ORIGINAL ARTICLE

Characterising the phenotype and mode of inheritance of patients with inherited peripheral neuropathies carrying *MME* mutations

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ABSTRACT

Background Mutations in the metalloendopeptidase (*MME*) gene were initially identified as a cause of autosomal recessive Charcot-Marie-Tooth disease type 2 (CMT2). Subsequently, variants in *MME* were linked to other late-onset autosomal dominant polyneuropathies. Thus, our goal was to define the phenotype and mode of inheritance of patients carrying changes in *MME*.

Methods We screened 197 index cases with a hereditary neuropathy of the CMT type or distal hereditary motor neuropathy (dHMN) and 10 probands with familial amyotrophic lateral sclerosis (fALS) using a custom panel of 119 genes. In addition to the index case subjects, we also studied other clinically and/or genetically affected and unaffected family members.

Results We found 17 variants in *MME* in a total of 20 index cases, with biallelic *MME* mutations detected in 13 cases from nine families (three in homozygosis and six in compound heterozygosis) and heterozygous variants found in 11 families. All patients with biallelic variants had a similar phenotype, consistent with late-onset axonal neuropathy. Conversely, the phenotype of patients carrying heterozygous mutations was highly variable [CMT type 1 (CMT1), CMT2, dHMN and fALS] and mutations did not segregate with the disease.

Conclusion *MME* mutations that segregate in an autosomal recessive pattern are associated with a late-onset CMT2 phenotype, yet we could not demonstrate that *MME* variants in heterozygosis cause neuropathy. Our data highlight the importance of establishing an accurate genetic diagnosis in patients carrying *MME* mutations, especially with a view to genetic counselling.

INTRODUCTION

''One of the most common inherited neurological disorders, Charcot-Marie-Tooth (CMT) disease, is clinically and genetically heterogeneous, with more than 90 disease-causing genes identified to date (http://neuromuscular.wustl.edu). Phenotypically, CMT is traditionally divided into demyelinating [CMT disease type 1 (CMT1)] or axonal [CMT disease type 2 (CMT2)] forms, the former lending itself to genetic characterisation in more than 90% of cases.[1–3] By contrast, in the majority of patients with the axonal form, no gene mutations have been identified that may account for their disease and causal genes still remain largely unknown.[1–3] The most frequent clinical CMT phenotype involves the development of progressive distal weakness and sensory loss in the first two decades of life.[4] Onset after the age of 40 is unusual and most late-onset forms are considered to be CMT2.[5] Diagnosing late-onset hereditary neuropathies is challenging as incomplete penetrance and small family size are frequently problems that hinder the identification of patients with genetic neuropathies. Suspected hereditary causes are even more difficult to define in sporadic cases and in fact, it is not infrequent for such cases to be considered as chronic idiopathic axonal polyneuropathy (CIAP) or chronic demyelinating neuropathies (CIDPs).[5 6]

In recent years, mutations in the metalloendopeptidase (*MME*) gene were identified as the cause of late-onset autosomal recessive (AR) CMT2 in some Japanese patients.[7] Likewise, several cases of autosomal dominant (AD) late-onset neuropathies in European and American families were linked to *MME* variants.[8] Here, we present data from a series of patients with CMT, distal hereditary motor neuropathy (dHMN) and familial amyotrophic lateral sclerosis (fALS) without genetic diagnosis, but who were studied using a panel of 119 genes causing neuropathy and/or ALS. The aim of this study was to shed light on the phenotype and mode of inheritance of the neuropathies caused by *MME* mutations.

MATERIALS AND METHODS

Patients and samples

We screened 197 index cases with CMT/dHMN and 10 patients with fALS using our custom panel of 119 genes (Neuro119: online supplementary table 1). Capture-based target enrichment was performed using custom probes and the SureSelectQXT kit (Agilent Technologies, Santa Clara, California, USA) suitable for Illumina sequencing (Illumina, San Diego, California, USA). All the patients were examined by experienced neurologists at six different Spanish hospitals. In most of the patients with CMT, a *PMP22* duplication or deletion and mutation of the most frequent genes

associated with these conditions (*MPZ*, *GJB1* and *GDAP1*) had been ruled out. In patients with fALS, *C9ORF72* expansion had been discarded. After sequencing the panel of genes, the clinical data and genetic findings from 20 families and 26 affected individuals carrying mutations in the *MME* gene were studied in more detail. In addition, 44 unaffected family members were included in this study for segregation analysis and/or for clinical assessment. In one family, peripheral blood derived mRNA was also characterised to assess *MME* splicing. In addition, muscle MRI was performed on five patients, obtaining standard axial sections at the level of the hip, thigh, lower leg and feet, as described previously.[9] Sural nerve biopsy was also performed on one individual (patient F4/II:1) when he was 53 years old. Written informed consent was obtained from all the patients included in this study. All the studies were carried out in accordance with the Helsinki declaration regarding experimentation on humans (World Medical Association, 1964).

Genetic analysis

Candidate variants were selected by excluding all variants with a minor allele frequency (MAF) higher than 1% in control databases (ESP6500, 1000G, ExAC and gnomAD). In addition, novel or less frequent nucleotide changes in coding exons (synonymous, missense, non-sense, frameshift and indels) and those affecting splice sites were prioritised. To predict the effects of the mutation on the protein, we generated a prediction score by running the following algorithms and programs: PROVEAN, SIFT, PolyPhen-2, GERP and PhyloP. After the initial filter process, interpretation of the variants was performed following the American College of Medical Genetics guidelines.[10] Candidate variants were validated by Sanger sequencing and they were tested for segregation analysis where possible.

RESULTS

In a total of 20 unrelated case subjects, 17 variants of *MME* gene were identified with the Neuro119 gene panel (table 1).

Families with biallelic mutations

Segregation analysis confirmed biallelic mutations in the *MME* gene in all 13 affected individuals from nine families (F1–F9) in which no consanguinity was reported (figure 1). In these nine families, 18 of the 27 healthy individuals examined carried a heterozygous *MME* change (figure 1); yet, no asymptomatic carriers of biallelic changes were found. The average age of the patients with biallelic mutations was 57 and the age of those carrying heterozygous changes was 60, while the average age of the non-carriers was 55. In total, we detected 11 different variants in the nine families with biallelic mutations, seven of which were not present in any of the control population databases consulted (table 1). Patients who carried the c.466delC change lived in the northwest of Spain, while those carrying the c.1342C>T substitution lived in the southeastern area, suggesting two independent founder events in our population.

In family 9, the c.1666C>T (p.Pro556Ser) variant was not found in either parent, suggesting that this mutation occurred de novo (figure 1, table 1). The analysis of *MME* mRNA extracted from one patient's peripheral blood (F4/II:2) revealed that the novel splice donor change c.196+1G>A caused the skipping of exon 2 (online supplementary figure 1), predicting an in-frame deletion variant (p.Asp54_Ser65del).

The Human Genome Variation Society (HGVS) nomenclature and RefSeq accession number NM_000902 were used for the description of genetic mutations identified in *MME*. Prediction and the conservation score was calculated according to algorithms and programmes shown in the online supplementary table 2 (ie, for the prediction and conservation score, values of 0/3, 1/3 and 1/2 estimate a tolerated effect on the protein, while values of 2/3–3/3 and 2/2 estimate a deleterious effect).

The clinical features and electrophysiological findings of all 13 patients are summarised in tables 2 and 3. The age at onset ranged from 35 to 73 years (median age of 44 years) and all the patients developed slowly progressive weakness and atrophy, which commenced in the distal lower limbs and spread to the distal upper limb muscles over a period of some years. The symptoms at the beginning of the disease were mainly motor, involving cramps and very frequent muscle contractures, although sensory symptoms were also prominent in the most evolved patients. Most patients needed assistance with walking within a few years of clinical onset.

There were several particularities among our patients that are worthy of further attention. Patient F1/II:1 had long-standing diabetes mellitus (DM) and a renal failure that required dialysis. His neuropathy developed after the age of 40 and it was first attributed to his DM; yet, a genetic cause was later considered due to the progressive atrophy in the limbs. Patients F2/II:5 and F4/II:2 were both diagnosed with CIDP, although treatment with different immunosuppressive and immunomodulatory therapies did not produce any clinical improvement. A nerve biopsy from patient F4/II:2 showed a loss of multifocal myelinated fibres of all diameters (data not shown). The clinical evolution of these patients generated suspicion of a genetic cause and furthermore, a second affected patient in family F2 was later found.

In two families (F6 and F7), there was a history of dementia, with the mother (F6/I:2) and maternal aunt (F6/I:3) of patient F6/II:4 having been diagnosed with Alzheimer's disease. The onset of dementia occurred in the eight decade of life and neither of them developed symptoms of neuropathy. The mother (F6/I:2) carried a change in *MME* that was detected in heterozygosis, whereas the aunt F6/I:3 had no change in *MME*. Patient F7/II:1 developed both neuropathy and dementia, commencing at the age of 40 and 67 years of age, respectively, and he was ultimately diagnosed with Alzheimer's disease. Patient F5/II:1, who carried the same mutation in homozygosis as patient F7/II:1, had no clinically evident cognitive impairment. Except for the patient with Alzheimer's disease, no other patients complained of memory loss or showed apparent cognitive impairment.

In nerve conduction and electromyography (EMG) studies, motor nerve conduction velocities (MNCVs) were compatible with a length-dependent axonal neuropathy (table 3). An intermediate (35–45 m/s) or even demyelinating (<38 m/s) range of MNCVs was recorded in some nerves although conduction to proximal muscles produced higher MNCV values (table 3). In the *MME* series, needle EMG revealed chronic neurogenic denervation. In some patients, positive sharp waves and fibrillation potentials were evident in the upper and lower limb muscles, particularly in the tibialis anterior muscle. Peripheral nerve hyperexcitability was recorded in a minority of patients, in the form of myokymia and fasciculations (table 3). The decrease in the sensory action potential commenced later than that in the compound motor action potential (CMAP), yet they tended to decrease first in the lower limbs and later in upper limbs as the disease evolved. Motor nerve amplitudes were initially conserved due to reinnervation, although such compensation was rapidly lost at some point and a progressive reduction of CMAP was then observed. In those patients (F2/II:5 and F4/II:2), in whom electrophysiological studies were performed at different stages of the disease, the spontaneous activity was initially frequent and

Table 1 Variants of *MME* identified in 20 families with Neuro119 gene panel

			Allele count (allele frequency)			Variant score			
Family/*MME* mutations	Type of variant	rs ID	ExAC	gnomAD	Ref.	Prediction score	Conservation score	Segregation/inheritance	Genotype/phenotype
F1/c.2067C>A (p.Asn689Lys); c.2242C>T (p.Arg748Trp)	Missense	rs146536523; rs141665432	29 (2.41E-04); 17 (1.40E-04)	47 (1.91E-04); 30 (1.22E-04)	n.r.; n.r.	3/3; 3/3	2/2	Positive/AR	CH/CMT2
F2/c.1342C>T (p.Arg448*)	Non-sense	rs149905705	4 (3.309E-05)	21 (7.6E-05)	8	LoF*	LoF*	Positive/AR	HMZ/CMT2
F3/c.2134C>T (p.His712Tyr); c.71G>A (p.Trp24*)	Missense; non-sense	This work; rs886039755	n.r.; n.r.	n.r.; n.r.	n.r./8	3/3; LoF*	2/2; LoF*	Positive/AR	CH/CMT2
F4/c.196+1G>A; c.1342C>T (p.Arg448*)	Splice donor; non-sense	This work; rs149905705	n.r.; 4 (3.309E-05)	n.r.; 21 (7.6E-05)	n.r./8	LoF*	LoF*	Positive/AR	CH/CMT2
F5/c.466delC (p.Pro156Leufs*14)	Frameshift	rs749320057	20 (1.66E-04)	66 (2.38E-04)	8	LoF*	LoF*	Positive/AR	HMZ/CMT2
F6/c.1342C>T (p.Arg448*); c.2248delT (p.Trp750Glyfs*24)	Non-sense; frameshift	rs149905705; this work	4 (3.309E-05); n.r.	21 (7.6E-05); n.r.	8/n.r.	LoF*	LoF*	Positive	CH/CMT2
F7/c.466delC (p.Pro156Leufs*14)	Frameshift	rs749320057	20 (1.66E-04)	66 (2.38E-04)	8	LoF*	LoF*	n.a./n.d.	HMZ/CMT2
F8/c.227delC (p.Thr77Leufs*16); c.1342C>T (p.Arg448*)	Frameshift; non-sense	Novel; rs149905705	n.r.; 4 (3.309E-05%)	n.r.; 21 (7.6E-05)	n.r./8	LoF*	LoF*	Positive/AR	CH/CMT2
F9/c.1666C>T (p.Pro556Ser); c.1972G>A (p.A658T)	Missense	This work; this work	n.r.; n.r.	n.r.; n.r.	n.r.;n.r.	3/3; 3/3	2/2	n.a./n.d.	CH/CMT2
F10/c.1342C>T (p.Arg448*)	Non-sense	rs149905705	4 (3.309E-05)	21 (7.6E-05)	8	LoF*	LoF*	Negative/n.d.	HET/CMT2+deafness
F11/c.1810G>A (p.Val604Ile)	Missense	rs200308077	35 (2.91E-04)	64 (2.31E-04)	n.r.	0/3	1/2	Negative/AR	HET/CMT2
F12/c.1883A>G (p.Asn628Ser)	Missense	rs181745819	4 (3.301E-05)	14 (5.0E-05)	n.r.	2/3	2/2	Negative/n.d.	HET/CMT1
F13/c.1495G>A (p.Glu499Lys)	Missense	rs201292663	7 (5.818E-05)	10 (4.071E-05)	n.r.	0/3	1/2	n.a./n.d.	HET/CMT2
F14/c.2248delT (p.Trp750Glyfs*24)	Non-sense	This work	n.r.	n.r.	n.r.	LoF*	LoF*	Negative/n.d.	HET/fALS
F15/c.773A>G (p.Gln258Arg)	Missense	rs763210226	1 (8.245E-06)	1 (4.07E-06)	n.r.	0/3	2/2	Negative/n.d.	HET/fALS
F16/c.1229G>A (p.Arg410His)	Missense	rs201238171	2 (1.649E-05)	3 (1.084E-05)	n.r.	1/3	2/2	n.a./n.d.	HET/possible-CMT
F17/c.674G>C (p.Gly225Ala)	Missense	rs147564881	176 (2.156E-03)	416 (1.533E-03)	n.r.	2/3	2/2	Negative/n.d.	HET/sensory dementia
F18/c.674G>C (p.Gly225Ala)	Missense	rs147564881	176 (2.156E-03)	416 (1.533E-03)	n.r.	2/3	2/2	Negative/n.d.	HET/dHMN
F19/c.674G>C (p.Gly225Ala)	Missense	rs147564881	176 (2.156E-03)	416 (1.533E-03)	n.r.	2/3	2/2	n.a./n.d.	HET/dHMN
F20/c.466delC (p.Pro156Leufs*14)	Frameshift	rs749320057	20 (1.66E-04)	66 (2.38E-04)	8	LoF*	LoF*	n.a./n.d.	HET/possible-CMT1

AR, autosomal recessive; CH, compound heterozygous; CMT, Charcot-Marie-Tooth; dHMN, distal hereditary motor neuropathy; fALS, familial amyotrophic lateral sclerosis; HET, heterozygous; HMZ, homozygous; LoF*, loss-of-function prediction; n.r., not reported; n.a., not available; n.d., not determined; n.r., not reported; rs ID, variant identification tag assigned by National Center for Biotechnology Information.

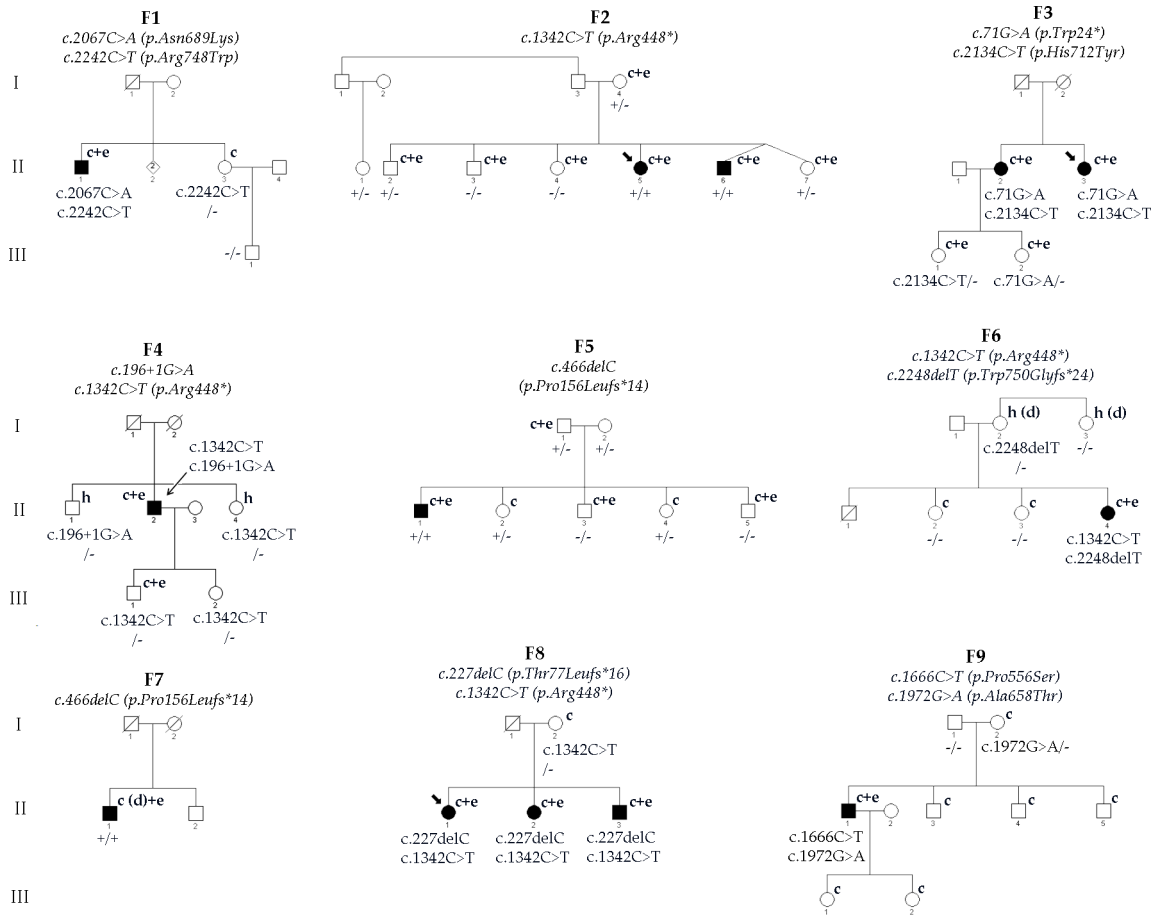

Figure 1 Pedigrees of the families with biallelic mutations in *MME*. *MME* variant genotypes of affected and unaffected individuals are shown below the pedigree symbols; an arrow in the upper right corner was used to indicate the genotype of the individual F4/II:2. Probands are indicated with an arrowhead. Patients and family members who received a clinical and/or electrophysiological evaluation are identified with the symbol (c) and (e), respectively. Family members with a clinical history only are indicated by an h. (d)=subjects diagnosed with dementia.

as the disease progressed, signs of chronic denervation become more evident (data not shown).

The clinical assessment of 12 heterozygous carriers (figure 1, symbol c) was normal, as were the results of the electrophysiological studies in seven of these (figure 1, symbol c+e). While a clinical examination was not carried out on the remaining relatives who were included in the segregation study, questions addressed to the index case or another family members did not suggest any neuropathic alterations (figure 1, symbol h).

MRI revealed a fatty infiltration that followed a length-dependent pattern. Indeed, the involvement of intrinsic muscles of the feet occurred at an earlier stage of the disease and was more severe than that in the muscles of the legs and thighs. The degree of fatty infiltration was similar in all the leg and thigh muscles at the same axial level (figure 2).

Families with heterozygous mutations in *MME*

In 11 families (F10–F20), the Neuro119 gene panel identified nine different heterozygous mutations in *MME*; all of them present in the control population databases (table 1). In seven families, a segregation analysis indicated that the mutations identified in the *MME* gene did not segregate with the disease (online supplementary figure 2A), while segregation analysis was not possible in the remaining four families (online supplementary figure 2B). In two families, F10 and F15, a novel *AIFM1* variant (p.Ile592Thr), that is probably damaging and a pathological missense mutation in *SOD1* p.Gly38Arg, were also identified (table 4; online supplementary figure 2).

The clinical features and pedigrees of all 11 patients are summarised in supplementary figure 2 and table 4. Two of the 11 probands that carried heterozygous mutations in *MME* belonged to families with fALS or ALS and frontotemporal dementia (F14 and F15). In the other nine probands, there was wide phenotypic variability, four of them presenting a CMT2 phenotype, two with distal motor neuropathy and the other three a demyelinating neuropathy. Interestingly, in family 11, the two affected members (F11/II:1 and F11/II:4) presented a phenotype that mimicked the AR *MME*-associated disease, although only F11/II:4 had the heterozygous variant p.Val604Ile in the *MME* gene. Families 17 and 20 had only mild sensory symptoms, although the electrophysiological study produced evidence of generalised demyelinating polyneuropathy. The patient in family 12 developed a sensory predominant neuropathy with late-onset symptoms that did not respond to immunomodulatory therapy.

DISCUSSION

Genetic testing of 207 patients with CMT/dHMN and ALS using targeted next-generation sequencing of 119 genes led to the identification of 17 different mutations in the *MME* gene in 20 families. Biallelic mutations in the *MME* gene were confirmed in nine families, while only heterozygous variants were found in 11 families. Four out of the 17 mutations were detected in more

Table 2 Clinical data from 13 patients with neuropathy due to biallelic mutations in *MME*

Family/ Patient	Phenotype	Sex/ current age (Y)	AOO (Y)	Initial symptoms	AOE (Y)	LL weakness (MRC) Proximal–distal (right/left)	UL weakness (MRC) Proximal– distal (right/ left)	Reflexes	Vibration sensation	Pinprick sensation	UL atrophy/ LL atrophy	Pes cavus	Walking aid	Cognitive impairment or dementia/other features
F1/II:1	CMT2	M/58	40	Difficulty in walking, legs' atrophy	58	4/4–0/0	5/5–3/3	Abs	Abs toes and ankles, red knees	Red knees	Hands/legs	No	One crutch since age 53. Stepagge gait	Diabetes mellitus, renal insufficiency, dialysis since age 54
F2/II:5	CMT2	F/53	44	Unsteadiness, cramps	53	5/5–<3/<3	5/5–3/3	Abs	Abs toes, red ankle and knee	Red knees	Hands/legs	Yes	Cane at 48, frame at 52	No
F2/II:6	CMT2	M/49	40	Unsteadiness, cramps	46	5/5–<3/<3	5/5–3/3	Abs	Red knees, red wrist	Red knees, red wrist	Hands/legs	Yes	Cane since age 46	No
F3/II:2	CMT2	F/66	58	Walking impairment	61	5/5–3/3	5/5-5/5	Abs	Reduced up to iliac crests	Reduced ankles	NA	NA	One crutch. Stepagge gait	No
F3/II:3	CMT2	F/80	73	Walking impairment and unsteadiness	78	5/5–<3/<3	5/5–5/5	Abs	Reduced up to iliac crests	Normal	NA	NA	Stepagge gait	No
F4/II:2	CMT2	M/65	44	Weakness in lower limbs, cramps	65	5/4-0/0	5/5–3/3	Abs	Abs toes and ankle, red knees	Red ankle, red ankle	Hands/legs	No	Leg orthosis. Uses one crutch.	No
F5/II:1	CMT2	M/59	53	Distal weakness in lower limbs	58	4/4–3/3	5/5–5/4	Abs	Reduced toes and ankle	Moderate	Legs and thighs	Yes	One crutch occasionally	No
F6/II:4	CMT2	F/39	35	Cramps, distal weakness LL	39	5/5–3/3	5/5–5/5	Decreased in UL, abs in LL	Abs toes	Red ankle	No/legs	Yes	No. Steppage gait	No
F7/II:1	CMT2	M/75	40	Fasciculations in all four limbs; weakness	61	NA	NA	NA	NA	NA	NA	NA	NA	Dementia since 2009; RMN: extensive diffuse white matter damage.
F8/II:1	CMT2	F/54	44	Distal weakness in lower limbs	53	4/4–2/2	5/5–3/3	Abs	Reduced up to iliac crests	Red ankle	Hands/legs	Yes	No/Stepagge gait	Trigeminal neuralgia
F8/II:2	CMT2	F/50	49	Distal weakness in lower limbs	50	5/5–4/4	5/5–5/5	Decreased in UL, abs in LL	Normal	Normal	No/legs	No	No	No
F8/II:3	CMT2	M/49	46	Distal weakness in lower limbs	49	5/5–4/4	5/5–5/5	Decreased in UL, abs in LL	Reduced up to iliac crests	Normal	No/legs	Yes	No/Stepagge gait	No
F9/II:1	CMT2	M/51	42	Walking impairment	51	5/5–3/3	5/5–5/5	Abs	Reduced toes and ankle	Red ankles	No/yes	No	No/Stepage gait	No

Abs, absent; AOE, age of examination; AOO, age of onset; CMT, Charcot-Marie-Tooth; F, female; LL, lower limbs; M, male; MRC, Medical Research Council; NA, not available; Red, reduced; UL, upper limbs; Y, years.

Table 3 Electrophysiological data of patients with neuropathy due to biallelic mutations in *MME*

			Median nerve				Ulnar nerve										
			Motor		Sensory		Motor		Sensory		Peroneal nerve, motor		Sural nerve		EMG		
Family/patient	Age	TDE	Amp	CV	Amp	CV	Amp	CV	Amp	CV	Amp	CV	Amp	CV	Distribution of chronic denervation	Spontaneous activity/distribution	Peripheral nerve hyperexcitability
F1/II:1*	56	16	1.6 5.6†	26.2 48.4†	NR		1.8	48.3	NR		NR		NR		Distal UL and proximal LL. Absent voluntary activity in distal LL	Yes/distal LL	Myokymia in distal UL and proximal LL
F2/II:5‡	47	3	8.3	46.6	3.75	36.1	12.8	48.6	4.8	42.6	NR	–	2.0	38.5	Distal UL and distal LL. Isolated motor units in distal LL	Yes/distal UL and distal LL	No
F2/II:6‡	43	3	2.9	35.7	1.7	23.6	12.9	43.9	6.1	50	NR		NR		ND	ND	ND
F3/II:2	61	3	6,6	48.4	5	49.3	8	46.2	3.2	47.1	1.8	36.4	0.7	31.1	Distal LL	No	No
F4/II:2	47	3	10.1	48.6	21	46.4	13.2	50.5	7.2	46.4	0.0	–	4.5	38.6	Distal UL and distal and proximal LL	Yes/distal LL	No
F5/II:1	56	3	11.2	45	11	40	9.5	50	18	44	0.1	34	5.0	37	Distal LL	Yes/distal LL	NA
F6/II:4	39	4	11.2	45.3	13	47.3	15.6	50.0	6.4	52.3	0.1	–	4.7	33.3	Distal and proximal UL and LL	Yes/distal UL and distal LL	Fasciculations in distal UL and proximal LL
F8/II:1	51	7	1.6	34.9	1.4	46.6	1.9	39.6	1.3	45.3	NR		NR		Distal UL and distal LL	NA	NA
F8/II:2	50	1	5.8	45.2	7.7	43.4	7.6	54.2	5.2	54.3	1.9	37.6	7.5	35.6	Distal LL	No	NA
F8/II:3	49	3	3.2	42.4	1.8	40.7	7.8	49.1	1.8	40.3	0.6	37	4.2	39.1	Distal LL	NA	NA
F9/II:1	50	8	4.2	40.7	3.9	38.3	8.7	41.9	3.25	46.2	0.02	32.2	1.08	38.6	Distal and proximal UL and LL	NA	NA

Electrophysiological studies were not available from patients F3/II:3 and F7/II:1
Spontaneous activity: fibrillations, positive sharp waves, high frequency repetitive discharges.
*Radial nerve, extensor indicis (elbow–forearm) CMAP: 2.4 mV, MNCV: 47.6 m/s.
†Flexor carpi radialis (axilla-elbow).
‡Patients diagnosed with carpal tunnel syndrome.
–, not calculated; CV, conduction velocity (m/s); EMG, electromyography; LL, lower limbs; Motor Amp, evoked motor potential amplitude (mV); ND, not done; NR, no response; Sensory Amp, sensory action potential amplitude (microV); TDE, time of disease evolution from clinical onset (years); UL, upper limbs.

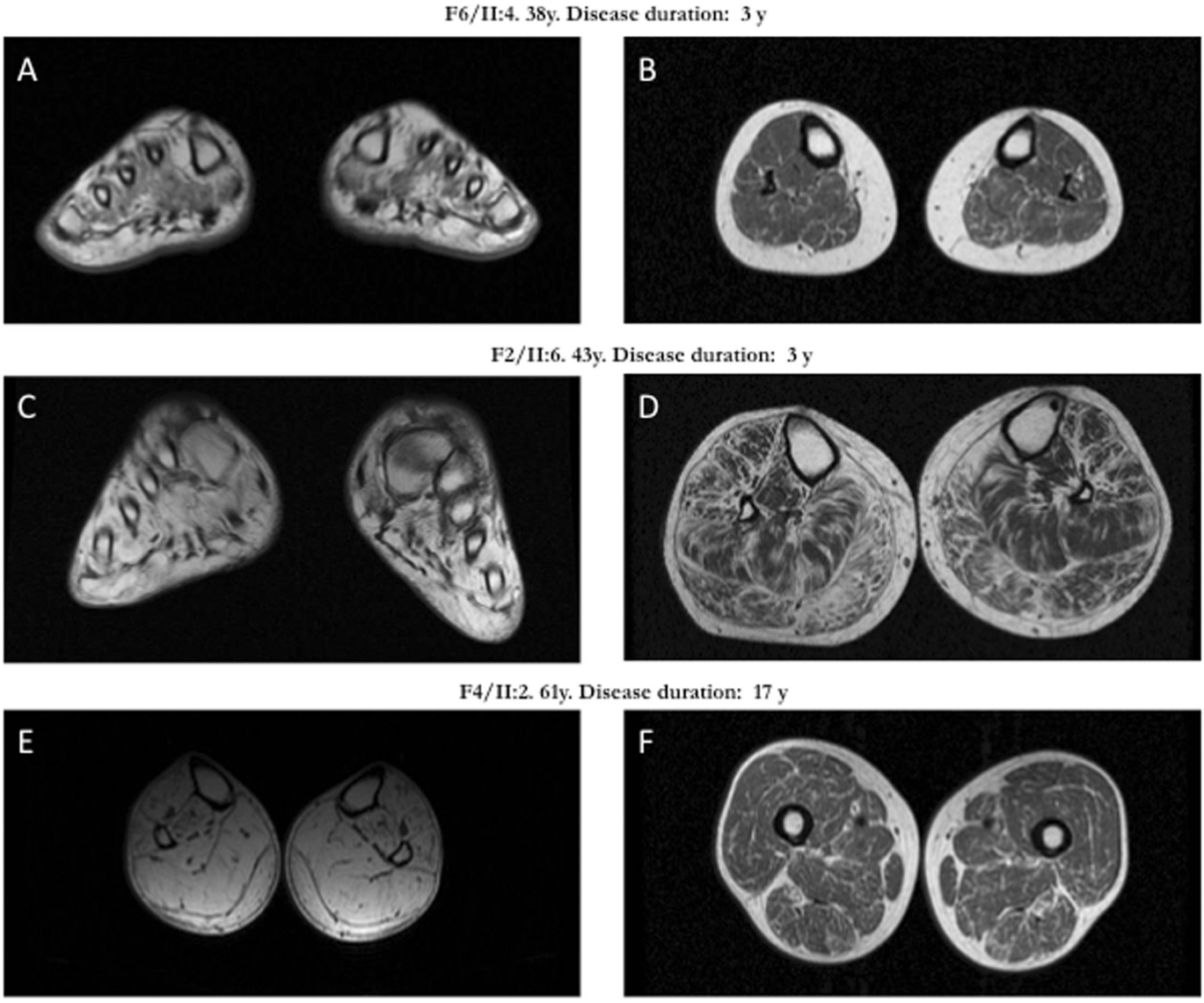

Figure 2 T1 weighted axial MRI images of the lower limbs from patients F6/II:4, F2/II:6 and F4/II:2. (A) and (C) show MRIs at the level of the feet; (B) (D) and (E) at the mid-calf level and (F) at the thigh level. Fatty infiltration followed a length-dependent pattern that was more severe in patients who were older or had a longer disease course. Note that in patient F6/II:4, there is a severe fatty infiltration in the intrinsic muscles of the feet (A) and only a subtle fatty infiltration in the lower leg muscles (B). In patient F2/II:6, there is complete fatty infiltration of the intrinsic muscles of the feet (C) and moderate–severe infiltration of the muscles at the level of the calf (D). In patient F4/II:2, there is complete fat replacement of the muscles in the lower legs (E) and a mild diffuse fatty infiltration of the thigh muscles (F).

than one family, while the other variants were found only once in 13 families. The changes that segregated with the neuropathy displayed an AR pattern of inheritance. Patients with biallelic mutations in *MME* had a homogeneous phenotype that consisted of late-onset axonal CMT, while those patients with heterozygous variants presented a more varied spectrum of phenotypes.

The p.Arg448* mutation was the most common in our *MME* cohort study and it was detected in five unrelated families. In four of these five families, this mutation was either homozygous (F2) or in trans with a second *MME* mutation (F4, F6 and F8). In the fifth family (F10), the p.Arg448* change was identified in heterozygosis and segregation analysis ruled out this change as the cause of the disease. However, a second and novel missense variant in the *AIFM1* gene could be responsible for the neuropathy and deafness in this patient. The second most common mutation p.Pro156Leufs*14 was found in homozygous in two families (F5, F7) with a late-onset CMT2 phenotype. In these families, heterozygous carriers of this mutation had normal clinical and electrophysiological features, including two individuals over 80 years old. A third loss of function (LoF) change (p.Trp750Glyfs*24) was found in two independent families (F6, F14). In the index case of family F6 (II:4), the presence of a second change in *MME in trans* (p.Arg448*) confirmed the diagnosis of an AR CMT2 phenotype associated with *MME*. In family F14, the p.Trp750Glyfs*24 was also identified in heterozygosity in patient II:3, who developed a slowly progressive ALS, as did his brother (II:4) who also had early onset dementia with no motor neuron impairment. The missense variant p.Gly225Ala was identified in heterozygosis in three different families (F17, F18 and 19). Taking into account the clinical variability of these patients, we classified this variant as a benign polymorphism since the segregation studies performed in two families (F17–18) were negative and the allele frequency in a control population database was high.

Table 4 Clinical features of 11 families (probands) with heterozygous variants in the *MME* gene

Family/patient	Change in *MME*	Phenotype	Inheritance	Sex/ current age (Y)	AOO (Y)	Initial symptoms	Functionality	Additional variants identified during the study
F10/II:3	c.1342C>T (p.Arg448*)	CMT2 and deafness	Sporadic	M/55	12	Weakness in LL	Uses foot orthoses and one crutch	*AIFM1* [c.1775T>C (p.Ile592Thr); novel]
F11/II:4	c.1810G>A (p.Val604Ile)	CMT2	AR	M/66	55	Unsteadiness, cramps	Stepagge gait	No
F12/I:1	c.1883A>G (p.Asn628Ser)	Demyelinating neuropathy	Sporadic	M/58	54	Unsteadiness	Uses a cane	No
F13/II:2	c.1495G>A (p.Glu499Lys)	CMT2	Sporadic	F/73	53	Sensory symptoms followed by LL weakness	Mild LL weakness	*HSPB1* [c.-109A>C; rs559109348]
F14/II:3	c.2248delT (p.Trp750Glyfs*24)	ALS	AD*	M/67	54	Right hand atrophy	Three regions affected. Beddriden	*MARS* [c.223T>C (p.Trp75Arg); rs760170215]; *DCTN2* [c.1198C>T (p.Arg400Trp); rs374783640]
F15/III:3	c.773A>G (p.Gln258Arg)	ALS	AD	F/58	30	LL, UL and respiratory muscles involvement	Slowly progressive ALS. Uses a wheelchair	*SOD1* (c.112G>A; p.Gly38Arg); Pathogenic in[11]
F16/I:1	c.1229G>A (p.Arg410His)	CMT2	Sporadic	M/63	51	LL weakness. Non progressive course.	Mild LL weakness.	*FUS* [c.676G>A (p.Gly226Ser); rs758970940]
F17/II:1	c.674G>C (p.Gly225Ala)	Demyelinating neuropathy	Sporadic†	F/41	39	Mild sensory symptoms in hands during pregnancy (CTS)	Mild sensory symptoms	No
F18/II:2	c.674G>C (p.Gly225Ala)	dHMN	Sporadic	M/50	12	LL weakness and atrophy	Stepagge gait	No
F19/II:1	c.674G>C (p.Gly225Ala)	dHMN	Sporadic	M/62	56	Distal lower limb weakness	Uses foot orthoses	No
F20/I:1	c. 466del C (*p.Pro156Leufs**)	Demyelinating neuropathy	Sporadic	M/54	78	Ankle sprain	Hypoesthesia in distal LL	No

*Familiar history of dementia with autosomal dominant (AD) inheritance.
†Her twin sister (F17/II:2; dHMN, distal hereditary motor neuropathy; supplementary figure 2) had the same electrophysiological changes
ALS, amyotrophic lateral sclerosis; AOO, age of onset; CMT2, Charcot-Marie-Tooth type 2; CTS, carpal tunnel syndrome; F, female; LL, lower legs; M, male; UL, upper legs; Y, years.

The rest of the changes detected were only identified in one family. Interestingly, the p.Val604Ile change was found in a patient with late-onset CMT2 (F11) with a similar clinical profile to patients with biallelic *MME* mutations. However, segregation analysis ruled out this change as the disease-causing mutation in this large pedigree. In family F15, the proband diagnosed with fALS carried the *MME* p.Gln258Arg variant. However, this variant was also found in two healthy relatives. In addition, a pathogenic mutation in the *SOD1* gene (p.Gly38Arg)[11] was detected in the proband using Neuro119 gene panel.

Our patients with biallelic mutations in *MME* have the same clinical characteristics as a cohort of Japanese patients published previously,[7] although there appear to be no common genetic variants between these series of patients. Conversely, three out of the 17 mutations detected in this study (p.Trp24*, p.Pro156Leufs*14 and p.Arg448*) were described previously in European and American patients, and linked to late-onset AD polyneuropathies.[8] In our cohort, the healthy relatives carrying the heterozygous LoF mutations (such as, p.Pro156Leufs*14, p.Arg448* and p.Trp750Glyfs*24), who were older than the affected individuals, did not present any signs of neuropathy. Indeed, it is notable that the allele frequency of the first two of these variants (p.Pro156Leufs*14 and p.Arg448*) has augmented from the ExAC to gnomAD databases. Likewise, according to constraint metrics from ExAC,[12] rare LoF changes are observed in *MME* gene, leading to a null LoF intolerance probability (PLI=0.00). Moreover, the number of missense variants for the *MME* gene is higher than expected, resulting in a negative Z-score of −1.12. All together, these findings support the hypothesis that *MME* is likely to be tolerant to both missense and LoF variants at least when in heterozygosis, strongly supporting an AR pattern of inheritance for *MME*.

Our patients diagnosed with a *MME*-associated neuropathy presented a late-onset and predominantly a motor neuropathy, with frequent cramps and muscle contractures accompanied by acute denervation evident in EMGs. Moreover, sensory impairment becomes apparent with disease evolution and it is an important cause of disability. In some of our patients, MNCV showed values compatible with an intermediate or even a demyelinating neuropathy, especially in the median nerve. In the patients described by Higuchi *et al*,[7] MNCV of the median nerve was indicative of an axonal neuropathy in all patients except in one in whom median MNCV was 37.4 m/s. The multifocal pattern of myelinated fibres loss found in the nerve biopsy of patient F4/II:2 has been described in other CMT2 neuropathies like *MORC2*.[13] In general, we consider that *MME*-CMT is predominantly an axonal neuropathy although in some patients, especially in the median nerve, there are both axonal and demyelinating features probably reflecting the complex interaction between axons and Schwann cells not infrequently found in other genetic neuropathies. Neuropathies caused by mutations in *MME* have a clinical phenotype that can be easily confused with an acquired neuropathy, especially due to their age of onset and their relatively rapid progression, unlike classic CMT. As a matter of fact, one patient in our series had been diagnosed with diabetic neuropathy and two other patients with CIDP and treated accordingly. Therefore, a possible diagnosis of neuropathy associated with mutations in *MME* should be considered in patients, who are diagnosed with CIDP but in whom the neuropathy is predominantly axonal and who show poor response to immunosuppressor or immunomodulatory treatments. In addition, fatty infiltration of muscles in the lower limbs is detected in the MRIs of patients diagnosed with CMT2 due to recessive mutations in *MME*, following a typical length-dependent pattern. Curiously, there is no clear preferential involvement of specific muscles or compartments, as occurs in other genetic neuropathies. With the data available, we conclude that the characteristic muscle MRI in *MME* patients involves muscle length-dependent fatty infiltration that appears to affect all muscles localised at the same axial level in a similar way.

It is unclear what are the pathological events driven by *MME* mutations in the peripheral nervous system (PNS). The Neprilysin (NEP) protein is expressed in many tissues,[14] including the PNS and central nervous system (CNS),[15 16] although NEP-deficient mice do not show any obvious abnormalities in motor performance or degeneration of their peripheral nerves.[8] Neurodegeneration in a length-dependent neuropathy may not be so evident in a mouse model given their short life span and the reduced vulnerability of shorter axons. In the CNS, NEP degrades Aβ amyloid (AB) and most studies have focused on its possible pathogenic role in Alzheimer's disease[17–19]; yet, to date *MME* mutations have not been associated to familiar Alzheimer's disease. Indeed, none of the Japanese patients with CMT2[7] were considered to be cognitively impaired after detailed neuropsychological testing. In our cohort, one patient who harboured a *MME* LoF mutation (p.Pro156Leufs*14) was diagnosed with Alzheimer's disease, although his cognitive impairment commenced nearly three decades after the neuropathic symptoms appeared. In family F6, the mother (I:2) and maternal aunt (I:3) of the proband were diagnosed with dementia; yet, this was not associated with the p.Trp750Glyfs*24 change, as this variant was not detected in the aunt. However, this change was also found in a second family with a history of ALS and dementia (F14/II:3 and F14/II:5, respectively). While it may still be feasible that p.Trp750Glyfs*24 is linked to dementia, the fact that one of the patients with dementia did not carry this change (F6/I:3) makes this hypothesis less likely. As such, the possible association of the *MME* gene with cognitive impairment remains elusive.

In conclusion, our findings confirm that *MME* does represent the most common causative gene responsible for late-onset AR-CMT2 in our population, but they do not provide support for the hypothesis that heterozygous mutations in *MME* are a direct cause of CMT. Our studies strengthen the importance of establishing a genetic diagnosis in order to avoid inappropriate immunomodulatory therapies and to ensure that appropriate genetic counselling is given, especially in the light of potentially new treatments for this condition.

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Contributors VL and TS conceived the study. VL, TS and MF participated in the study design and in the draft of the manuscript. VL, AS-M, MDM-R and CE performed, collected and implemented the genetic studies. MF, ALP-N, TG-S, MJS, JP, MM, JG-G, MJS, MJC, JJV and JFV-C performed, collected and implemented the clinical studies. All authors approved the final version of the manuscript.

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