0.05$.

Results

The retrospective study includes 50 patients: 26 had CMT4B1, 19 CMT4B2, and 5 CMT4B3. The main clinical features of the commoner subtypes, CMT4B1 and CMT4B2, subjects are summarized in Tables 1 and 2, whereas the details of the rarer CMT4B3 patients are described in the text.

CMT4B1 and CMT4B2

Among the 26 CMT4B1 patients, 21 from nine families had a positive family history (three had consanguineous parents) and five were sporadic cases (three with consanguineous parents). Among the 19 CMT4B2 subjects, five were sporadic and 14 were from 12 families that had either affected family members (n = 8, including two sib couples) and/or consanguineous parents (n = 7). The study included more than one affected member, and up to six, from the same family in five CMT4B1 and two CMT4B2 families.

Clinical findings

CMT4B1 patients had a significantly earlier age at onset than had CMT4B2 patients. The first symptoms were related to lower limb weakness in all CMT4B1 patients except for one who was hypotonic at birth; four patients from the same family had vocal cord symptoms since disease onset. In CMT4B2, the heralding symptom was lower limb weakness with walking difficulties in all patients but two - one with hand weakness and tremor and the other with congenital glaucoma. A patient with toe walking during infancy was subsequently found to have congenital glaucoma. All patients became eventually able to walk but one-half of the CMT4B1 patients showed delay in motor milestones as compared to one-fifth of CMT4B2. The majority of patients in both subtypes developed foot deformities (pes cavus or, particularly in CMT4B1, pes planus) and about half of the patients had undergone foot surgery. Scoliosis or kyphoscoliosis was frequent, particularly in CMT4B2.

Age at last visit was considerably higher for CMT4B2 subjects and this must be taken into account when comparing disease severity. Of the 26 CMT4B1 patients, eight ambulatory subjects used ankle foot orthoses (AFOs), one needed AFOs and support for walking, while 12 were wheelchair-bound; the remaining five patients were children still ambulant without using AFOs, one of them used shoe inserts. The mean age of first AFO use was 14 years (range 7-28) and that of regular wheelchair use was 24.5 years (range 17-39); they were known in more than one-half of the CMT4B1 patients.

Among the 19 CMT4B2 subjects, three did not use any orthotics or inserts, two had only shoe inserts, eight ambulatory subjects used AFOs or orthopaedic shoes, two walked with unilateral support and AFOs or inserts, and four patients needed a wheelchair (two regularly, one only for outside and one intermittently). Regarding the upper limb function, 22 CMT4B1 and 18 CMT4B2 subjects had difficulties with buttons and/or eating utensils.

Vocal cord palsy and dysphonia were equally frequent in the two subtypes, being reported in about 40% of the cases. On the other hand, respiratory involvement was more common and relevant in CMT4B1 subjects, with eight patients showing some degree of insufficiency, of whom four were on non-invasive ventilation (NIV), and one with a tracheostomy owing to

severe vocal cord palsy. Two patients with CMT4B1 died because of respiratory insufficiency. In comparison, only three CMT4B2 subjects showed respiratory impairment, including one patient on NIV at age 48, one almost needing it at age 25, and one with mild vital capacity reduction at age 40.

Six CMT4B2 patients had overt glaucoma (bilateral in all but one instance), congenital in two cases, diagnosed at age 16 months or during adolescence in the rest; four subjects had also buphthalmia, one became blind unilaterally, two developed cataracts. While no CMT4B1 subject had glaucoma, two CMT4B1 siblings had coincidental Usher syndrome with retinal degeneration and deafness, and one had blindness related to keratoconus. No other CMT4B1 subject had hearing problems, whereas five CMT4B2 subjects had partial hearing loss. Other cranial nerve involvement was reported in both types, including masticatory muscle weakness in two CMT4B1 subjects and facial weakness which was particularly common in CMT4B1. Three CMT4B1 sibs had also Chiari I malformation and syringomyelia shown at MRI, while one CMT4B2 patient had an autism spectrum disorder with normal brain MRI and another one showed learning disability. Table 1 summarizes also data on muscle weakness, deep tendon reflexes, and sensory involvement. CMT4B1 patients had proximal involvement more commonly, whereas sensory loss was relatively less disabling.

Table 2 reports the comparison between the two subtypes of total MRC scores of the nine muscle pairs, and of CMTES, CMTNS, motor CMTES, and sensory CMTES, with ages at examination. Age at examination was lower in CMT4B1 patients, significantly for the MRC score. Scores were worse for CMT4B1, significantly for MRC, CMTNS, and motor CMTES scores, indicating more severe disease despite younger age.

When comparing members from the same family and taking into account the age at examination, for CMT4B1 we observed only a small disease severity variability as far as disease onset, clinical scores, and walking ability are concerned. Two pairs of CMT4B2 sibs showed differences in age at onset (2 vs 20 and 2 vs 10 years, respectively) but only in the latter pair of sibs there were differences in disease course (one chair-bound with respiratory involvement at age 47 and the other still ambulant without support at age 37).

Values of MCV and CMAP amplitudes for ulnar and median nerves are also reported in Table 2; motor nerve conduction velocities were reduced to a similar extent in both CMT4B types and there were no significant differences as far as CMAP amplitude values, when obtainable, are concerned. Six CMT4B1 patients had recordable sensory action potentials (SAPs) in the upper limbs, while sural nerve SAP was absent in all tested patients; all 18 CMT4B2 patients examined had absent SAPs in upper and lower limbs.

Nerve biopsy was performed in 12 CMT4B1 and eight CMT4B2 patients and showed the typical myelin outfoldings in all cases[11, 15, 19, 24, 46–48].

MTMR2 and MTMR13 mutations

All the variants identified in CMT4B1 and CMT4B2 patients are shown in Fig. 1. Overall, we identified 17 mutations in *MTMR2* in CMT4B1 patients, located in different domains of

the protein, of which nine have been reported, whereas five are novel and three are novel disease associations of known rare variants[7, 11, 15, 16, 19–21, 24] (Fig. 1A and Table 3). Of note, 15 mutations represent loss-of-function alleles, such as nonsense mutations or frameshifts leading to premature truncated proteins, whereas two are missense mutations. Moreover, 11 mutations were found in homozygosity, whereas six represent compound heterozygous novel variants including four loss-of-function alleles, and one loss-of-function allele in combination with a missense mutation on the other allele (p.W206*/p.W583*; p.S296Rfs*8/p.A629Efs*31; p.R459*/p.T537I).

Loss-of-function alleles are predicted to result in a truncated MTMR2, which should be degraded. The p.G103E variant is the only missense mutation identified in homozygosity[24].

We identified 24 mutations in *MTMR13/SBF2*, responsible for CMT4B2, of which five have been previously reported, 16 are novel variants and three represent novel disease associations of known rare variants[8, 12, 18, 30] (Fig. 1B and Table 4). Among the 24 mutations, 21 represent loss-of-function alleles and three are missense variants. Ten out of 24 are inherited in homozygosity. The remaining 14 variants are inherited as compound heterozygous mutations and represent loss-of-function alleles such as frameshifts, two large intragenic deletion and duplication, and nonsense mutations. In three genotypes, p.G207V/c.2536+1G>A; p.E739K/exons14-27del; and p.D1477G/c.862-1G>A, one loss-of-function allele is in combination with missense mutations on the other allele, which are predicted to be damaging at the protein level (Table 4).

Disease severity and correlation with age and mutation type

In Figs. 2 and 3 we report the correlation between the disease severity scores (MRC, CMTES, and motor CMTES) and the age at examination, with the corresponding *MTMR2* and *MTMR13* mutations. The slopes are steeper for CMT4B1 than for CMT4B2. Regression analyses showed that disease severity according to MRC and motor CMTES is significantly associated with age in CMT4B1, with a worsening by 1.9 point/year (MRC; $p<0.0001$) and 0.17 point/year (motor CMTES; $p<0.05$), respectively. In CMT4B2, the slopes are less steep (0.5 point/year, MRC; 0.05 point/year, motor CMTES) and the association of disease severity with age was not significant. These cross-sectional data indicate that for increasing ages the scores worsen more in CMT4B1 than CMT4B2 patients. When adjusted for age, mean CMT4B1 scores were worse than those of CMT4B2 by 24 points for the MRC ($p<0.001$, age-adjusted) and 4.5 for the motor CMTES ($p<0.001$, age-adjusted).

Almost all the *MTMR2* mutations do not influence the correlation between disease severity and age, in agreement with the hypothesis of a complete loss of function of the MTMR2 protein for such mutations, including the compound heterozygous p.R459*/p.T537I and the C-terminal p.F551Lfs*17 variant, which removes both the coiled-coil and the PDZ (PSD-95/Dlg/ZO-1)- binding domains (see Fig. 1A). The p.F551Lfs*17 MTMR2 protein, if expressed, should be unable to homo- and heterodimerize and to dephosphorylate phospholipid substrates similarly to more upstream loss-of-function alleles[49]. The homozygous missense p.G103E variant does not influence correlation between disease severity and age, similarly to loss-of-function alleles. This observation is in agreement with

the finding that p.G103E significantly reduces MTMR2 phosphatase activity on PtdIns3*P* and PtdIns(3,5)P_2 phospholipids similar to nonsense mutations[50].

Interestingly, the three scores - MRC (Fig. 2A), CMTES (Fig. 2B) and motor CMTES (Fig. 2C) - differ from those expected only for the p.R628Pfs*18 mutation, and indeed the patient has a milder CMT4B1 phenotype compared to all other cases reported. The p.R628Pfs*18 mutation removes the most C-terminal PDZ-binding domain of MTMR2, which should result in an expressed MTMR2 protein unable to bind PDZ-domain containing interactors such as Dlg1 (Discs Large 1)[7, 51].

Similarly, *MTMR13* mutations do not influence the correlation between disease severity and age. This includes the three compound heterozygous genotypes carrying one missense mutation, which likely impact on MTMR13 function similarly to homozygous and compound heterozygous loss-of-function alleles. Such observations are in agreement with the *in silico* prediction of the deleterious effect of these mutations on the protein function (Table 4).

CMT4B3

We also collected data from three patients of a Korean family[36] and from the two siblings of a Syrian family[34] with CMT4B3. There were no novel data with respect to those already published. The phenotype of the first family is consistent with CMT4B with a demyelinating progressive sensory-motor neuropathy with myelin outfoldings without associated features; the three affected sisters were compound heterozygotes for two missense mutations, p.M417V and p.T1590A[36], both predicted as benign or tolerated by *in silico* analyses and therefore with a mild impact on the protein. Conversely, the Syrian family had an axonal motor neuropathy with microcephaly, intellectual disability, multiple cranial neuropathies, and “the fork and the bracket” signs on brain MRI showing the abnormal cranial nerves[52]; the two affected siblings carried a homozygous mutation, p.L335P, located next to the critical dDENN domain and predicted to be highly deleterious for the protein function[34]. The fork and the bracket signs, first described by Megarbane and colleagues[52] and later by Flusser et al. in another CMT4B3 family[32], have not been reported in other conditions thus far. They are unusual, possibly non-specific, imaging findings consisting in abnormal hyperintensities in T2-weighted images in the pons and mesencephalon, respectively, presumably resulting from degeneration of the VI and VII cranial nerves (fork) and of the oculomotor nerves fibers and nuclei as well as the medial longitudinal fasciculus (bracket).

Discussion

International collaborative studies are fundamental for tackling rare diseases, enabling the collection of relevant data that better defines their clinical phenotypes, natural histories, phenotype-genotype correlations, and prognosis, thereby improving management and paving the way for clinical trials. In this multinational retrospective study involving 18 centres in four continents, we were able to collect detailed data on 50 patients affected by CMT4B, a rare CMT subtype with only 89 affected subjects previously reported in the literature in 29 papers. Among 50 patients, 21 are described in this study for the first time. Overall, we found 41 mutations in *MTMR2* and *MTMR13*, responsible for CMT4B1 and B2,

respectively, of which 21 (50%) are novel variants and six represent novel disease association of known rare variants; 36 represent loss-of-function alleles and five are missense variants, which are predicted to have a deleterious effect on the protein function. Of note, six out of 17 mutations in *MTMR2* and 14 out of 24 in *MTMR13* are compound heterozygous mutations. Interestingly, genetically unrelated patients and patients from the same geographical area carry different mutations for *MTMR2* or *MTMR13*, thus excluding a founder effect.

Our data show that CMT4B1 is significantly more severe than CMT4B2, with earlier onset, more frequently delayed motor milestones, more severe walking disability and wheelchair needs, more common and severe respiratory involvement, and worse MRC and motor CMTES scores. The correlation between disease severity scores (MRC, CMTES, and motor CMTES) and age indicates that deterioration slopes are steeper for CMT4B1. Regression analyses confirm that the MRC and motor CMTES scores are much worse in CMT4B1 and the association between severity and age is stronger in CMT4B1. Therefore, results from both univariate and multivariate analyses are consistent with a more rapid disease progression in CMT4B1, which however should be confirmed in a longitudinal study. The slope of deterioration of clinical scores in both subtypes allow also important prognostic predictions (see Figs. 2 and 3).

The observation that CMT4B1 is more severe than CMT4B2 is in agreement with what has been reported so far on MTMR2 and MTMR13 function. MTMR2 is a catalytically active phospholipid phosphatase acting on PdtIns3*P* and PtdIns(3,5)P_2 [10]. Several studies suggested that PtdIns(3,5)P_2 could be the preferential substrate of MTMR2 *in vivo*[42, 49, 50, 53, 54]. MTMR13, a catalytically inactive myotubularin, interacts with MTMR2 through the coiled-coil domain and MTMR13/MTMR2 heterodimers are thought to possess a higher enzymatic activity as compared to MTMR2 homodimers[38, 39]. Therefore, while in CMT4B1 the MTMR2 catalytic activity is lost, in CMT4B2 the MTMR2 catalytic activity can be partially preserved, thus resulting in a milder clinical phenotype as compared to CMT4B1. Another possibility is that, in the absence of MTMR13, MTMR2 heterodimerizes with MTMR5 and dephosphorylates substrates although less efficiently than MTMR2/MTMR13. How many homodimers or heterodimers of catalytically active and inactive myotubularins are physiologically present in different cell types and what are their specific biochemical properties and membrane localizations is unclear. Notably, there was very little phenotypic variability within CMT4B1 families, while disease severity appeared less homogeneous in two CMT4B2 sibs couples.

Vocal cord palsy with dysphonia is a frequent finding for both CMT4B1 and CMT4B2 and may be another clue to diagnosis[11]. It may range in degree from a hoarse voice to life-threatening stridor leading to respiratory difficulties, requiring ventilatory support and even tracheostomy; laser treatment proved effective in more than one case. Respiratory involvement is possible in both types but occurs particularly in CMT4B1. Regular laryngeal and respiratory monitoring are therefore recommended for both diseases.

Cranial nerve involvement is common in both subtypes, but facial weakness (62% vs 16%) and tongue atrophy or weakness (27% vs 5%) are much more frequent in CMT4B1 while hearing loss appears to be more common in CMT4B2 (26%).

Our data also confirm that glaucoma and related eye complications occur exclusively in CMT4B2, as these were found in 32% of our cases, a rate only slightly lower than that reported (40%) by Lassuthova and colleagues[12]. In accord with their findings, we confirm that glaucoma may be either congenital or start later in life and that regular ocular pressure assessment is recommended. MTMR2 and MTMR13 co-localize and co-fractionate at intracellular membranes[38, 39]. Interestingly, MTMR13 has been found to be associated with heavy membrane fractions, which do not contain MTMR2, suggesting the possibility that MTMR13 has other MTMR2-independent functions[39]. For instance, MTMR13 through DENN (Differentially Expressed in Neoplastic versus Normal cells) domains can act as a GEF (Guanine Exchange Factor) of Rab21 GTPase, to coordinate the efflux of membranes from early endosomal compartment toward recycling compartments and autophagosomes, thus promoting autophagic flux[55, 56]. Thus, it is possible to speculate that loss of MTMR13 also affects other tissues in addition to nerves, where MTMR2-independent functions are more physiologically relevant.

Autism and learning disability occurred in 2/19 CMT4B2 subjects, a rate too low to suggest a causal association. Interestingly, MTMR2 has been suggested to negatively regulate endocytosis and to contribute to the maintenance of excitatory synapses in neurons *in vitro*[57].

Both CMT4B1 and CMT4B2 patients had reduced motor and sensory nerve conduction velocity slowing to a similar extent; the mean CMAP amplitude was lower in CMT4B1 but not significantly different than in CMT4B2. Interestingly, all examined CMT4B2 patients had absent SAPs, while a few CMT4B1 patients had still recordable SAPs in their upper limbs, and the clinical sensory scores were slightly, though not significantly, worse in CMT4B2 patients. When performed, nerve biopsies consistently showed the typical myelin outfoldings in several nerve fibers.

Our data also indicates that there is no correlation between *MTMR2* or *MTMR13* mutations type, genotypes, domain localization, and clinical scores. Almost all mutations behave similarly regarding the correlation between clinical scores and age at examination, suggesting that all variants act as complete loss-of-function alleles and produce similar disease severity within each CMT4 subtype. The only exception is the p.R628Pfs*18 mutation in the *MTMR2* gene, which is associated with a milder phenotype in CMT4B1[15]. Interestingly, the p.R628Pfs*18 mutation is predicted to remove only the most C-terminal PDZ-binding domain. Thus, this truncated MTMR2 protein should retain, at least in part, the ability to localize at membranes through the PH-GRAM (Pleckstrin Homology-Glucosyltransferases, Rab-like GTPase activators and Myotubularins) domain and to dephosphorylate phospholipid substrates.

In contrast, tentative genotype-phenotype correlations have been made for CMT4B3. For *MTMR5/SBF1*, the mutation type and localization show correlation with disease severity.

Only two compound heterozygous missense mutations co-segregating in three siblings from the Korean family have been associated so far with a demyelinating CMT4B3 neuropathy with myelin outfoldings and slow disease progression[36]. These variants are predicted to be benign with a mild impact on MTMR5 protein function. On the contrary, loss-of-function mutations or (like in the Syrian family) homozygous missense mutations, which are located in DENN domains or in the region between DENN and GRAM domains at the N-terminus, are associated with a more complex syndrome with axonal neuropathy complicated by central nervous system and skeletal abnormalities[32–35]. This observation might suggest that in neurons and axons MTMR5 partners with a different catalytically active MTMR and complete loss of MTMR5 cannot be compensated by the presence of MTMR2 or MTMR13, which might not exert a relevant function in neurons. However, further cases will be necessary to confirm these associations as CMT4B3 is even more rare than CMT4B1 and B2.

Of note, two therapeutic strategies have been recently proposed for CMT4B, of which one has already been investigated at the pre-clinical level *in vivo*. NRG1 (Neuregulin) type III is one of the main signals regulating Schwann cell development and myelination[58]. Importantly, the amount of NRG1 type III on the axonal surface determines myelin thickness in Schwann cells. NRG1 type III is negatively regulated by TACE (Tumor necrosis factor-α Converting Enzyme) secretase, whose activity is enhanced by Niacin-Niaspan, an FDA approved drug[41]. Thus, we recently tested the hypothesis that Niaspan, by activating TACE and decreasing NRG1 type III signalling, could be effective in reducing the amount of myelin formed in hypermyelinating neuropathies, including CMT4B1. We observed that this drug significantly rescues myelin outfoldings *in vitro* and *in vivo* in *Mtmr2*-null mice, a model of the CMT4B1 neuropathy[41].

Another approach is to rebalance PtdIns(3,5)P_2 phospholipid levels, which are elevated in *Mtmr2*-null cells[42]. PIKfyve is the kinase that produces PtdIns(3,5)P_2 and its activity can be inhibited by small molecule compounds[59, 60]. We already reported that myelin outfoldings *in vitro* are significantly rescued by inhibiting PIKfyve activity, likely because of rebalanced levels of PtdIns(3,5)P_2 in Schwann cells[42].

The present retrospective study, with its intrinsic limitations, is a first step in the pathway to clinical trial readiness, which needs to be followed by longitudinal natural history studies, the selection of appropriate outcome measures and the design of interventional studies.

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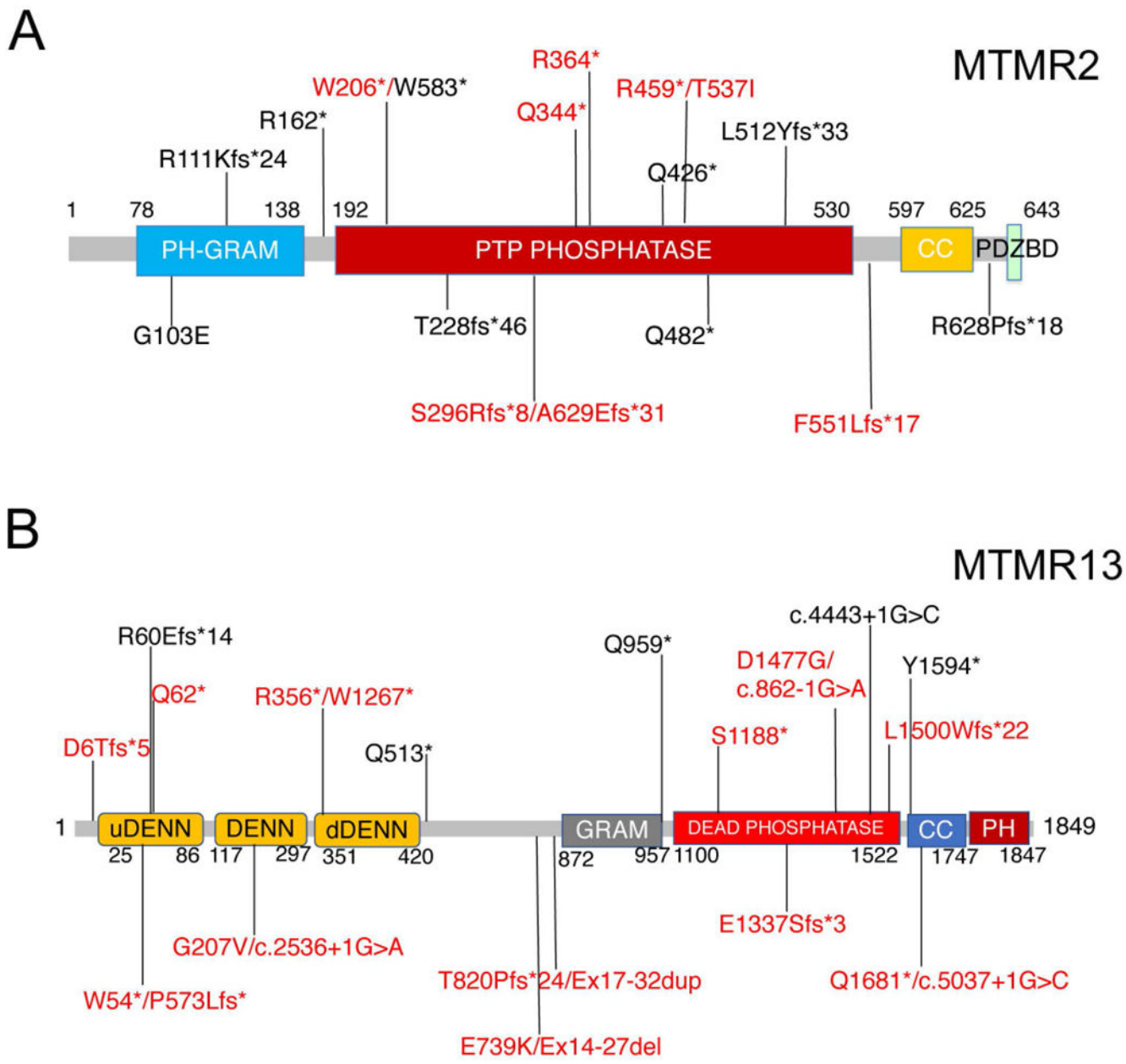

Fig. 1.

Schematic representation of MTMR2 and MTMR13 proteins and localization of mutations reported in this study. MTMR2 domains are PH-GRAM: pleckstrin homology–glucosyltransferases, rab-like GTPase activators and myotubularins; PTP Phosphatase: protein tyrosine phosphatase/dual specificity phosphatase (PTP/DSP) domain; CC: coiled coil domain; PDZ-BD: PDZ binding domain (**A**). MTMR13 domains are DENN: differentially expressed in neoplastic versus normal cells; GRAM: glucosyltransferases, rab-like GTPase activators and myotubularins; DEAD phosphatase: catalytically inactive phosphatase domain; PH: Pleckstrin homology domain (**B**). In red, novel variants or novel disease associations of known rare variants (described in Tables 3 and 4); in black are variants already reported.

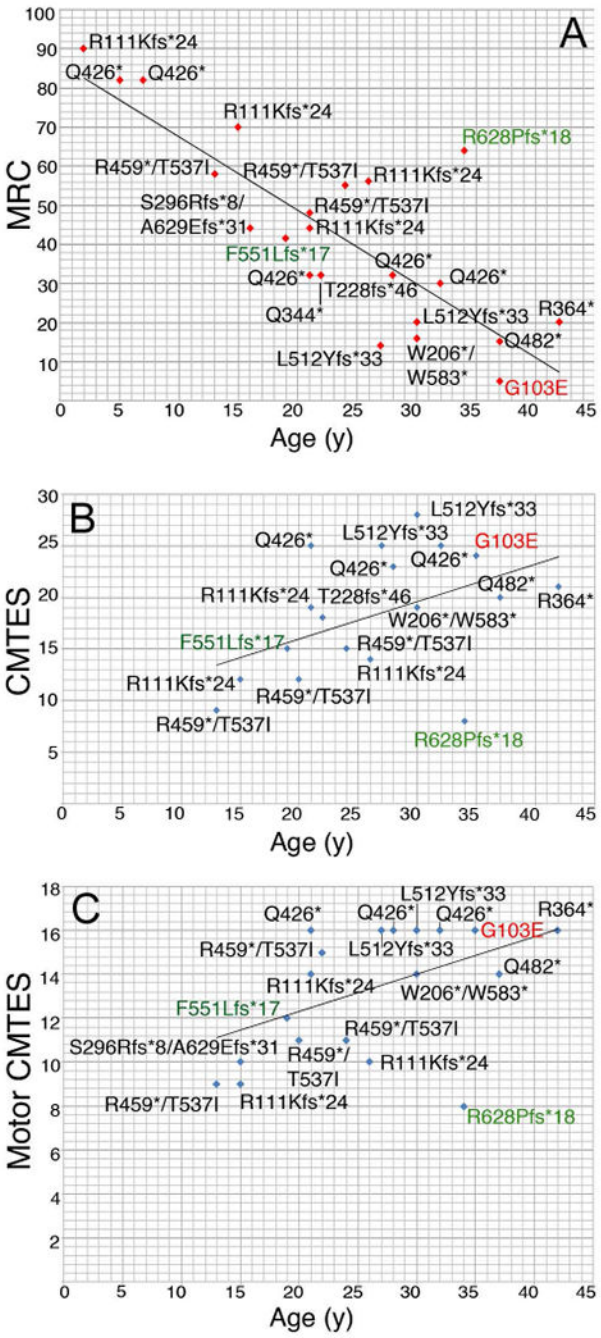

Fig. 2.

Correlation between the disease severity scores and age for CMT4B1. MRC in (**A**), CMTES in (**B**) and motor CMTES in (**C**) scores. For each patient the corresponding mutation(s) is (are) shown. In red, homozygous missense mutation; in green, mutations at C-terminal domains.

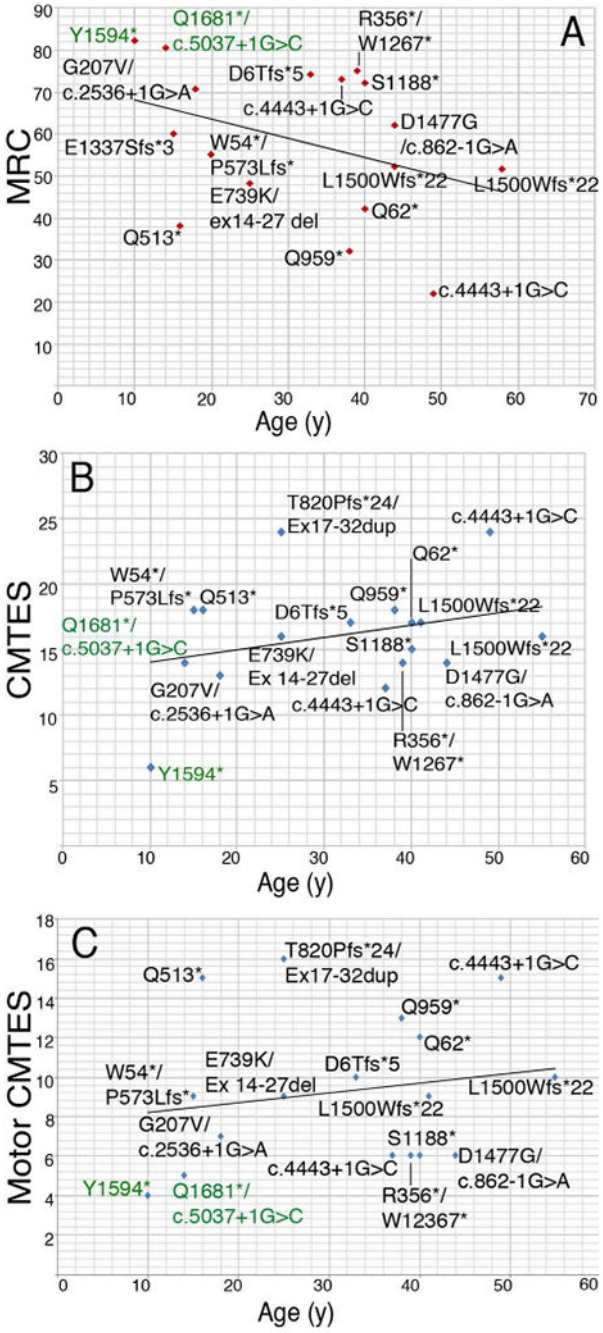

Fig. 3.

Correlation between the disease severity scores and age for CMT4B2. MRC in (**A**), CMTES in (**B**) and motor CMTES in (**C**) scores. For each patient the corresponding mutation(s) is (are) shown. In green, mutations at C-terminal domains.

Table 1.

Comparison of clinical findings in CMT4B1 and CMT4B2

	CMT4B1	CMT4B2	Significance
Patients, n (females)	26 (10)	19 (11)	
Age of onset (years) (mean ± SD, range)	2.8 ± 2.8; 0-13	6.7 ± 5.0; 1-20	**p<0.01**
Age at last visit (years) (mean ± SD, range)	21.9 ± 12.6; 3-48	31.7 ± 13.5; 8-58	**p = 0.02**
Delay in motor milestones	13 (50%)	4 (21%)	p = 0.07
Vocal cord palsy/dysphonia	10 (38%)	8 (42%)	p = 1.00
Respiratory involvement	8 (31%)	3 (16%)	p = 0.31
Eye disease	Glaucoma = 0	Glaucoma = 6 (32%)	**p<0.01**
	Usher syndrome = 2 Keratoconus blindness = 1	Optic atrophy = 2 (11%)	p = 1.00
Ptosis	6 (23%)	2 (11%)	p = 0.44
Facial weakness	16 (62%)	3 (16%)	**p<0.01**
Hearing loss	2 (Usher Syndrome)	5 (partial) (26%)	p = 0.11
Tongue atrophy/weakness	7 (27%)	1 (5%)	p = 0.11
Foot deformities	22 (85%) (pes planus in 8)	16 (84%) (pes planus in 3)	p = 1.00
Foot surgery	11 (42%)	10 (53%)	p = 0.56
Scoliosis	9 (35%)	11 (58%)	p = 0.14
AFO or orthopaedic shoes only	8 (31%)	8 (42%)	p = 0.53
Unilateral Support	1 (4%)	2 (11%)	p = 0.57
Wheelchair use	12 (46%)	4 (21%)	p = 0.12
Difficulties with buttons/eating with utensils	22 (85%)	18 (95%)	p = 0.62
Weakness	UL proximal 14 (54%)	UL proximal 5/18 (28%)	p = 0.13
	UL distal 22 (85%)	UL distal 18/18 (100%)	p = 0.13
	LL proximal 21 (81%)	LL proximal 9/18 (50%)	p = 0.05
	LL distal 25 (96%)	LL distal 18/18 (100%)	p = 1.00
DTR	Absent UL 22 (85%)	Absent UL 14 (74%)	p = 0.46
	Absent LL 26 (100%)	Absent LL 18 (95%)	p = 0.42
Sensory symptoms			
- paraesthesias	8 (31%)	5 (26%)	p = 1.00
- distal sensory loss	15 (58%)	14 (74%)	p = 1.00

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	CMT4B1	CMT4B2	Significance
- ulcers	1 (4%)	1 (5%)	p = 0.35

DTR = deep tendon reflexes; LL = lower limbs; SD = Standard deviation; UL = upper limbs.

Values in bold indicate significant differences between CMT4B1 and CMT4B2.

Table 2.

Comparison of clinical scales and electrophysiological findings in CMT4B1 and CMT4B2

Mean ± SD (N, min-max)	CMT4B1 (n = 26)	CMT4B2 (n = 19)	Significance
MRC score (9 muscle pairs)	44.3 ± 24.6 (24, 5-90)	58.21 ± 17.8 (17, 22-82)	**p = 0.04**
Age at examination MRC	22.3 ± 10.8 (24, 2-42)	31.7 ± 14.5 (17, 10-58)	**p = 0.02**
CMTES	18.1 ± 6 (19, 8-28)	16.1 ± 4.3 (17, 6-24)	p = 0.26
CMTNS	30.1 ± 4.7 (10, 19-36)	23.4 ± 4.5 (16, 17-32)	**p = 0.001**
Motor CMTES	13.2 ± 2.9 (20, 8-16)	9.3 ± 3.8 (17, 4-16)	**p = 0.001**
Sensory CMTES	5.0 ± 3.4 (19, 0-12)	6.8 ± 2.2 (17, 2-10)	p=0.07
Age at examination CMTES/CMTNS	25.6 ± 7.9 (20, 13-42)	31.6 ± 13.8 (17, 10-55)	p = 0.11
Ulnar Nerve MCV (m/s)	18.2 ± 5.9 (9, 9.6-27,2)	18.2 ± 5.5 (12. 11-29)	p=1.00
Ulnar nerve CMAP amplitude (mV)	2.4 ± 2.2 (9, 0.1-6)	3.2 ± 2.1 (12, 0.1-6)	p=0.41
Median Nerve MCV (m/s)	19.7 ± 6.1 (12, 13-34)	14.7±1.6 (7, 13-18)	p=0.05
Median nerve CMAP amplitude (mV)	1.7 ± 1.3 (12, 0.1-4.6)	2.0 ± 1.7 (8, 0.1-4.5)	p=0.66

CMAP = compound muscle action potential; CMTES = CMT Examination Score; CMTNS = CMT Neuropathy Score, MCV = motor conduction velocity; MRC = Muscle Research Council, strength manual testing; SD = standard deviation.

Values in bold indicate significant differences between CMT4B1 and CMT4B2.

Table 3.

Novel variants identified in the *MTMR2* gene.

Nucleotide variant	Protein variant	Patient ID	Patient Origin	Haplotype	GnomAD Allele Frequency (AF)	Provean	Polyphen-2
c.618G>A	p.W206*	1-F	Paris 6, French*	Biallelic, *in trans*	0		
c.1090C>T	p.R364*	1-S	Portuguese	Homoz	1.062e-5		
c.1030C>T	p.Q344*	1-D, 1-E	Mauritius Island*	Homoz	0		
c.1375C>T	p.R459*	1-A, 1-B, 1-C	Paris 1,2,3 French*	Biallelic, *in trans*	3.984e-6		
c.1610C>T	p.T537I	1-A, 1-B, 1-C	Paris 1,2,3 French*	Biallelic, *in trans*	0	Deleterious −5.524	Probably damaging 0.996
c.878_887dup	p.S296Rfs*8	1-T	Ashkenazi Jewis, Eastern Europe	Biallelic, *in trans*	0		
c.1849_1852dup	p.A629Efs*31	1-T	Ashkenazi Jewis, Eastern Europe	Biallelic, *in trans*	2.839e-5		
c.1653delC	p.F551Lfs*17	1-Q	Bangladesh	Homoz	0		

Legend: Novel variants (AF=0, not reported) and novel disease association of known rare variants (AF<0.01), which have not previously associated with CMT4B and identified in the *MTMR2* gene.

*Details pertaining to patients 1-A, 1-B, 1-C, 1-D, 1-E, and 1-F will be included in a manuscript in preparation. Biallelic, *in trans,* was inferred on the basis of the segregation analysis from the parents.

Table 4.

Novel variants identified in the *MTMR13* gene.

Nucleotide variant	Protein variant	Patient ID	Patient Origin	Haplotype	GnomAD Allele Frequency (AF)	Provean	Polyphen-2
c.16_19del	p.D6Tfs*5	2-A	Turkish*	Homoz	0		
c.184C>T	p.Q62*	2-G	Italian	Homoz	0		
c.1066 C>T	p.R356*	2-S	Albanian	Biallelic, segregation N/A	8.041e-6		
c.3800 G>A	p.W1267*	2-S	Albanian	Biallelic, segregation N/A	0		
c.3563C>A	p.S1188*	2-B	Maltese	Homoz	0		
c.4430A>C	p.D1477G	2-J	Maghreb	Biallelic, *in trans*	0	Deleterious-6.351	Probably damaging 1.000
c.862-1G>A	Splicing	2-J	Maghreb	Biallelic, *in trans*	0		
c.4499delT	p.L1500Wfs*22	2-C, 2-D	Italian	Homoz	0		
c.620G>T	p.G207V	2-R	British	Biallelic, *in trans*	0	Deleterious-7.556	Probably damaging 1.000
c.2536+1G>A	Splicing	2-R	British	Biallelic, *in trans*	0		
c.161G>A	p.W54*	2-I	British	Biallelic, *in trans*	3.987e-6		
c.1718delC	p.P537Lfs*	2-I	British	Biallelic, *in trans*	0		
c.2457delT	p.T820Pfs*24	2-K	USA	Biallelic, *in trans*	0		
[hg19] 11p15.4(9,829, 246 - 9,917,849) ×3	Ex 17-32dup	2-K	USA	Biallelic, *in trans*	0		
c.2215G>A	p.E739K	2-O	British	Biallelic, *in trans*	0	Deleterious-3.797	Probably damaging 0.972
[hg19] 11p15.4 (9,853,777 – 10,003,829)	Ex 14-27del	2-O	British	Biallelic, *in trans*	0		
c. 4009_4016del	p.E1337Sfs*3	2-Q	Pakistani	Homoz	0		
c.5041C>T	p.Q1681*	2-N	British	Biallelic, *in trans*	3.191e-5		
c.5037+1G>C	Splicing	2-N	British	Biallelic, *in trans*	0		

Legend: Novel variants (AF=0, not reported) and novel disease association of known rare variants (AF<0.01), which have not previously associated with CMT4B and identified in the *MTMR13* gene.

* Details pertaining to patient 2-A will be included in a manuscript in preparation. Biallelic, *in trans,* was inferred on the basis of the segregation analysis from the parents. N/A= Not assessed.