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RESEARCH REPORT

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Clinical and genetic features of CMT2T in Italian patients confirm the importance of *MME* pathogenic variants in idiopathic, late-onset axonal neuropathies

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Abstract

Background and Aims: Since 2016, biallelic mutations in the membrane metalloendopeptidase (*MME*) gene have been associated with late-onset recessive CMT2 (CMT2T). More recently, heterozygous mutations have also been identified in familial and sporadic patients with late-onset axonal neuropathy, ranging from subclinical to severe. This indicates that the heterozygous *MME* variants may not be fully penetrant, or alternatively, that they may be a potential risk factor for neuropathy. Here, we describe the clinical, neurophysiological, and genetic findings of 32 CM2T Italian patients.

Alessandro Geroldi and Andrea La Barbera contributed equally to this study.

Methods: The patients were recruited from four different Italian referral centers. Following a comprehensive battery of neurological, electrophysiological, and laboratory examinations, the patients' DNA was subjected to sequencing in order to identify any variants in the gene. Bioinformatic and modeling analyses were performed to evaluate the identified variants' effects.

Results: We observe a relatively mild axonal sensory-motor neuropathy with a greater impairment of the lower extremities. Biallelic and monoallelic patients exhibit comparable disease severity, with an earlier onset observed in those with biallelic variants. When considering a subgroup with more than 10 years of disease, it becomes evident that biallelic patients exhibit a more severe form of neuropathy. This suggests that they are more prone to quick progression.

Interpretation: CM2T has been definitively defined as a late-onset neuropathy, with a typical onset in the fifth to sixth decades of life and a more rapidly progressing worsening for biallelic patients. CMT2T can be included in the neuropathies of the elderly, particularly if *MME* variants heterozygous patients are included.

KEYWORDS

CMT, genotype–phenotype correlation, late-onset peripheral neuropathies, *MME*, neprilysin

1 | INTRODUCTION

Charcot–Marie–Tooth (CMT) disease is a clinically and genetically heterogeneous group of neuromuscular diseases involving all types of Mendelian inheritance patterns.

Historically, the classification of CMT has been based on neurophysiological findings. Specifically, the upper limb motor nerve conduction velocity (MNCV) of 38 m/s is used to distinguish between demyelinating CMT (CMT1) (MNCV ≤ 38 m/s) and axonal CMT (CMT2) (MNCV > 38 m/s).[1,2] Moreover a third class of intermediate CMT has been considered with MNCV ranging from 25 to 45 m/s.[3]

Over 100 genes with different modes of inheritance have been associated with CMT mainly due to the increasing use of next-generation sequencing (NGS) technologies.[4]

However, some forms of CMT, such as adult-onset CMT2, remain genetically undiagnosed. A correct diagnosis may be delayed or never established due to late onset and unremarkable family history. Late-onset disorders were previously considered most likely autosomal dominant (AD) with age dependent penetrance, but more recently, late-onset recessive conditions have also been described.[5]

In particular, biallelic mutations in the membrane metalloendopeptidase *MME* gene have been identified as the most common cause of autosomal recessive CMT2 in Japanese populations, with disease onset occurring in the fifth decade.[4] In the same way in the Spanish population, biallelic mutations in the *MME* gene were identified in patients with a late-onset motor predominant neuropathy with sensory involvement in later stages of the disease. In both the Spanish and Japanese cohorts, heterozygous carriers did not exhibit any symptoms of neuropathy and clinical and neurophysiological assessments were normal[6,7] suggesting that mutations in *MME* do not cause AD CMT2.

More recently, heterozygous mutations in *MME* were also found in familial and sporadic patients with late-onset axonal neuropathy in Europe and North America.[8] The phenotype exhibited a wide range of neuropathy severity, from subclinical to severe axonal neuropathy indicating an age-dependent penetrance. Senderek et al. demonstrated a higher frequency of *MME* variants in cases compared with controls, indicating that monoallelic variants in *MME* may be considered a risk factor.[9]

The *MME* gene encodes neprilysin (NEP), a zinc-dependent metalloprotease that is widely expressed on the surface of cells in many tissues, including the peripheral nervous system (PNS).[10,11]

The role of NEP in the PNS is still unclear, but it appears to be important in regulating neuropeptide levels. Deficiency of NEP leads to exacerbation of pain and neurogenic inflammation after nerve injury.[11]

In this paper, we described 32 Italian patients carrying mono or biallelic *MME* variants comparing their clinical, neurophysiological, and genetic findings.

2 | PATIENTS AND METHODS

The patients described in this study were recruited from CMT routine diagnostic activities at four different Italian referral centers for inherited neuropathies diagnosis, namely Genoa, Florence, Rome, and Naples.

Patients underwent a neurological objective examination, followed by nerve conduction studies (NCSs) in their upper and lower

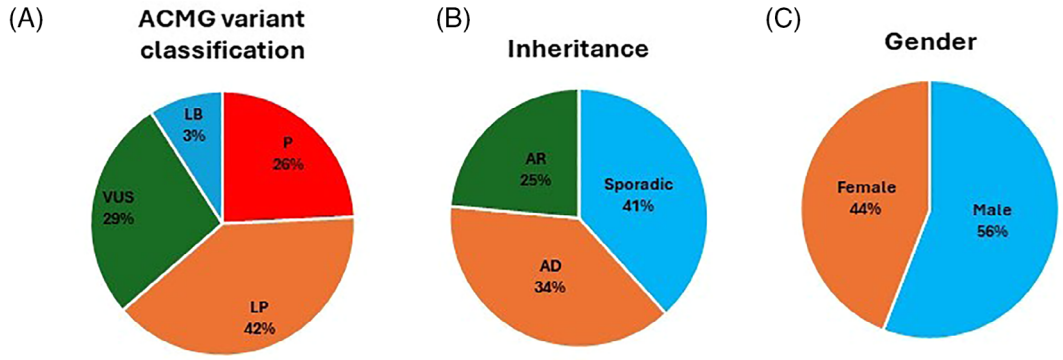


FIGURE 1 ACMG-based variants pathogenicity (A), inheritance (B), and gender (C) distribution among the 32 patients.

limbs (LLs) using standard techniques. At the same time, extensive laboratory studies were conducted to rule out acquired causes of neuropathy.

The genomic DNA was extracted from a peripheral blood sample of the patients using standard methods after obtaining their written informed consent. *MME* molecular analysis was performed using Sanger sequencing or NGS targeted analysis.

Variants interpretation and classification were performed following the Guidelines of the American College of Medical Genetics and Genomics (ACMG).[12]

After sequencing, the clinical data, neurophysiological and genetic findings of affected individuals carrying mutations in the *MME* gene were studied in more detail.

After clinical reevaluation patients' disability was classified, according to CMT neuropathy score (CMTES).

Mini–Mental State Examination (0–30 scale, normal > 24) was performed to evaluate cognitive impairment.

All procedures were conducted in compliance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

The significance of the results was determined using a *t*-student test with a *p*-value of less than .05.

The MME PDB structure was downloaded from the Protein Data Bank (pdb code 7AE1). Missense variants were evaluated using Chimera X, selecting the most probable conformation.

3 | RESULTS

During routine diagnostic activities, the participating centers identified 27 index cases and five affected relatives carrying *MME* variants for a total of 32 cases. To classify their variants, the ACMG guidelines were followed, defining them as Pathogenic (P) in 8 patients, likely pathogenic (LP) in 13, a variant of unknown significance in 10 and likely benign (LB) in the remaining one (Figure 1). The gender distribution is almost equal, 18 male and 14 female patients with 59% of familial cases and 41% of sporadic cases (Figure 1).

Clinical and neurophysiological data of these patients are summarized in Tables 1 and 2.

Disease onset ranges from 24 to 75 years with a mean onset of 54.7 years (median 55, mode 48) (Figure 2) with a mean disease duration of 10.6 years (median 9.5, mode 10) (Figure 2).

The difference between the mean disease onset of mono and biallelic patients is slightly greater than the statistical threshold for significance (49.6 years vs. 59.2 years, $p = .052$). The duration of illness follows the same pattern (10.5 years vs. 11.5 years, $p = .7$). However, when we exclude the values for variants predicted as LB, a significant difference (49.6 years vs. 61.4 years, $p = .0099$) in onset is observed.

Onset symptoms are predominantly motor (53%), and less frequently purely sensory (25%) or mixed (22%). Among the motor symptoms, gait impairment is the most common, while paresthesia/distal tingling and neuropathic pain are the most commonly identified purely sensory symptoms (Figure 3).

Clinically, most patients present with predominantly distal LL neuropathy of mild-to-moderate severity with a mean CMTES of 10, ranging from 1 to 21 (Figure 4A).

The CMTES for heterozygous and homozygous patients are comparable, while it can be seen that this value depends more on the duration of the disease, as shown in Figure 4. Specifically, CMTES is statistically smaller in patients with less than 10 years of disease compared with those with more than 10 years (CMTES 8 vs. 13, $p = .044$ with $p < .05$). This difference becomes more significant when we exclude patients presenting the variants predicted LB (CMTES 8 vs. 13, $p = .019$) (Figure 4A).

A difference in CMTES score between heterozygous and homozygous patients is observed only in subgroups with more than 10 years of disease duration. In this case, homozygous patients exhibit greater disease severity, although it is not statistically significant due to a single outlier value (Figure 4A).

Overall, 69% of patients show moderate to severe LL weakness while 81% show no or only mild strength deficit at the upper limb. On the sensory side, about 2/3 of MME patients have mild to moderate loss of sensation to touch, with 62% reduced pinprick and 63% reduced light touch. LL deep tendon reflexes (DTRs) are absent or reduced in 91% of patients, meanwhile UL DTRs are abnormal in 56% (Figure 4B).

When considering individual clinical parameters such as reflex, weakness, and sensory involvement separately, the differences

TABLE 1 Clinical features.

Case	Substitution	Zygosity	ACMG	Family history	Sex	Age at onset (years)	Age at evaluation (years)	Disease duration (years)	Onset symptom	DTRs		Weakness		Sensory involvement		CMTES	Comorbidity
										UL	LL	UL	LL	Pinprick	Touch		
Pt1	c.1229G>T p.Arg410Leu	Htz	VUS	NO	F	72	82	10	Walking difficulties and cramps	na	-	-	++	-	+	na	Paroxysmal atrial fibrillation, post-thyroidectomy hypothyroidism for goiter, cholecystectomy, TIA
Pt2	c.1946T>G p.Ile649Ser	Hmz	LP	AD	M	48	57	10	LL weakness, gait difficulties	-	-	-	+	-	-	3	Hypertension
Pt3	c.156C>G p.Tyr52Ter; c.1497+5G>A	N.A.	(LP) (VUS)	NO	M	48	52	6	LL weakness	-	–	+	++	+	+	na	NO
Pt4	c.877C>T p.Arg293Ter	Htz	P	NO	M	69	74	5	Walking difficulties	+	–	+	++	+	+	16	Multiple myeloma
Pt5	c.1040A>G p.Tyr347Cys	Htz	VUS	AD	F	61	80	19	Neuropathic pain and frequent falls	+	-	+	-	-	-	4	Hypertension, essential thrombocythemia, osteoarthritis, multi-infarct encephalopathy
Pt6	c.[2144G>A p.Gly715Asp	Htz	VUS	AD	M	72	73	1	Neuropathic pain	-	–	-	-	-	++	5	Allergic asthma, seminoma, benign prostatic hyperplasia, multinodular goiter, hypercholesterolemia
Pt7	c.877C>T p.Arg293Ter	Htz	P	NO	M	55	64	9	Sensory deficit—walking difficulties	+	-	-	++	+	+	10	NO
Pt8	c.1188G>A p.Lys396Lys	Htz	VUS	AD	M	74	76	2	Neuropathic pain	-	–	-	++	-	-	8	Mild hypercholesterolemia, benign prostatic hypertrophy
Pt9	c.2077-1G>C	Htz	LP	NO	F	62	64	2	Muscle cramps	+	–	-	+	-	-	3	Psoriasis, Hashimoto's thyroiditis, hypercholesterolemia
Pt10-I	c.467delC p.Pro156fs*14	Hmz	P	AD	M	50	70	20	Walking difficulties and cramps	–	–	++	+++	+	-	21	NO
Pt10-II	c.467delC p.Pro156fs*14	Htz	P	AD	M	65	78	13	Neuropathic pain	-	–	+	+++	+	-	14	NO
Pt11	c.467delC p.Pro156fs*14	Htz	P	NO	F	40	59	19	Walking difficulties	+	+	-	++	+	+	7	NO
Pt13	c.1946T>C p.Ile649Thr	Hmz	LP	NO	F	59	60	1	Walking difficulties	-	–	+	+	-	-	6	NO
Pt14-I	c.2153+5G>A	Hmz	LP	AR	M	50	61	11	Walking difficulties	–	–	na	na	++	++	17	Glaucoma
Pt14-II	c.2153+5G>A	Hmz	LP	AR	M	40	62	22	Walking difficulties	+	-	-	+	-	-	7	Keratocono, undifferentiated connectivitis
Pt14-III	c.2153+5G>A	Hmz	LP	AR	M	60	66	6	Distal tingling	+	-	-	-	-	-	1	NO

(Continues)

TABLE 1 (Continued)

Case	Substitution	Zygosity	ACMG	Family history	Sex	Age at onset (years)	Age at evaluation (years)	Disease duration (years)	Onset symptom	DTRs		Weakness		Sensory involvement		CMTES	Comorbidity
										UL	LL	UL	LL	Pinprick	Touch		
Pt15	c.2154-5_2160del	Htz	VUS	NO	M	62	69	7	Distal tingling	-	–	+	+	++	++	11	NO
Pt16	c.467delC p.Pro156Leufs*14	Hmz	P	NO	M	33	41	8	Distal tingling	-	–	-	++	++	+++	15	Asthma
Pt17	c.440-2A>C	Hmz	P	NO	F	48	66	10	Gait impairment	–	–	++	+++	++	++	17	NO
Pt18	c.1437-1446delinsCAATAG p.Arg479Serfs*14	Hmz	LP	NO	F	35	44	8	Gait impairment	–	–	-	++	+	+	2	NO
Pt19	c.1810G>A p.Val604Ile	Htz	LB	AD	M	24	33	7	Cramp and gait impairment	–	–	+	++	+	++	4	NO
Pt20	c.2076G>C p.Gln692His	Htz	LP	AD	M	30	54	20	Gait impairment	+	+	+	+	-	-	4	NO
Pt21	c.2077-1G>C	Htz	LP	AD	F	73	85	10	Gait impairment and paresthesia	–	–	++	+++	+	+	7	NO
Pt22	c.2154-5_2160del	Hmz	VUS	AR	M	68	77	8	Paresthesia	–	–	++	+++	++	++	12	NO
Pt23	c.467delC p.Pro156fs*14	Hmz	P	AR	F	55	68	13	Gait impairment, fatigue	-	+	+	+++	+	+++	15	NO
Pt24	c.869_879dupCTGAAGATCGA p.Asn294Leufs*14	Hmz	LP	AR	F	43	65	22	Gait impairment	–	–	+	+++	++	++	16	Primary biliary cholangitis
Pt25-I	c.1040A>G p.Tyr347Cys	Htz	VUS	AD	F	47	58	11	Gait impairment and distal paresthesia	+	–	-	++	+	++	9	Rheumatoid Arthritis
Pt25-II	c.1040A>G p.Tyr347Cys	Htz	VUS	AD	F	56	86	30	Gait impairment	+	–	+	+++	+	++	16	Celiac disease
Pt26	c.1040A>G p.Tyr414Cys	Htz	VUS	NO	F	75	83	8	Gait impairment	+	-	-	+++	+	+	14	Sjogren syndrome
Pt27-I	c.2011G>T p.Glu671Ter	Hmz	LP	AR	F	51	57	6	Gait impairment	+	–	-	++	-	+	5	Thyroiditis
Pt27-II	c.2011G>T p.Glu671Ter	Hmz	LP	AR	M	55	57	2	Gait impairment	+	–	-	++	-	+	9	Multiple Sclerosis
Pt28	c.2067C>A p.Asn689Lys	Htz	VUS	NO	M	70	93	23	Gait impairment	–	–	++	+++	++	++	20	NO

Note: DTRs (normal: +; reduced: -; absent: –); weakness and sensory involvement (severe: +++; moderate: ++; mild: +; normal: -);
Abbreviations: AD, autosomal dominant; AR, autosomal recessive; DTR, deep tendon reflexe; Hmz, homozygous; Htz, heterozygous; LL, lower limb; NO, sporadic; UL, upper limb, VUS, variant of unknown significance.

TABLE 2 Upper limb (UL) nerve conduction studies (NCSs).

		UL NCS				UL NCS	
Case	Neuropathy	CMAP (mV)	MNCV (m/s)	Case	Neuropathy	CMAP (mV)	MNCV (m/s)
Pt1	Sensory motor axonal	3.7	51	Pt16	Sensory motor axonal	8.1	33
Pt2	Sensory motor axonal	15.5	54	Pt17	Sensory motor axonal	4.6	46
Pt3	Sensory motor axonal	8	51	Pt18	Motor axonal	11	59
Pt4	Sensory motor axonal	6.8	59	Pt19	Sensory motor axonal	2.2	58
Pt5	Sensory motor axonal	6.1	66	Pt20	Motor axonal	0.2	nr
Pt6	Sensory motor axonal	na	na	Pt21	Sensory motor axonal	5.6	55
Pt7	Sensory motor axonal	11.4	68	Pt22	Sensory motor axonal	1.9	31
Pt8	Sensory motor axonal	6.9	55	Pt23	Sensory motor axonal	12.9	51
Pt9	Sensory motor axonal	na	na	Pt24	Sensory motor axonal	12.1	48
Pt10-I	Sensory motor axonal	2.7	40	Pt25-I	Sensory motor axonal	11.6	51
Pt10-II	Sensory motor axonal	11.7	57	Pt25-II	Sensory motor axonal	6	47
Pt11	Sensory motor axonal	9.6	56	Pt26	Sensory motor axonal	12.3	52
Pt13	Sensory motor axonal	13.4	38	Pt27-I	Sensory motor axonal	15	50
Pt14-I	Sensory motor axonal	10.7	54	Pt27-II	Sensory motor axonal	10.9	50
Pt14-II	Sensory motor axonal	17.5	53	Pt28	Sensory motor axonal	9	57
Pt14-III	Sensory motor axonal	10.9	54				
Pt15	Sensory motor axonal	9.1	53				

Abbeviations: CMAP, compound motor amplitude potential; MNCV (m/s), motor nerve conduction velocity.

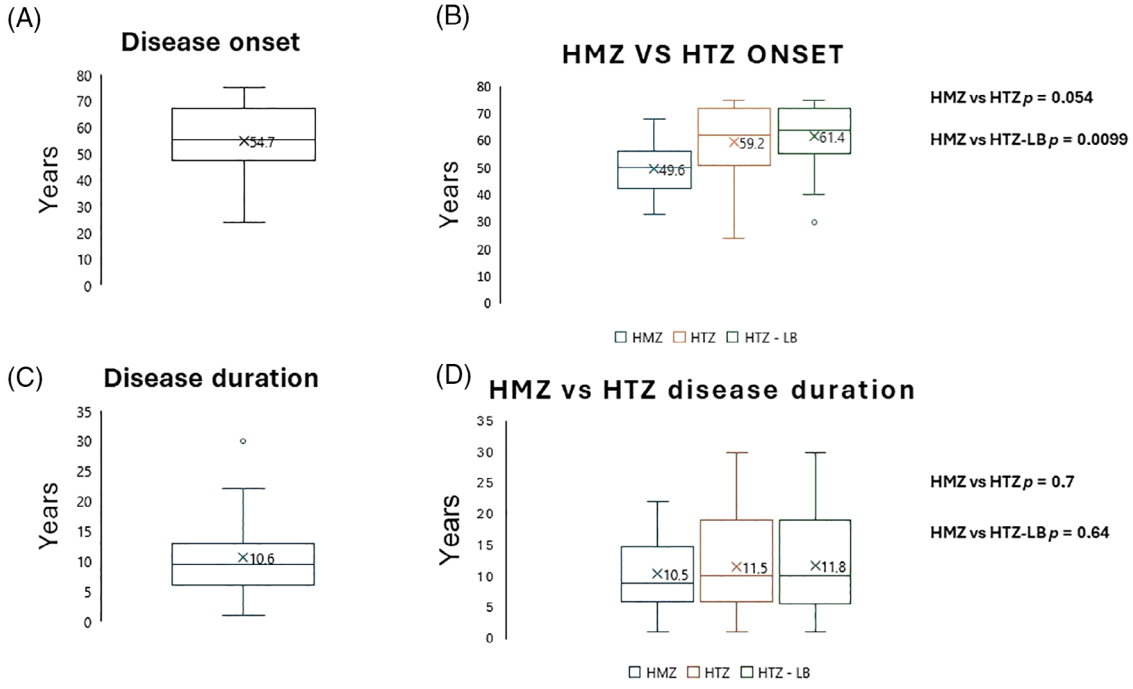
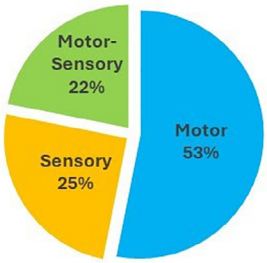


FIGURE 2 The box plots show the disease onset (A) of the whole group and compare the homozygous (HMZ) and heterozygous (HTZ) groups (B). The HTZ group experiences a statistically significant later disease onset when deprived of likely benign variants (HTZ-LB) ($p = .0099$; $p < .05$). (C) The disease duration of the entire group. (D) Comparisons of the homozygous (HMZ) and heterozygous (HTZ) groups. No statistically significant differences were found between the three groups.

between homozygotes and heterozygotes are more dispersed. This trend does not follow the same pattern as CMTES, even in subgroups with more or less than 10 years of disease (data not shown).

No signs of central nervous system involvement have been identified and none of the evaluated patients had apparent cognitive impairment.



ONSET	ONSET DETAILS	NUMBER OF PATIENTS
Motor	gait impairment and LL weakness or fatigue	6.3%
	LL weakness	6.3%
	walking difficulties/gait impairment	43.8%
Sensory	neuropathic pain	9.4%
	cramps	3.1%
	paresthesia or distal tingling	12.5%
Motor-Sensory	walking difficulties and cramps	6.3%
	walking difficulties and neuropathic pain	3.1%
	walking difficulties and sensory deficit	3.1%
	gait impairment and cramps	3.1%
	gait impairment and paresthesia	6.3%

FIGURE 3 Distribution and type of symptoms at onset.

The patient's medical history did not reveal any risk factors for peripheral neuropathies, such as diabetes or vitamin deficiencies. However, analysis of other comorbidities identified autoimmune diseases of several types in the 23.5% of the patients. No differences in inheritance, onset, or disease severity were observed between this group and the remaining patients.

Neither the duration of the disease nor the mono or biallelic state of MME variants seems to influence the presence of comorbidities.

NCSs show axonal motor and sensory polyneuropathy in most cases (78%), with only seven cases showing a different pattern. In detail, we can observe two patients with prevalent motor neuropathy, two patients with prevalent sensory neuropathy and three patients with intermediate motor sensory neuropathy. Upper limb nerve MNCV ranged from 31 to 68 m/s with a mean of 52 m/s and a median of 53 m/s (Figure 5) Upper limb nerve compound motor nerve amplitude has a mean value of 8.9 mV (median 9.35) with a maximum of 17.50 mV and a minimum of 0.2 mV (Figure 5). Details on the values of individual patients are shown in Table 2. No evident difference has been observed between homozygous and heterozygous patients with NCS, regardless of disease duration.

Of the 32 patients described in this study, 13 have *MME* variants in biallelic state, 18 in mono allelic state, while one patient has two heterozygous variants whose allelic phase could not be determined (Figure 6). No variants are shared between the homozygous and heterozygous groups, except for c.467delC and c.2154-5_2160del, which are present in both groups (Table 3). In detail, c.467delC is present in five patients from four families and c.2154-5_2160del in two patients from two families.

We observe a total of 21 different variants (Table 3). Among them missense (9 variants in 11 patients) is the most represented category, followed by loss of function (6 variants in 11 patients) and splicing (6 variants in 10 patients) (Figure 7).

Six of the nine missense variants result in amino acid changes that lead to loss or gain of H-bonds or to collisions between side chains of nearby residues, while three variants have no apparent consequence on protein structure (Table 3 and Figure 7).

In detail, p.Val604Ile results in a change between two hydrophobic aliphatic amino acids without loss or gain of strong interaction between nearby amino acids. Again, the substitutions p.Arg410Leu and the already described p.Ile649Ser[8] do not create or remove boundaries. In these latter cases, Grantham distance between the amino acids involved is more relevant and in particular, in p.Ile649Ser, the inclusion of a polar and phosphorylatable or glycosylatable residue may suggest a damage not directly related to the structure. The same hypothesis may be applied to the removal of an arginine in p.Arg410Leu, an amino acid often involved in catalysis.

Out of the six variants with a probable effect on the structure, four have not been described in the literature. Of these, p.Gln692His seems to have the most relevant effect on the three-dimensional structure, with multiple losses and gains of hydrogen bonds and collisions between adjacent amino acids (Figure 8). In contrast, p.Tyr414Cys and p.Ile649Thr lead to a single loss of a hydrogen bond between Tyr414 and Asn514 and a gain of a hydrogen bond between Ile649 and Leu644, respectively. Finally, p.Gly715Asp is predicted to affect only steric hindrance with a probable clash between Gly715 and Trp694.

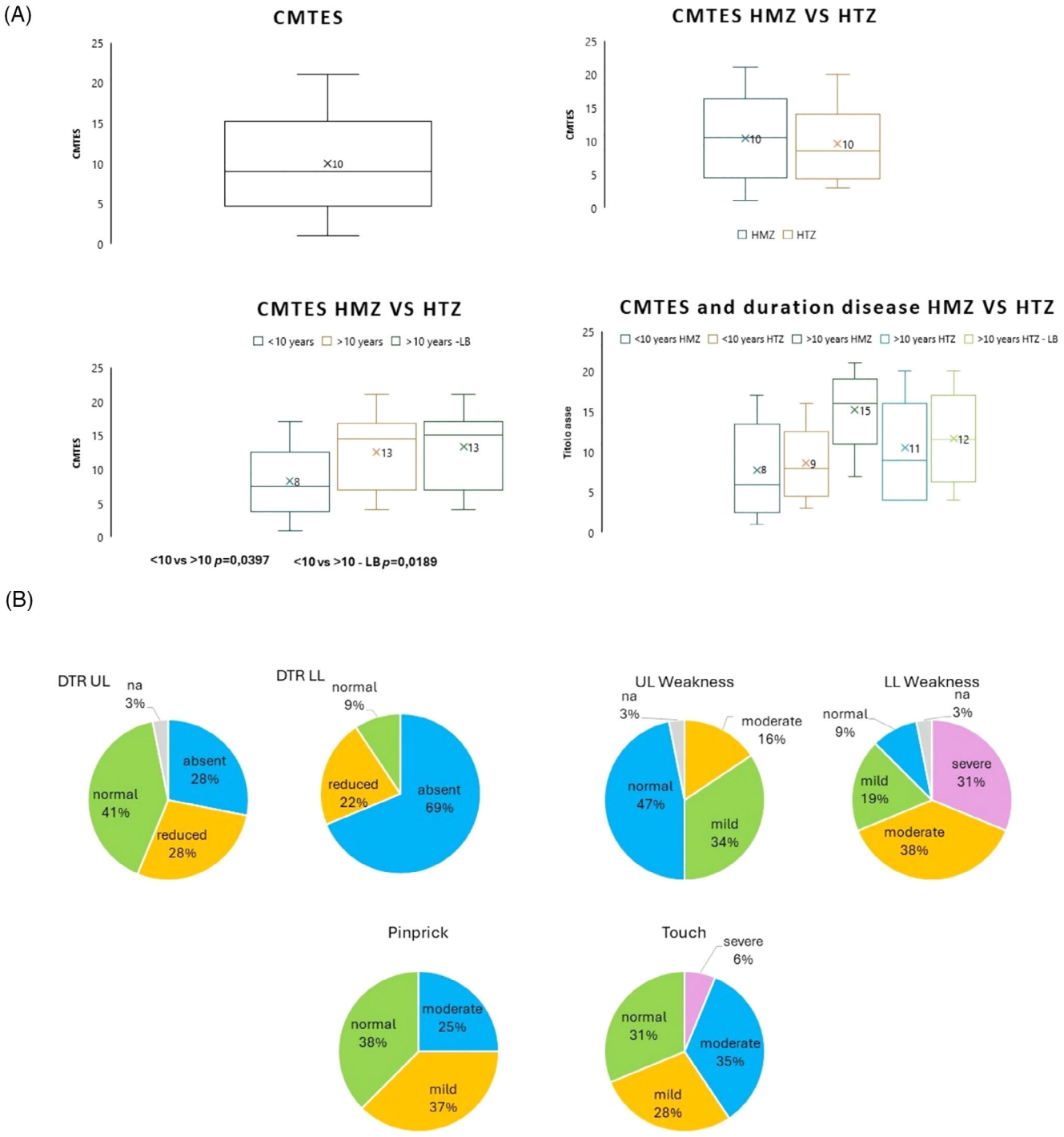


FIGURE 4 (A) Comparison between disease severity in Heterozygous (HTZ) and homozygous (HMZ) patients, patients with more than 10 (>10) and less than 10 (<10) years of disease duration, heterozygous and homozygous patients with more than 10 and less than 10 years of disease duration (HTZ > 10, HTZ < 10 and HMZ > 10, HMZ <10, - likely benign variants excluded). (B) Overall clinical parameters.

An additional missense variant, p.Gly224Ala, has been identified in two relatives (Pt 12-I and 12-II not described in detail and not included in the statistics). This variant results in the replacement of two non-polar, uncharged amino acids, with only a slight increase in steric hindrance, and no clashes between nearby residues. These observations, in conjunction with the variant's high minor allele frequency (0.19%) and atypical phenotypic findings (onset at only 15 years of age), suggest that this variant may be a suitable exemplar of a benign variant. Therefore, Pt 12-I and 12-II are not affected by CMT2T.

All nonsense or frameshift variants are predicted to undergo nonsense-mediated decay (NMD), as are all splicing variants. The only

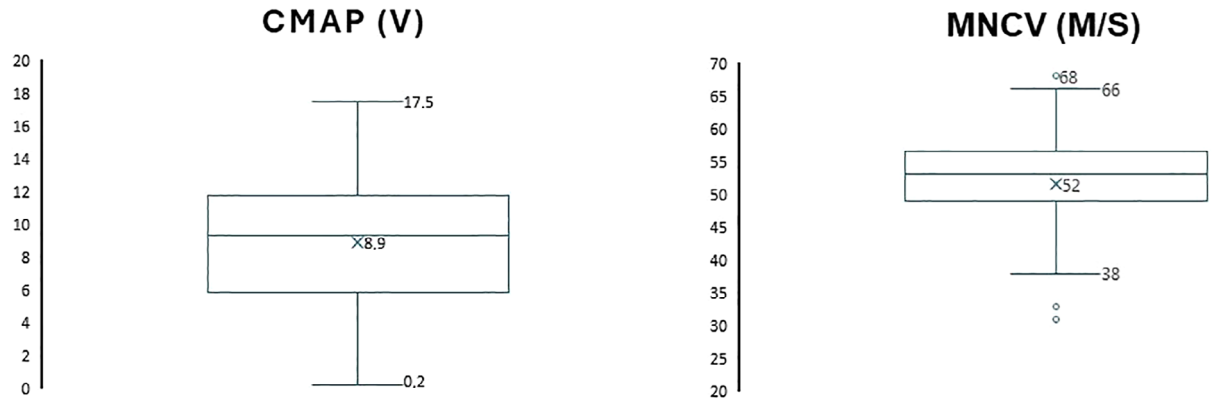


FIGURE 5 Overall values of upper limb nerve conduction studies. CMAP, compound motor amplitude potential; MNCV (m/s), motor nerve conduction velocity.

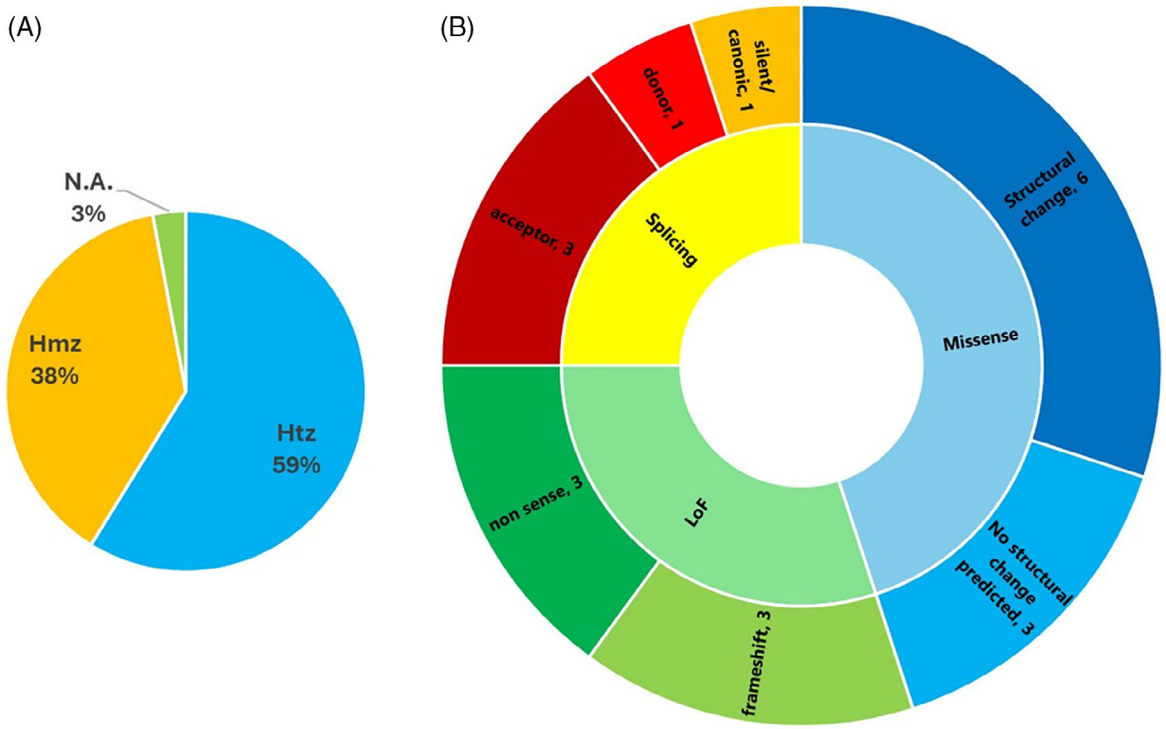


FIGURE 6 (A) Heterozygous (Htz) versus homozygous (Hmz) variant. (B) The distribution of variants based on their typology.

exceptions are c.1497+5G>A, which is predicted to result in an in-frame deletion of exon 15, and c.2154-5_2160del, which is predicted to result in a 16 aa shorter protein (p.Ile719Thrfs*15) (Table 4).

No mRNA study or NEP dosage has been performed to confirm these predictions.

4 | DISCUSSION

In this study, we describe a cohort of CMT patients carrying *MME* variants in both monoallelic and biallelic states to characterize the main clinical and neurophysiological features of CMT2T in the Italian population. We also provide insight into the pathological mechanism underlying the identified variants by in silico analysis.

Over the past 8 years, multiple papers have demonstrated that *MME* is the most significant gene in the development of late-onset sensorimotor axonal neuropathies.[5–9] Furthermore, it has been observed that another considerable proportion of late-onset hereditary neuropathies, especially those with a predominant sensory component, are caused by expansions in the *RFC1* gene, which is also associated with CANVAS phenotype.[14] Our results confirm an advanced age of onset, with a mean of 52 years, with no significant difference between males and females.

TABLE 3 Variants identified in MME gene.

Case	HGVS	Exon	Zygosity	rs Id	GnomAD NFE	Category	Predicted impact on protein	ACMG criteria	ACMG	Reference
Pt3	c.156C>G p.Tyr52Ter; c.1497+5G>A p.?	2	N.A.	-	0	LoF; Spl	NMD	(PVS1, PM2) (PP3, PM2)	(LP) (VUS)	-
Pt17	c.440-2A>C p.?		HMZ	rs200435950	0.0035%	Spl	Exon 6 skipping	PVS1, PM2, PS4m, (PP5)	P	[1]
Pt23	c.467delC p.Pro156fs*14	6	HMZ	rs749320057	0.0319%	LoF	NMD	PVS1, PS4m, PM2, (PP5)	P	[1]
Pt10-I	c.467delC p.Pro156fs*14	6	HMZ	rs749320057	0.0319%	LoF	NMD	PVS1, PS4m, PM2, (PP5)	P	[1]
Pt10-II	c.467delC p.Pro156fs*14	6	HTZ	rs749320057	0.0319%	LoF	NMD	PVS1, PS4m, PM2, (PP5)	P	[1]
Pt11	c.467delC p.Pro156fs*14	6	HTZ	rs749320057	0.0319%	LoF	NMD	PVS1, PS4m, PM2, (PP5)	P	[1]
Pt16	c.467delC p.Pro156fs*14	6	HTZ	rs749320057	0.0319%	LoF	NMD	PVS1, PS4m, PM2, (PP5)	P	[1]
Pt12-I	c.674G>C p.Gly225Ala	8	HTZ	rs147564881	0.19%	Ms	No apparent consequence on protein structure	PP3, BS1, BP6	LB	-
Pt12-II	c.674G>C p.Gly225Ala	8	HTZ	rs147564881	0.19%	Ms	No apparent consequence on protein structure	PP3, BS1, BP6	LB	-
Pt24	c.869_879dup p.Asn294Leufs*14	10	HMZ	-	0	LoF	NMD	PVS1, PM2	L P	-
Pt7	c.877C>T p.Arg293Ter	10	HTZ	rs764060752	0	LoF	NMD	PVS1, PS4m, PM2, (PP5)	P	[2,3]
Pt4	c.877C>T p.Arg293Ter	10	HTZ	rs764060752	0	LoF	NMD	PVS1, PS4m, PM2, (PP5)	P	[2]
Pt5	c.1040A>G p.Tyr347Cys	11	HTZ	rs138218277	0.1%	Ms	Loss of a H-bond between Tyr347 and Asp209	PP5	VUS	[1,4]
Pt25-I	c.1040A>G p.Tyr347Cys	11	HTZ	rs138218277	0.1%	Ms	Loss of a H-bond between Tyr347 and Asp209	PP5	VUS	[1,4]
Pt25-II	c.1040A>G p.Tyr347Cys	11	HTZ	rs138218277	0.1%	Ms	Loss of a H-bond between Tyr347 and Asp209	PP5	VUS	[1,4]
Pt8	c.1188G>A p.Lys396Lys	12	HTZ	-	0.0002%	Sil/Spl	Reading frame disrupted	PM2, PP3m	VUS	
Pt1	c.1229G>T p.Arg410Leu	13	HTZ	-	0	Ms	No apparent consequence on protein structure	PM2	VUS	-
Pt26	c.1241A>G p.Tyr414Cys	13	HTZ	rs202095767	0.0009%	Ms	Loss of a H-bond between Tyr414 and Asn514	PM2, PP3	VUS	-

(Continues)

TABLE 3 (Continued)

Case	HGVS	Exon	Zygosity	rs Id	GnomAD NFE	Category	Predicted impact on protein	ACMG criteria	ACMG	Reference
Pt18	c.1437-1446 delinsCAATAG p.Arg479Serfs*14	15	HMZ	-	0	LoF	NMD	PVS1, PM2	LP	-
P19	c.1810G>A p.Val604Ile	19	HTZ	-	0.0023%	Ms	No apparent consequence on protein structure	PM2, BP4, BP6	LB	-
Pt13	c.1946T>C p.Ile649Thr	20	HMZ	rs184666602	0.0031%	Ms	New H-bond between Ile649 and Leu644	PM2, PP3s	LP	-
Pt2	c.1946T>G p.Ile649Ser	20	HMZ	rs184666602	0.0219%	Ms	No apparent consequence on protein structure	PM2, PP3s	LP	[4]
Pt27_I	c.2011G>T p.Glu671Ter	21	HMZ	-	0	LoF	NMD	PVS1, PM2	LP	-
Pt27_II	c.2011G>T p.Glu671Ter	21	HMZ	-	0	LoF	NMD	PVS1, PM2	LP	-
Pt28	c.2067C>A p.Asn689Lys	21	HTZ	rs146536523	0.0133%	Ms	Clashes between Lys689 and Leu573. loss of a H-bond between Asn689 and Arg68, Leu573, Pro563	PM2, PP3m	VUS	[5]
Pt20	c.2076G>C p.Gln692His	21	HTZ	-	0	Ms	Loss of a H-bond between Gln692 and Asn72, Arg68, Cys88, Leu69. Gain of H-bond between His692 and Leu 688. Clashes between His692 and Arg68, Asn689	PM2, PP3s	LP	-
Pt9	c.2077-1G>C p.?	22	HTZ	rs1217067826	0.0009%	Spl	NMD	PVS1, PM2, PP5	LP	-
Pt21	c.2077-1G>C p.?	22	HTZ	rs1217067826	0.0009%	Spl	NMD	PVS1, PM2, PP5	LP	-
Pt6	c.2144G>A p.Gly715Asp	22	HTZ	-	0	Ms	Clash between Gly715 and Trp694	PM2, PP3	VUS	-
Pt14-I	c.2153+5G>A p.?	22	HMZ	rs771921345	0	Spl	NMD	PM2, PP3, PP1m	LP	-
Pt14-II	c.2153+5G>A p.?	22	HMZ	rs771921345	0	Spl	NMD	PM2, PP3, PP1m	LP	-
Pt14-III	c.2153+5G>A p.?	22	HMZ	rs771921345	0	Spl	NMD	PM2, PP3, PP1m	LP	-
Pt22	c.2154-5_2160del p.?	23	HMZ	-	0	Spl	Reading frame disrupted without predicted NMD	PVS1m, PM2	VUS	-
Pt15	c.2154-5_2160del p.?	23	HTZ	-	0	Spl	Reading frame disrupted without predicted NMD	PVS1m, PM2	VUS	-

Abbreviations: GnomAD NFE, non-Finnish European population frequency based on GnomAD database; HMZ, homozygous; HTZ, heterozygous; LoF, loss of function; Ms, missense; N.A., not available; NMD, non-sense mediated decay; rs Id, single-nucleotide polymorphism identification number; Sil, silent; Spl, splicing, VUS, variant of unknown significance.

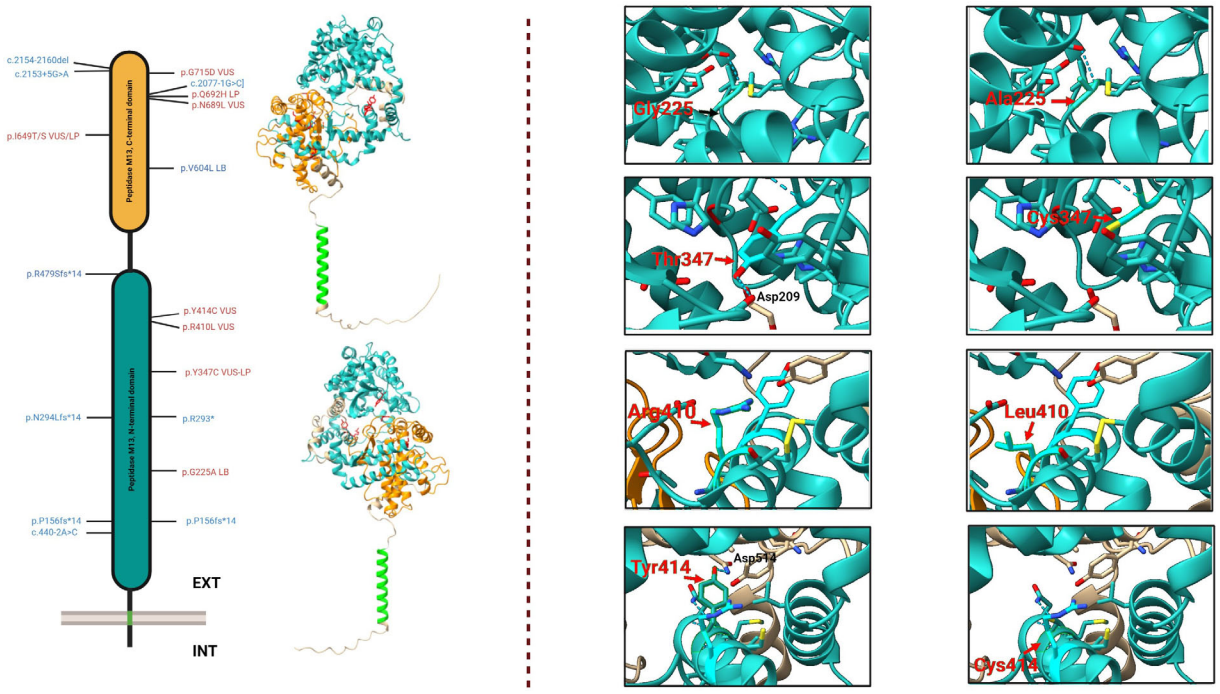


FIGURE 7 On the left schematic and 3D (front and back) representation of MME protein. In cyan peptidase M-13, N-terminal domain, in dark yellow Peptidase M-13, C-terminal domain, in green transmembrane domain. Identified variants are marked in red (missense) and cyan (loss of function). On the right localization and interaction with nearby amino acids of the wild-type (SX) and mutant (DX) codons involved in missense variant. H-Bonds are depicted with a cyan dotted line; Gly225Ala: No apparent consequences on protein structure; Thr347Cys: Loss of a H-Bond with Asp209; Arg410Leu: No apparent consequences on protein structure; Tyr414Cys: Loss of a H-Bond with Asn514.

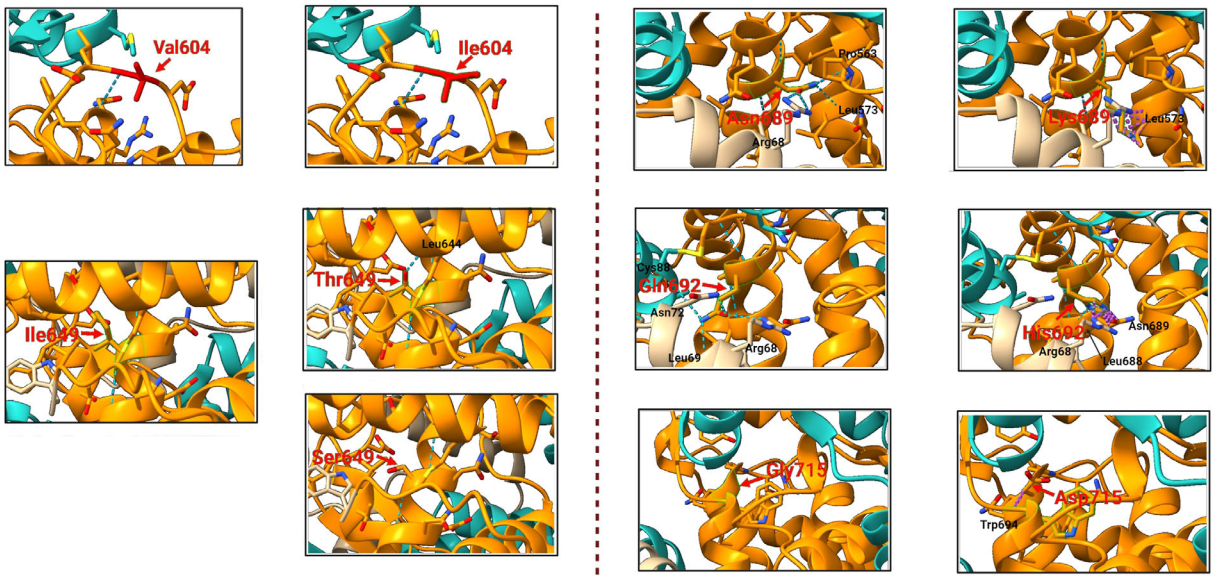


FIGURE 8 Localization and interaction with nearby amino acids of the wild-type (SX) and mutant (DX) codons involved in missense variant. H-Bonds are depicted with a cyan dotted line—clashes between residues are depicted in purple dotted line; Val604Ile: No apparent consequences on protein structure; Ile649Thr: New H-Bond between Thr649 and Leu644; Ile649Ser: No apparent consequences on protein structure; Asn689Lys: Loss of H-bonds with Arg68, Leu573, and Pro563—Clashes with Leu573; Gln692His: Loss of H-Bonds with Ans72, Arg68, Cys88 and Leu69—New H-Bonds between His692 and Leu 688—Clashes between His692, Arg68, and Asn689; Gly715Asp: Clashes between Asp715 and Trp694.

TABLE 4 Splicing variants and their predicted consequence on mRNA (based on MobiDetails).[13]

MME (NM_007289.4)	SPLICING	RISK	Strenght variation due to the variant (MaxEnt score)	Cryptic site activation	Bp from canonic site	Cryptic vs. WT site	Predicted consequence
c.1497+5G>A	Alteration of the consensus splice site	96.71%	5′ [11.08 GAGgtaagt → 8.39 GAGgtaaat] (−24.28%)	NO	NO	NO	Exon 15 deletion?
c.440-2A>C	Alteration of the consensus splice site	98.41%	3′ [6.11 ccaaaagCTG → −1.93 ccaaacgCTG] (−131.59%)	NO	NO	NO	Exon 6 skipping, NMD?
c.2077-1G>C	Alteration of the consensus splice site	98.41%	3′ [11.76 tatctctagGTG → 3.70 tatctctacGTG] (−68.54%)	NO	NO	NO	Exon 22 skipping, NMD?
c.2153+5G>A	Alteration of the consensus splice site	98.41%	5′ [9.73 CAGgtgcgt → 8.39 CAGgtgcat] (−49.74%)	NO	NO	NO	Intron 22–23 retain, NMD? P. V693Dfs*42, NMD?
c.2154-5_2160del	Alteration of the consensus splice site	98.41%	3′[9.15 ttttttttgtttgtttcaaag GAT→ ttttttttgtttgtttgggacTTT −11.87] (−229.73%) 3′[−22.61 gtttgtttcaaaggattattGGG → gtttgtttgggactttgcagAAC 6.2] (+127.42%)	YES	19	0.80 vs. 0.84	p.Ile719Thrfs*15, no NMD?
c.1188G>A	Alteration of the consensus splice site	98.41%	5′[5.73 AAGgtgaag →−2.530AAAgtgaag] (−144.15%)	NO	NO	NO	Intron 12–13 retain, NMD?

Overall, this is a mild neuropathy compared with the more common forms of CMT, but it is still more severe than other age-related neuropathies.

Despite what is described in the literature,[9] the CMTES scores for monoallelic and biallelic patients are comparable, so that most of the severity of the disease seems to depend only on the duration of the disease. A significant difference in the CMTES score between monoallelic and biallelic patients occurs when we consider a subgroup of patients with more than 10 years of disease duration. In this case, biallelic patients have greater disease severity, confirming that homozygous patients progress more rapidly.

Although most patients present with sensory motor neuropathy, the onset of symptoms is predominantly motor and results in gait impairment.

As previously described,[8,9] our patients presented with a length-dependent neuropathy, with upper limbs only mildly affected, whereas the LL showed moderate to severe distal weakness, often associated with sensory deficits. Almost all patients have impaired LL reflexes, whereas upper limb reflexes are preserved in 41% of cases.

Neuropsychological testing revealed no cognitive impairment in our patients, in agreement with previous reports[5,6,8,9,15] thus confirming that *MME* mutations are not sufficient to cause Alzheimer's disease.

The neurophysiological study demonstrated the presence of an axonal neuropathy, with no significant differences observed between biallelic and monoallelic patients or based on disease duration. While in the subgroup with more than 10 years of disease, greater clinical severity was observed in biallelic compared with monoallelic samples, the NCS values did not appear to be indicative of this more severe phenotype.

Many of the identified variants lead to a mRNA NMD identifying loss of function (LoF) mechanisms as the most likely to be pathogenic. These mechanisms can include nonsense, frameshift, but also splicing variants. Within these categories, we have identified most of the variants in homozygosity, the only ones so far that are definitely associated with late-onset neuropathy. The variant p.Pro156fs*14, described by Auer-Grumbach and colleagues in 2016,[7] was particularly significant. It was identified in five patients (four index cases) in both biallelic and monoallelic states. Interestingly, within the same family, Pt10-I and Pt10-II present the variant in bi and monoallelic states, respectively, and Pt10-I consistently shows a 15-year earlier onset. On the contrary, in another family, Pt27-I and Pt27-II show a comparable disease onset and both harbor the homozygous p.Glu671Ter.

Among the identified variants involving splice sites, four (c.440-2A>C, c.2077-1G>C, c.2153+5G>A, and c.1188G>A) are predicted to undergo NMD and two (c.1497+5G>A and c.2154-5 2160del) are likely to bypass this machinery but still result in a non-functional protein lacking the C-terminal tail of the peptidase M13 domain (peptidase-M13_N and peptidase-M13_C, respectively), confirming LoF as the main pathological mechanism. Interestingly, the variant c.2153+5G>A is shared by three siblings, and although its predicted variation in 5′ splicing score is not as strong, it is defined as Likely Pathogenic by the overall ACMG criteria. The different ages of onset in the three siblings may reflect a case in which factors other than the genetic background might be added to the latter in determining the phenotype of the patients.

More controversial is the role of missense mutations. In silico analysis shows relevant structural consequences in six of the

10 different missense variants identified, while four seem to have no noticeable effect on the 3D structure of the protein. Ile649 seems to be an important codon harboring the only two missense homozygous changes Ile649Thr and Ile649Ser, the latter already described by Senderek and colleagues.[9] Although both substitutions have deleterious in silico predictions, p.Ile649Thr seems to have a stronger impact on the tertiary structure of the protein. If we postulate an LoF mechanism also for missense variants, it is easy to assume that the greater the structural damage, the greater the chance that the protein will lose its function.

In contrast, p.Gly224Ala has no relevant effect on the protein structure except for a slight increase in steric hindrance. Moreover, the relatively high frequency in the population and the early age of onset in the two patients carrying this variant suggest a benign role for this variant. The same can be postulated for p.Val604Ile, despite the lower minor allele frequency and the later disease onset of the patient carrying this variant.

If the role of homozygous missense or nonsense *MME* variants in late-onset peripheral neuropathy has been established, the contribution of heterozygous variants, especially missense variants, is still unclear. A causal role has been hypothesized at low penetrance or as an AD risk factor as describe by Senderek and colleagues.[9] The presence of a variant in heterozygosity in *MME* could thus be a necessary, but not sufficient condition for the onset of neuropathy, which would require a second event, either genetic or environmental, to manifest itself.

In this case, to determine the pathogenicity of a variant, we should more carefully evaluate some factors, such as the frequency in the general population and the co-segregation.

Within the heterozygous missense variants, a debate should be stimulated about those that are classified as benign (B) /LB by the ACMG. In literature, some variants are considered a risk factor despite being LB or B, for example, *GLUCOSIDASE*, *BETA*, and *ACID* variants for Parkinson's disease.

Even if CMT is not a complex disease, in this hypothesis, their benignity may not be a criterion on which to exclude their role in the pathogenesis of the neuropathy. Conversely, if the hypothesis of marked age-dependent penetrance is accepted, LB or B variants should not be considered to have a role in the development of the disease. Our data show that by excluding LB variants from the analyzed cohort, many of the clinical characteristics of the group become more consistent. This leads us to favor the age-dependent penetrance hypothesis.

The late onset of the disease obviously complicates this scenario, as younger generations are more likely to present asymptomatically. A large case–control study may be useful to address this issue.

CMT2T is confirmed to be a mild motor sensory neuropathy compared to other inherited forms. The mean disease onset is in the early years of the 6th decade of life and therefore it may have some relevance to the overall incidence of peripheral neuropathy in the elderly, particularly in populations with a high average age.

Long-term follow-up studies may be useful to monitor the disease progression and to determine how much it contributes to the clinical picture of patients, in addition to other age-related disorders.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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