ARTICLE

The genetic landscape of axonal neuropathies in the middle-aged and elderly

Focus on *MME*

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Abstract

Objective

To test the hypothesis that monogenic neuropathies such as Charcot-Marie-Tooth disease (CMT) contribute to frequent but often unexplained neuropathies in the elderly, we performed genetic analysis of 230 patients with unexplained axonal neuropathies and disease onset ≥35 years.

Methods

We recruited patients, collected clinical data, and conducted whole-exome sequencing (WES; n = 126) and *MME* single-gene sequencing (n = 104). We further queried WES repositories for *MME* variants and measured blood levels of the *MME*-encoded protein neprilysin.

Results

In the WES cohort, the overall detection rate for assumed disease-causing variants in genes for CMT or other conditions associated with neuropathies was 18.3% (familial cases 26.4%, apparently sporadic cases 12.3%). *MME* was most frequently involved and accounted for 34.8%

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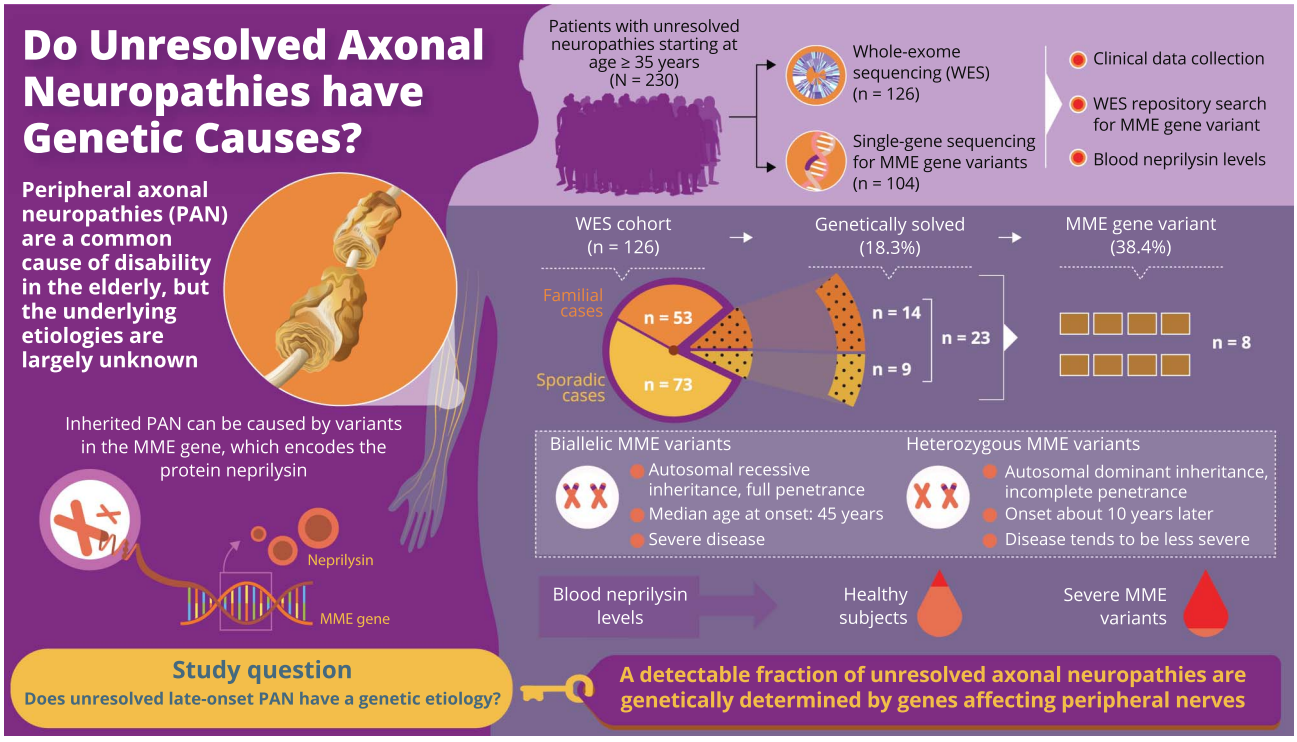
Glossary

ALS = amyotrophic lateral sclerosis; **CMT** = Charcot-Marie-Tooth disease; **IQR** = interquartile range; **MAF** = minor allele frequency; **WES** = whole-exome sequencing.

of genetically solved cases. The relevance of *MME* for late-onset neuropathies was further supported by detection of a comparable proportion of cases in an independent patient sample, preponderance of *MME* variants among patients compared to population frequencies, retrieval of additional late-onset neuropathy patients with *MME* variants from WES repositories, and low neprilysin levels in patients' blood samples. Transmission of *MME* variants was often consistent with an incompletely penetrant autosomal-dominant trait and less frequently with autosomal-recessive inheritance.

Conclusions

A detectable fraction of unexplained late-onset axonal neuropathies is genetically determined, by variants in either CMT genes or genes involved in other conditions that affect the peripheral nerves and can mimic a CMT phenotype. *MME* variants can act as completely penetrant recessive alleles but also confer dominantly inherited susceptibility to axonal neuropathies in an aging population.



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The prevalence of peripheral neuropathies rises with age, amounting to up to 8% among individuals >65 years of age.[1] This makes these conditions a common cause of progressive disability in the elderly.[2] Acquired reasons that are detectable in relevant proportions of patients include diabetes mellitus, systemic immune disorders, toxic causes, and inflammatory neuropathies.[3] Nevertheless, even after appropriate and extensive diagnostic workup, the underlying etiologies often remain unknown, and half of the polyneuropathies are considered idiopathic.[4] However, variants in disease genes for hereditary peripheral neuropathies, for example, axonal Charcot-Marie-Tooth disease (CMT2), can be associated with late disease onset, suggesting that at least some missing causes might be genetic. In earlier studies, a genetic cause has been documented in few or even single patients or families.[5–9] More recently, 3 studies reported variants in *MME* (encoding the metalloprotease neprilysin) in patients with late-onset axonal neuropathies characterized by a severe and rapidly progressive course and predominant manifestation in the lower limbs.[10–12] Biallelic *MME* variants resulted in autosomal-recessive CMT2 with age-related complete penetrance in Japanese families,[10] whereas heterozygous *MME* variants conferred risk to develop late-onset neuropathies in 20 unrelated families from Western and central Europe and the United States.[11,12] Analysis of

patient samples with heterozygous *MME* variants and in vitro studies were consistent with decreased tissue availability and impaired enzymatic activity of the gene product neprilysin.[12]

Here, we performed comprehensive genetic analysis in an extended series of patients with late-onset axonal neuropathies to assess the possible contribution of genetic causes. To further explore the mode of inheritance and variant spectrum of *MME*-related neuropathies, we performed variant screening in additional cases and followed up *MME* variants retrieved from whole-exome sequencing (WES) databases and clinical testing laboratories.

Methods

Study participants

Two-hundred thirty unrelated probands (PED1–PED230) from Austria (n = 137), Germany (n = 33), the Czech Republic (n = 39), and Poland (n = 21) were recruited between 2012 and 2016 during visits at our institutions. Inclusion criteria were progressive sensorimotor peripheral neuropathy with disease onset ≥35 years and axonal or mixed pattern of nerve conduction studies in the lower limbs. Patients complained of motor and sensory deficits or had isolated motor or sensory symptoms combined with clinical signs and electrophysiologic evidence indicating involvement of the other part of the peripheral nervous system. Exclusion criteria were acquired risk factors and causes of peripheral neuropathies, except for cases with diabetes mellitus and onset of diabetes later than diagnosis of peripheral neuropathy. Diabetes was considered unlikely as the relevant cause of the neuropathy when at least 3 of the following findings were recorded: mild diabetes, predominant motor involvement in the beginning, unexpectedly fast progression, or positive family history for a peripheral neuropathy. Moreover, we considered patients with a previous diagnosis of possible chronic inflammatory demyelinating polyneuropathy without further supporting evidence if immunomodulatory therapy proved inefficient or familial aggregation of the disease was recorded. Some of these individuals (n = 51) have already been included in our study that identified *MME* variants as risk alleles for late-onset peripheral neuropathies.[12]

Of the 230 index cases, 126 (PED1–PED126) underwent WES. Fifty-three probands had a family history compatible with mendelian inheritance of the neuropathy; 73 were isolated cases or probands for whom no sufficient information on family history was available. The remaining 104 index patients (PED127–PED230) were screened for *MME* variants by Sanger sequencing or multigene panel sequencing. Eighty-three individuals were screened for variants in the entire *MME* gene (72 by Sanger sequencing and 11 by multigene panel sequencing). In the remaining 21 cases, only *MME* exons 2, 6, and 11 were analyzed by Sanger sequencing.

WES repositories (GENESIS database; Helmholtz Zentrum München, ANZAC Research Institute, Germany) and databases of the authors' institutional clinical genetic testing laboratories were queried for *MME* variants. Medical records of 56 patients (PED231–PED286) could be followed up. Patients originated from Norway, Sweden, Germany, Austria, Hungary, France, the United Kingdom, the United States, Brazil, and Australia.

Standard protocol approvals, registrations, and patient consents

Written informed consent was obtained from all study participants. The study protocol was approved by the ethics committee of the Medical University of Vienna and the participating institutions. Data from Norwegian families (PED264–PED281) were provided in anonymized form after approval by the Norwegian Centre for Research Data.

Clinical and electrophysiologic studies

All probands were examined by experienced neurologists and neurophysiologists at their primary care centers. To define the degree of disease severity, we used a score from 1 to 4 (1 = mild: sensory loss in distal lower limbs, no remarkable gait disturbances; 2 = moderate: motor [and sensory] disturbances in distal lower limbs, mild to moderate steppage gait; 3 = severe: complete paralysis of foot extensors, steppage gait, ataxia; 4 = very severe: unable to walk, uses 2 crutches or is wheelchair dependent). Only patients who had been symptomatic for >5 years were scored.

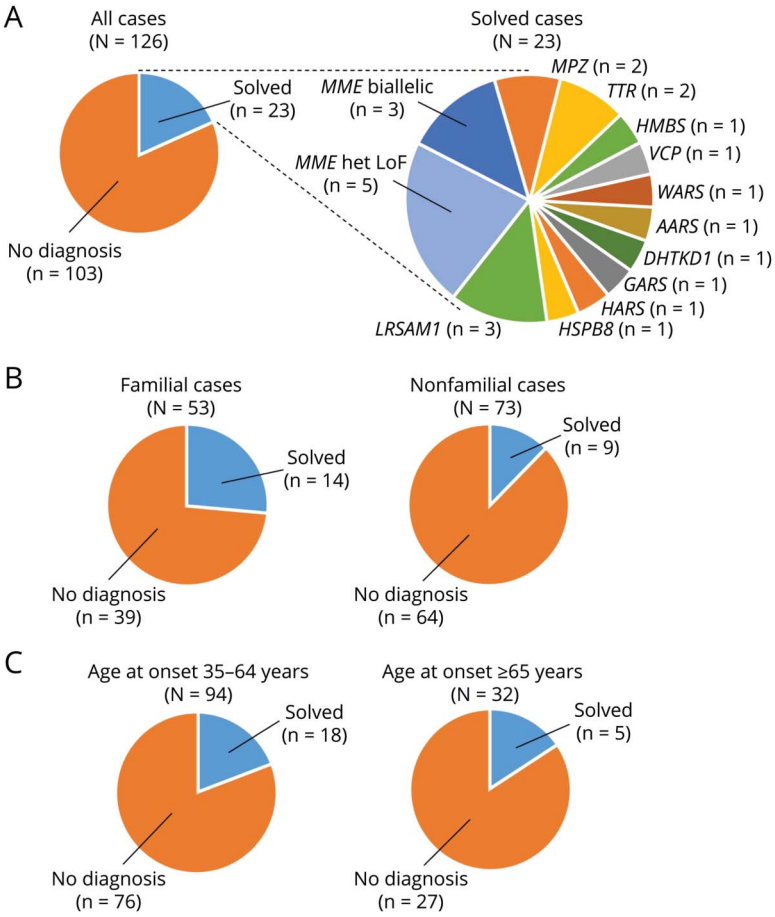
Genetic analysis

WES and Sanger sequencing were performed as reported previously.[12] For multigene panel sequencing, patients were screened with an Agilent (Santa Clara, CA) SureSelect–based, custom-designed panel covering all coding exons of *MME* and 101 additional CMT genes. Sequencing was performed on an Illumina (San Diego, CA) HiSeq 4,000 sequencer, and data were analyzed according to GATK recommendations.[13] Thresholds of minor allele frequencies (MAFs) were set at <0.001 for autosomal-dominant traits and at <0.01 for autosomal-recessive traits (MAF according to gnomAD all exomes[14]). We accepted higher MAFs than conventionally used to account for adult-onset conditions with incomplete penetrance. For classification of missense variants, in silico predictions, conservation scores, potential consequences on the protein, and experimental data were considered. For comparison of allele frequencies among cases and controls, data were statistically analyzed with the Fisher exact test with Bonferroni correction for multiple comparisons.

Quantification of neprilysin levels

Blood samples were collected from 80 individuals. Fifty-eight probands were diagnosed with late-onset peripheral neuropathy. This group was further subdivided into non-*MME* cases (late-onset axonal neuropathy unrelated to *MME* variants, n = 34), *MME* cases (late-onset axonal neuropathy associated with *MME* variants, n = 15), and p.Met8Val *MME* cases (late-onset axonal neuropathy carrying only the p.Met8Val polymorphism, n = 9). Twenty-two participants were healthy controls. Blood plasma was obtained from centrifugation of

Figure 1 Genetic findings in late-onset axonal neuropathies



(A) Contribution of genetic causes to the etiology of late-onset axonal neuropathies as identified by whole-exome sequencing of 126 patients. (B) Comparison of variant detection rates in familial and nonfamilial cases. (C) Comparison of variant detection rates specified by age at disease onset.

whole blood anticoagulated with EDTA. Neprilysin levels were determined in duplicate with DuoSet ELISA Development System and DuoSet Ancillary Reagent Kit 2 (R&D Systems, Minneapolis, MN) using a FLUOstar OPTIMA microplate reader (BMG Labtach GmbH, Offenburg, Germany). Data were statistically analyzed with Shapiro-Wilk test and Kruskal-Wallis test with Dunn post hoc test and Bonferroni correction for multiple comparisons. Outliers (3× interquartile range [IQR]) were not included in the statistical analyses.

Data availability

Anonymized data will be made available on reasonable request.

Results

Genetic causes of late-onset axonal neuropathies identified by WES

In 23 of 126 probands (18.3%), WES identified rare nonsynonymous likely pathogenic variants in genes related to CMT or conditions featuring peripheral neuropathy as part of the phenotypic spectrum (figure 1A). The variant detection rate among cases with a positive family history for a peripheral neuropathy was 26.4% (14 of 53). This fraction was lower at 12.3% (9 of 73) among patients without or with inconclusive family history (figure 1B). The diagnostic yield among patients with an age at onset between 35 and 64 years was 19.1% (18 of 94) and lower at 15.6% in the group of patients with an age at onset of ≥65 years (5 of 32, age range 65–84 years) (figure 1C).

The most frequently involved gene was *MME* (figures 1A and 2A and table 1). Three patients carried rare biallelic variants consistent with autosomal-recessive inheritance, while 5 patients carried single heterozygous loss-of-function (nonsense, frameshift, or splice) variants (figure S1 and table S1, doi.org/10.5061/dryad.66t1g1jzv). Beyond probably pathogenic heterozygous loss-of-function *MME* variants, we identified rare heterozygous missense variants in 5 additional patients. These latter cases were not included in the group of genetically solved cases. Whenever tested, segregation of heterozygous *MME* variants was consistent with an adult-onset condition with incomplete penetrance (figure S1) except for the p.Glu441Lys missense variant (found in 1 pedigree). Available DNA samples allowed us to exclude this variant in 2 patients in the family. Two index patients with rare heterozygous loss-of-function

Table 1 *MME* variants reported in this study

cDNA change	Protein change	Function	dbSNP	PolyPhen-2/ SIFT/CADD	Allele count (MAF) gnomAD all exomes	Allele count (frequency) in 230 cases	HGMD	Comment	Estimated consequence
Presumed causal *MME* variants									
c.35A>C	p.Asp12Ala	Missense	NA	B/D/23.5	0 (0)	1 (0.0022)	CM1610200	Dominant risk factor	Possibly pathogenic
c.68G>A	p.Arg23Gln	Missense	rs201850855	D/T/25.9	38 (0.0001511)	NA[b]	NA	Dominant risk factor	Possibly pathogenic
c.71G>A	p.Trp24*	Nonsense	rs886039755	NA	0 (0)	1 (0.0022)	CM1610199	Dominant risk factor[a]	Probably pathogenic
c.202C>T	p.Arg68*	Nonsense	rs201692212	NA	6 (0.00002387)	NA[b]	CM1610208	Dominant risk factor	Probably pathogenic
c.298delA	p.Thr100Profs*11	Frameshift	rs753679568	NA	1 (0.000003978)	1 (0.0022)	CD1610206	Dominant risk factor	Probably pathogenic
c.358G>A	p.Asp120Asn	Missense, splice region	NA	D/B/23.5	3 (0.00001195)	2 (0.0043)	NA	Recessive allele; last base of the exon; BDGP: splice donor 0.99 (wt) vs 0.41 (mut)	Probably pathogenic
c.440-2A>C	p.(?)	Splice site	rs200435950	NA	4 (0.00001604)	3 (0.0065)	CS1610204	Recessive allele and dominant risk factor	Pathogenic
c.467delC	p.Pro156Leufs*14	Frameshift	rs749320057	NA	65 (0.0002599)	3 (0.0065)	CD042876	Recessive allele and dominant risk factor[a]	Pathogenic
c.531delA	p.Lys177Asnfs*15	Frameshift	NA	NA	0 (0)	NA[b]	NA	Dominant risk factor	Probably pathogenic
c.536-1G>A	p.(?)	Splice site	rs759072209	NA	1 (0.000003999)	NA[b]	NA	Dominant risk factor	Probably pathogenic
c.599delT	p.Ile201Leufs*13	Frameshift	NA	NA	0 (0)	NA[b]	NA	Dominant risk factor	Probably pathogenic
c.667C>T	p.Arg223*	Nonsense	rs201328537	NA	1 (0.000004101)	NA[b]	NA	Dominant risk factor	Probably pathogenic
c.725G>T	p.Cys242Phe	Missense	NA	D/T/24.6	0 (0)	NA[b]	NA	Dominant risk factor; expected to alter disulfide bridge formation	Possibly pathogenic
c.818T>C	p.Met273Thr	Missense	NA	D/D/26.1	0 (0)	NA[b]	NA	Dominant risk factor	Possibly pathogenic
c.957+1delG	p.Lys319Asnfs*6	Frameshift	rs776678738	NA	2 (0.000007979)	NA[b]	CD1610207	Dominant risk factor	Probably pathogenic

Continued

Table 1 *MME* variants reported in this study *(continued)*

cDNA change	Protein change	Function	dbSNP	PolyPhen-2/ SIFT/CADD	Allele count (MAF) gnomAD all exomes	Allele count (frequency) in 230 cases	HGMD	Comment	Estimated consequence
c.958-4_958-3delinsG	p.(?)	Splice site	NA	NA	0 (0)	2 (0.0043)	NA	Dominant risk factor	Probably pathogenic
c.1040A>G	p.Tyr347Cys	Missense	rs138218277	D/D/23.6	133 (0.0005293)	8 (0.0174)	CM1610201	Recessive allele and dominant risk factor; impairs enzyme function	Probably pathogenic
c.1042G>C	p.Ala348Pro	Missense	rs199567914	D/D/27.6	0 (0)	1 (0.0022)	CM1610202	Dominant risk factor	Possibly pathogenic
c.1265C>A	p.Ala422Asp	Missense	rs777476150	D/D/29.9	5 (0.00001993)	1 (0.0022)	CM1610203	Dominant risk factor; impairs enzyme function	Possibly pathogenic
c.1282G>A	p.Val428Met	Missense	rs201362716	D/D/31	0 (0)	NA[b]	NA	Recessive allele	Probably pathogenic
c.1342C>T	p.Arg448*	Nonsense	rs149905705	NA	20 (0.00007978)	3 (0.0065)	CM042749	Recessive allele and dominant risk factor[a]	Pathogenic
c.1345G>T	p.Glu449*	Nonsense	NA	NA	0 (0)	1 (0.0022)	NA	Dominant risk factor	Probably pathogenic
c.1400dupA	p.Arg468Glufs*3	Frameshift	rs751149568	NA	8 (0.00003199)	NA[b]	NA	Recessive allele and dominant risk factor	Pathogenic
c.1505A>C	p.Tyr502Ser	Missense	NA	D/D/28.5	0 (0)	1 (0.0022)	NA	Dominant risk factor	Possibly pathogenic
c.1564C>T	p.Gln522*	Nonsense	NA	NA	0 (0)	2 (0.0043)	NA	Recessive allele	Pathogenic
c.1640C>T	p.Ser547Phe	Missense	rs200973878	D/D/33	1 (0.000003984)	NA[b]	NA	Dominant risk factor	Possibly pathogenic
c.1706A>C	p.Gln569Pro	Missense	NA	D/T/24.4	1 (0.000003983)	1 (0.0022)	NA	Dominant risk factor	Possibly pathogenic
c.1747G>A	p.Gly583Arg	Missense	rs764485980	D/D/33	1 (0.000003985)	NA[b]	NA	Dominant risk factor	Possibly pathogenic
c.1771G>A	p.Asp591Asn	Missense	rs1028833096	D/D/35	1 (0.000003986)	NA[b]	NA	Dominant risk factor	Possibly pathogenic
c.1914+1G>A	p.(?)	Splice site	rs1003705057	NA	2 (0.000007970)	NA[b]	NA	Dominant risk factor	Probably pathogenic
c.1946T>G	p.Ile649Ser	Missense	rs184666602	D/D/34	29 (0.0001160)	1 (0.0022)	NA	Recessive allele	Probably pathogenic
c.2050C>T	p.Gln684*	Nonsense	rs200678412	NA	0 (0)	NA[b]	NA	Dominant risk factor	Probably pathogenic

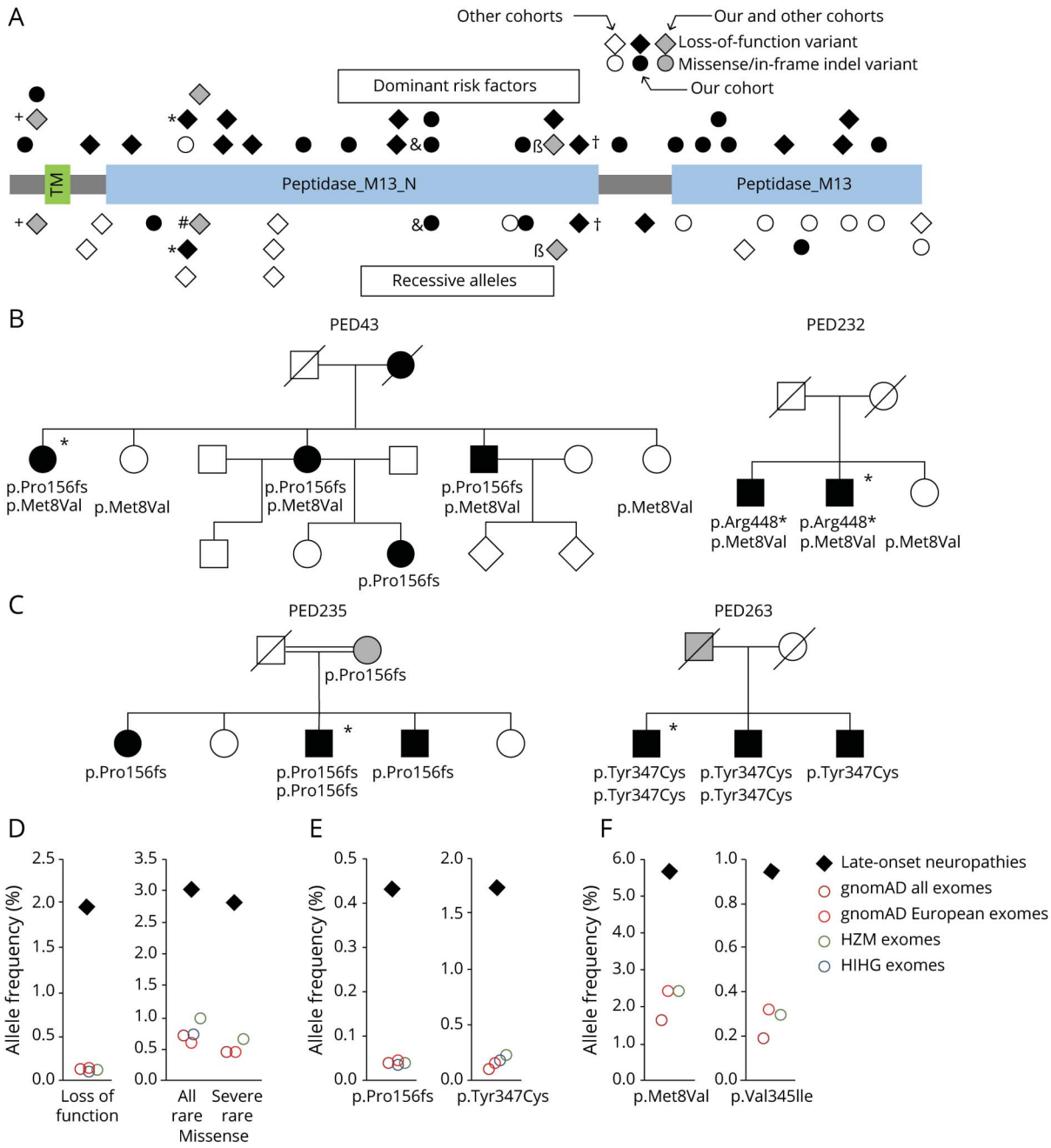
Continued

Table 1 *MME* variants reported in this study *(continued)*

cDNA change	Protein change	Function	dbSNP	PolyPhen-2/ SIFT/CADD	Allele count (MAF) gnomAD all exomes	Allele count (frequency) in 230 cases	HGMD	Comment	Estimated consequence
c.2082delG	p.Trp694Cysfs*48	Frameshift	NA	NA	0 (0)	NA[b]	NA	Dominant risk factor	Probably pathogenic
c.2137A>C	p.Ser713Arg	Missense	NA	D/D/32	0 (0)	NA[b]	NA	Dominant risk factor	Possibly pathogenic
Additional rare and low-frequency *MME* variants									
c.22A>G	p.Met8Val	Missense	rs61762319	B/D/22	4,119 (0.01638)	26 (0.05652)	NA	MAF >0.01	Likely benign
c.917C>T	p.Ala306Val	Missense	NA	B/T/14.74	1 (0.000003984)	NA[b]	NA	Mild missense variant	Likely benign
c.1033G>A	p.Val345Ile	Missense	rs61758194	B/T/14.32	482 (0.001919)	4 (0.008696)	NA	MAF >0.001, mild missense variant	Likely benign
c.1154G>A	p.Arg385Gln	Missense	rs200791566	B/T/20.6	7 (0.00002791)	NA[b]	NA	Mild missense variant	Likely benign
c.1321G>A	p.Glu441Lys	Missense	rs773654354	B/T/21.4	5 (0.00001995)	1 (0.0022)	NA	Mild missense variant; not found in 2 affected relatives	Likely benign
c.1511A>T	p.Glu504Val	Missense	rs201239248	B/T/22.9	4 (0.00001596)	NA[b]	NA	Not strictly conserved	Likely benign
c.1914C>T	p.(=)	Splice region	rs199578318	NA	8 (0.00003188)	NA[b]	NA	Last base of the exon; BDGP splice site prediction: no strong effect	Likely benign

Abbreviations: BDGP = Berkeley Drosophila Genome Project; CADD = Combined Annotation Dependent Depletion; HGMD = Human Gene Mutation Database; MAF = minor allele frequency; mut = mutation; NA = not available/ not applicable; SIFT = Scale-Invariant Feature Transform; wt = wild=type.
PolyPhen-2[22] scores >0.446 predict a damaging (D) effect of an amino substitution. Scores ≤0.446 are considered benign (B). SIFT[23] scores <0.05 are assigned the prediction deleterious (D). Higher scores are labeled tolerated (T). CADD[24] phred-like rank scores >20 are considered damaging.
[a] Also reported as recessive allele in another study.[25]
[b] Variants found only in cases retrieved from whole-exome sequencing repositories, not observed in our study populations.

Figure 2 MME variants associated with late-onset neuropathies



(A) Schematic representation of neprilysin and distribution of variants identified in this and other studies.[10–12,25] Functionally relevant protein domains are indicated. Variants acting as autosomal-dominantly inherited risk factors are shown above and variants inherited as autosomal-recessive alleles are shown below the protein. Variants found in both groups of patients are shown twice and labeled with individual symbols (+, *, #, &, β, †). (B) Detection of serious *MME* variants and the p.Met8Val polymorphism in 2 pedigrees. Women are represented by circles; men are represented by squares. Symbols of affected individuals are filled (black, clinically affected; gray, probably affected by history or subclinical disease); those of unaffected individuals are empty. Crossed symbols represent deceased persons. Asterisks indicate index patients whose data are presented in tables S1, S3, and S4. (C) Pedigree of 2 families with both dominantly and recessively inherited late-onset CMT2 due to homozygous and heterozygous *MME* variant p.Pro156Leufs*14 and p.Tyr347Cys. (D) Comparison of cumulative allele frequencies for *MME* loss-of-function and rare missense variants between this study and whole-exome sequencing (WES) databases. Severe missense = predicted damaging/disease causing by at least 2 out of 3 in silico algorithms (PolyPhen-2, Scale-Invariant Feature Transform, Combined Annotation Dependent Depletion). (E) Individual frequencies of recurrent *MME* variants with a minor allele frequency (MAF) (gnomAD all exomes) between 0.0001 and 0.001 in this study and WES databases. (F) Individual frequencies of *MME* variants with an MAF (gnomAD all exomes) >0.001 in this study and WES databases. TM = transmembrane domain.

variants carried an additional low-frequency variant, p.Met8Val (MAF [gnomAD all exomes] = 0.01641), which was also present in 2 of 3 additional affected members in one of the pedigrees (figure 2B and table S1). Another 15 cases were found to carry only *MME* variants with MAF (gnomAD all exomes) above the thresholds set for recessively (0.01) or dominantly (0.001) inherited variants (p.Met8Val: 14 cases; p.Met8Val and p.Val345Ile: 1 patient).

Table 2 Presumed causal variants in non-*MME* cases

Gene	cDNA change	Protein change	Function	dbSNP	PolyPhen-2/SIFT/CADD	gnomAD all exomes MAF	HGMD	Comment	Estimated consequence
AARS	c.1079C>G	p.Ala360Gly	Missense	rs561829699	D/D/24.9	0.000007954	NA	Variant affects tRNA binding motif that contains a known pathogenic variant	Probably pathogenic
DHTKD1	c.1160-2A>G	p.(?)	Splice site	NA	NA	0.000003981	NA	Known CMT-related *DHTKD1* variants are truncating	Probably pathogenic
GARS	c.383T>G	p.Leu128Arg	Missense	NA	D/D/32	0	NA	Variant p.Glu125Gly close to this position is pathogenic	Probably pathogenic
HARS	c.612A>G	p.Ile204Met	Missense	NA	D/D/13.23	0	NA	Variant in catalytic domain	Probably pathogenic
HMBS	c.499C>T	p.Arg167Trp	Missense	rs118204,101	D/D/34	0.00003838	CM920343	Previously reported variant, functional studies	Pathogenic
HSPB8	c.59C>T	p.Pro20Leu	Missense	NA	B/D/26.4	0	NA	Similar CMT-related variants in *HSPB1*, residue conserved in *HSPB1/8*	Probably pathogenic
LRSAM1	c.2032T>G	p.Cys678Gly	Missense	NA	D/D/27	0	NA	Dominant missense and in-frame insertion variants in the same region	Probably pathogenic
LRSAM1	c.2047-1_2047dupGG	p.(?)	Splice site	NA	NA	0	NA	Truncating dominant variants reported	Probably pathogenic
LRSAM1	c.2060T>C	p.Phe687Ser	Missense	NA	D/D/31	0	NA	Dominant missense and in-frame insertion variants in the same region	Probably pathogenic
MPZ	c.181dupG	p.Asp61Glyfs*42	Frameshift	NA	NA	0	NA	Truncating *MPZ* variants can cause axonal CMT	Probably pathogenic
MPZ	c.186C>G	p.Ile62Met	Missense	rs121913605	D/T/23.3	0.00001194	CM033405	Previously reported variant	Pathogenic
TTR	c.148G>A	p.Val50Met	Missense	rs28933979	D/D/25.3	0.0001034	CM870020	Multiple reports	Pathogenic
VCP	c.283C>T	p.Arg95Cys	Missense	rs121909332	B/D/27.1	0.000003977	CM087382	Multiple reports, functional evidence available	Pathogenic
WARS	c.1228G>A	p.Asp410Asn	Missense	NA	D/D/35	0.000003977	NA	Variant in catalytic domain	Probably pathogenic

Abbreviations: CADD = Combined Annotation Dependent Depletion; CMT = Charcot-Marie-Tooth disease; MAF = minor allele frequency; NA = not available/not applicable; SIFT = Scale-Invariant Feature Transform. PolyPhen-2[22] scores >0.446 predict a damaging (D) effect of an amino substitution. Scores ≤0.446 are considered benign (B). SIFT[23] scores <0.05 are assigned the prediction deleterious (D). Higher scores are labeled tolerated (T). CADD[24] phred-like rank scores >20 are considered damaging.

Table 3 Frequencies of *MME* variants in cases and controls

	Late-onset neuropathies, n	gnomAD all exomes, n	gnomAD European exomes, n	HZM exomes, n	GENESIS exomes, n
No. of samples (alleles)	230 (460)	123,136 (246,272)	55,860 (111,720)	11,225 (22,500)	3,793 (7,586)
Loss-of-function variants[a]	9 (0.01957)	172 (0.0007024) *p* = 9.35E-11	91 (0.0008187) *p* = 4.33E-10	15 (0.0006667) *p* = 4.82E-10	3 (0.0004653) *p* = 1.14E-9
Rare missense variants[b]	14 (0.03043)	1,562 (0.006361) *p* = 2.36E-6	592 (0.005319) *p* = 3.22E-7	206 (0.009156) *p* = 0.00015	50 (0.006591) *p* = 0.00001
Rare, serious missense variants[c]	13 (0.02826)	926 (0.003771) *p* = 4.78E-9	414 (0.003720) *p* = 3.70E-8	131 (0.005822) *p* = 6.89E-6	NA
p.Pro156Leufs*14	2 (0.004348)	62 (0.0002525) *p* = 0.00652	33 (0.0002965) *p* = 0.0092	6 (0.0002667) *p* = 0.01	2 (0.0002636) *p* = 0.018
p.Tyr347Cys	8 (0.01739)	128 (0.0005202) *p* = 2.64E-10	117 (0.001049) *p* = 5.88E-8	41 (0.001822) *p* = 5.36E-6	10 (0.001318) *p* = 2.83E-6
p.Met8Val	26 (0.05652)	4,041 (0.01641) *p* = 8.76E-8	2,719 (0.02435) *p* = 0.0001	544 (0.02418) *p* = 0.00025	NA
p.Val345Ile	4 (0.008696)	463 (0.001882) *p* = 0.012	354 (0.003174) *p* = 0.061	67 (0.002978) *p* = 0.054	NA
p.Gly225Ala	0 (0)	393 (0.001635) *p* = 0.99	230 (0.002126) *p* = 0.99	78 (0.003467) *p* = 0.99	NA

Abbreviations: GENESIS exomes = exome data sets of individuals with various genetic and acquired disease (except polyneuropathies) contained in the GENESIS database; gnomAD all exomes = all exome datasets in gnomAD; gnomAD European exomes = exome datasets of individuals of European descent in gnomAD; HZM exomes = exome data sets of individuals with various genetic and acquired disease (except polyneuropathies) contained in the whole-exome sequencing repository of the Helmholtz Zentrum München; NA = not available.
The *p* values for comparisons of the case group with control groups are given as exact values (Fisher exact test). Late-onset neuropathies = screening cohort used in this study (230 Austrian, German, Polish and Czech index cases).
[a] Nonsense, frameshift, and splice variants with minor allele frequency (gnomAD all exomes) <0.05.
[b] Minor allele frequency (gnomAD all exomes) <0.001.
[c] Minor allele frequency (gnomAD all exomes) <0.001 and predicted damaging/disease-causing by at least 2 in silico algorithms (PolyPhen-2,[22] Scale-Invariant Feature Transform,[23] Combined Annotation Dependent Depletion[24]).

Beyond *MME*, probably pathogenic variants were identified in 8 additional CMT2 genes (figure 1A, table 2, and figure S2 and table S2, doi.org/10.5061/dryad.66t1g1jzv). Three individuals had novel heterozygous *LRSAM1* variants, all altering the C-terminal RING finger motif of the encoded protein where known missense or in-frame indel variants in *LRSAM1* are located.[15–18] Two patients carried heterozygous *MPZ* variants, one of which had been reported earlier.[5] Single cases had novel heterozygous variants in *AARS, DHTKD1*, *GARS*, *HARS*, *HSPB8*, and *WARS*. We also observed known pathogenic variants in 3 genes for conditions that feature peripheral neuropathy as part of a wider clinical spectrum (figure 1A, table 2, figures S2, and table S2). Two individuals carried the common p.Val50Met Met *TTR* variant, which leads to familial amyloid polyneuropathy with or without cardiomyopathy.[19] Another patient had a variant in *HMBS* known to cause acute intermittent porphyria.[20] This 65-year-old woman had never experienced attacks of porphyria. She presented with a mild CMT phenotype consisting of wasting and weakness in distal lower limbs together with absent ankle jerks. She also had sensory ataxia, numbness in distal upper and lower limbs, and impaired temperature perception in the hands. Finally, a *VCP* variant was identified in a patient with peripheral neuropathy and progressive muscle pain. *VCP* variants have been reported to cause several conditions affecting the skeletal muscle or the CNS and peripheral nervous system.[21]

Targeted sequencing of the *MME* gene

Analysis of the *MME* gene in an independent series of 104 additional index cases revealed private or rare variants (figure 2A and table 1) in 14 patients (figure S3 and table S3, doi.org/10.5061/dryad.66t1g1jzv). One pedigree was consistent with autosomal-recessive inheritance; 4 patients carried single rare heterozygous loss-of-function variants; and 9 patients carried rare heterozygous missense variants, which segregated in accordance with an adult-onset trait with incomplete penetrance whenever tested. Three index patients with rare heterozygous variants also displayed an additional low-frequency variant, p.Met8Val or p.Val345Ile (MAF [gnomAD all exomes] = 0.001882). Another 8 cases were found to carry only *MME* variants with MAF (gnomAD all exomes) above the thresholds set for recessively (0.01) or dominantly (0.001) inherited variants (p.Met8Val: 6 cases; p.Val345Ile: 2 patients).

Table 4 Synopsis of clinical manifestations of *MME* variants

	Cases with biallelic *MME* variants[a]	Cases with heterozygous *MME* variants
AAD, median/range, y (n)	45/26–73 (30)	55.5/21–70 (52)
Initial presentation, % (n)		
Motor	90.0 (30)	63.8 (47)
Sensory	6.7 (30)	46.8 (47)
Pain	3.3 (30)	8.5 (47)
Motor, % (n)		
UL	57.9 (19)	51.1 (45)
LL	100 (30)	95.7 (47)
Sensory, % (n)		
UL	33.3 (6)	17.8 (45)
LL	82.1 (28)	95.7 (46)
Pain, % (n)	50.0 (4)	29.7 (37)
Absent/decreased PTR, % (n)	100.0 (24)	87.2 (39)
Median nerve NCS		
MNCV, mean/range, m/s (n)	44.0/34.9–53.3 (26)	45.4/38.5–55.2 (25)
CMAP, mean/range, mV (n)	6.4/1.6–14.0 (26)	6.8/0.8–12.5 (25)
Severity score, mean (n)	2.83 (30)	2.48 (48)

Abbreviations: AAD = age at diagnosis; CMAP = compound muscle action potential; LL = lower limbs; MNCV = motor nerve conduction velocity; NCS = nerve conduction studies; PTR = patellar tendon reflexes; UL = upper limbs.
[a] Cases with biallelic variants also include the series of patients previously reported.[10, 25]

MME variants followed up from WES repositories and clinical testing laboratories

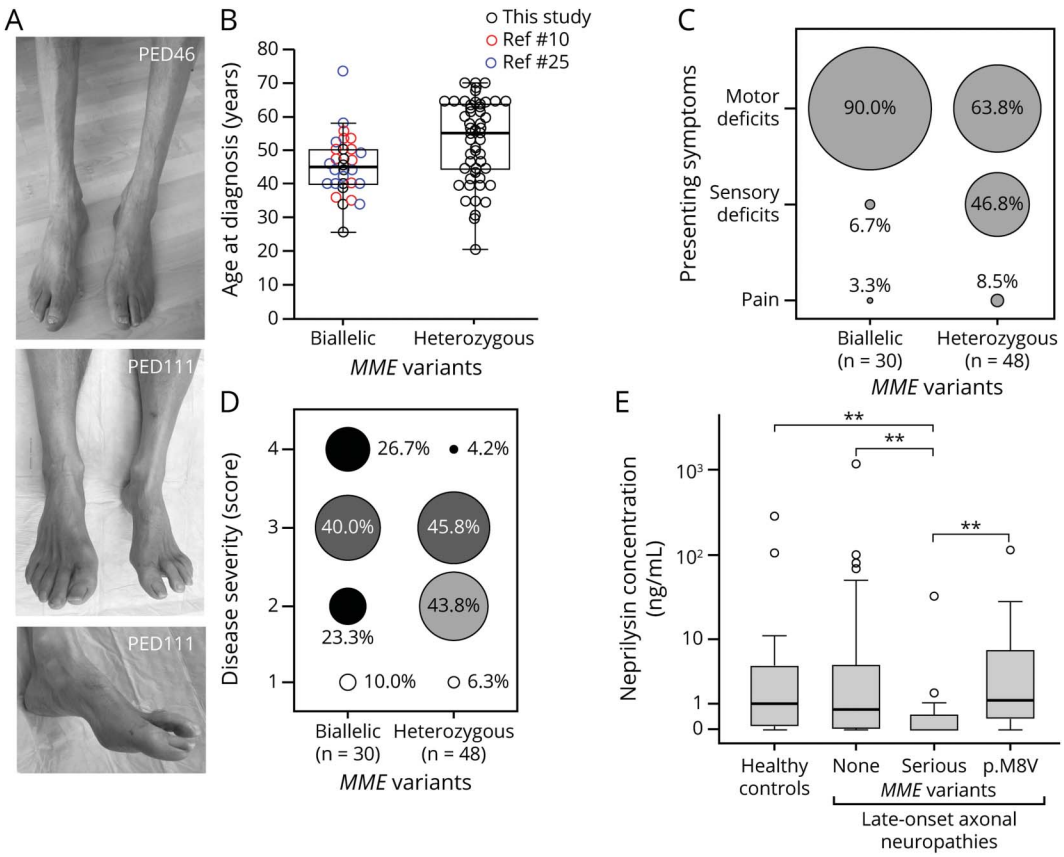
For 43 cases with presumed disease-related *MME* variants (figure 2A and table 1) retrieved from WES repositories and clinical testing laboratories, clinical and family history data were available (figure S4 and table S4, doi.org/10.5061/dryad.66t1g1jzv). Two index patients represented cases with autosomal-recessive inheritance, while transmission of *MME* variants in 2 families was consistent with both autosomal-recessive and incompletely penetrant dominant inheritance (figure 2C and table S4). Twenty-eight individuals carried single rare heterozygous loss-of-function variants, and 11 patients carried rare heterozygous missense variants. Heterozygous variants segregated in accordance with an adult-onset trait with incomplete penetrance whenever tested, except for the p.Ile201Leufs*13 variant (1 family). One proband did not carry this variant but had peripheral neuropathy. Age at onset (at 38 years) was ≈15 years earlier than in affected relatives, and symptoms were slowly progressive (still mild deficits after 15-year disease duration), leaving open the possibility of a phenocopy due to another cause for a peripheral neuropathy. Three index patients with rare heterozygous variants also carried an additional low-frequency variant p.Met8Val and 1 further case carried 2 additional rare missense variants that were classified as benign changes (figure 2B and table S4). Another 13 cases carried only *MME* variants that were classified as likely benign or had MAF (gnomAD all exomes) above the thresholds for recessively (0.01) or dominantly (0.001) inherited variants.

Distribution of heterozygous *MME* variants among cases and controls

Heterozygous *MME* loss-of-function variants (MAF [gnomAD all exomes] <0.05) showed a statistically significant enrichment in our screening cohort compared to control datasets, even when a Bonferroni adjustment was applied to correct for the approximate number of genes (n = 20,000) targeted by WES (nominal *p* values between 9.35E-11 and 1.14E-9, Fisher exact test) (figure 2D and table 3). Similarly, we observed a tendency toward enrichment of rare missense variants (MAF [gnomAD all exomes] <0.001) in the patient group, although it did not reach genome-wide significance for all control cohorts (*p* values between 3.22E-7 and 0.00015, Fisher exact test). When missense variants that were predicted to be disease causing by at least 2 in silico algorithms were considered,[22–24] nominal *p* values became smaller but again did not reach the genome-wide threshold for all control groups (figure 2D and table 3).

For individual variants that were not private, singletons, or ultrarare (MAF [gnomAD all exomes] between 0.0001 and 0.001), allele frequencies among cases and controls were also

Figure 3 MME-related late-onset neuropathies



(A) Distal muscle atrophy and pes cavus deformity in individuals with heterozygous p.Trp24* (PED46) and heterozygous p.Thr100Profs*11 (PED111) *MME* variants. (B) Comparison of the age at disease onset in cases with autosomal-recessive and assumed autosomal-dominant inheritance of *MME* variants reported in this study and previous studies.[10,25] Median, quartiles, and whiskers corresponding to 1.5 times the interquartile range (IQR) are shown. (C) Distribution of presenting symptoms in cases with autosomal-recessive and assumed autosomal-dominant inheritance of *MME* variants. Cases with biallelic variants also include the series of patients reported by other studies.[2,10,25] Diameters of the circles correspond to the proportion of cases with the respective symptom. (D) Distribution of disease severity scores from 1 (mild) to 4 (very severe) in cases with autosomal-recessive and assumed autosomal-dominant inheritance of *MME* variants. Cases with biallelic variants also include the series of patients reported by previous studies.[10,25] Diameters of the circles correspond to the proportion of cases with the respective severity score. (E) Boxplots comparing neprilysin levels in EDTA plasma obtained from healthy controls (n = 22), patients with late-onset neuropathy without *MME* variants (n = 34), those with serious *MME* variants (n = 15), and patients with the p.Met8Val low-frequency polymorphism (n = 9). Outliers (3× IQR) are depicted as open circles and were not included in the statistical analyses. ***p* < 0.01 (Kruskal-Wallis test with Dunn post hoc test and Bonferroni correction).

directly compared. Again, such variants (p.Pro156Leufs*14 and p.Tyr347Cys) were overrepresented among cases, although not reaching genome-wide significance for all control cohorts (figure 2E and table 3). Similarly, 2 variants with an MAF (gnomAD all exomes) >0.001 (p.Met8Val and p.Val345Ile) were also enriched among cases (figure 2F and table 3). As mentioned, in several patients, these likely benign variants were combined with rare heterozygous variants, sometimes confirmed to reside *in trans* yet usually not segregating with the disease phenotype (figure 2B). In contrast, another low-frequency variant contained in gnomAD, p.Gly225Ala (MAF [gnomAD all exomes] = 0.001635), had similar allele frequencies in all control groups but was not detected among cases (table 3).

Clinical manifestations of *MME* variants

Clinical data of index patients are displayed in tables S1, S3, and S4 and are summarized in table 4. Consistent with previous reports,[10–12,25] patients with *MME* variants from our study generally presented with a uniform, recognizable phenotype. Despite advanced age at onset, the neuropathy was rather severe and rapidly progressive, leading to muscle wasting and weakness predominantly in the lower legs (particularly loss of foot dorsiflexion, figure 3A), gait disturbances, or even wheelchair dependence within a few years. Sensory deficits, primarily in the lower limbs, might be the only presenting symptom and were reported in almost all patients during the disease course. About 30% of patients complained of neuropathic pain. Findings from nerve conduction studies showed median motor nerve conduction velocity around the lower limit (45 m/s) and compound muscle action potential amplitudes usually below the normal range (<10 mV), consistent with an axonal neuropathy. Laboratory studies disclosed mildly to moderately elevated creatine kinase levels in several patients.

Clinical presentation of patients carrying 2 rare serious *MME* variants and patients with apparently heterozygous variants was largely similar (table 4). However, in patients with a single *MME*

variant, the disease started ≈11 years later (figure 3B and table 4) and more frequently manifested with sensory and less commonly with motor deficits (figure 3C and table 4). Moreover, the mean disease severity score and the proportion of cases with very severe disease were higher among patients with biallelic *MME* variants compared to patients with apparently heterozygous variants (figure 3D and table 4). Observations in 2 particular families displaying both autosomal-recessive and incompletely penetrant dominant transmission of an *MME* variant (figure 2C) were also in agreement with a more severe effect of biallelic variants. In family PED235, the index patient had severe muscle weakness in the lower limbs and gait disturbance since the age of 39 years. He was homozygous for the p.Pro156Leufs*14 variant, while 2 siblings who showed a similar but less severe phenotype with later disease onset (at 42 and 54 years) both carried this variant in the heterozygous state. The disease was apparently nonpenetrant in the parents, who were obligate heterozygotes, although the mother had mild neurophysiologic abnormalities at age 82 years, indicating possible subclinical involvement. The father was not available for examination. In family PED263, 2 brothers carrying a homozygous p.Tyr347Cys variant had disease onset at age 48 and 40 years and progressive, severe neuropathy, while another brother had age at onset of 66 years and milder disease. The parents, who were obligate heterozygotes, were reportedly unaffected, but no detailed clinical information was available.

Among the 43 patients with rare presumed causal *MME* variants recruited from other research and clinical testing laboratories, 11 cases displayed a broader spectrum of phenotypes (table S4, doi.org/10.5061/dryad.66t1g1jzv). Three patients had an earlier age at onset (at 21, 26, and 30 years). In another family, the disease was classified as distal hereditary motor neuropathy, and patients in a fourth family presented with brisk reflexes. Three index patients had been diagnosed as having amyotrophic lateral sclerosis (ALS) with bulbar dysfunction, although one of them had an unusually long duration of the disease. Affected individuals from 2 families had a clinical diagnosis of sensory ataxia. One index patient had been classified as a case of hereditary neuropathy with liability to pressure palsies.

Neprilysin levels in EDTA blood plasma samples

Individuals with truncating or other serious heterozygous *MME* variants had a median neprilysin concentration of 0 ng/mL (IQR 0.02, n = 12) (figure 3E). In 75% of these samples, levels were below the detection threshold of the assay. Samples from healthy controls and individuals with late-onset axonal neuropathies without *MME* variants or only the low-frequency p.Met8Val variant yielded similar median neprilysin levels of 0.55 ng/mL (IQR 3.33, n = 20), 0.45 ng/mL (IQR 1.8, n = 30,) and 1.08 ng/mL (IQR 5.89, n = 8), respectively. About 20% of these samples had neprilysin levels below the detection threshold. The difference between individuals with serious *MME* variants and the other groups was statistically significant ($p < 0.01$, Kruskal-Wallis test with Dunn post hoc test and Bonferroni correction). The difference between other groups failed to reach statistical significance.

Discussion

We determined the contribution of genetic causes in a group of patients with unresolved late-onset axonal sensorimotor neuropathies. Application of WES revealed a diagnostic yield of 18.3% and might be even higher depending on consideration of single heterozygous *MME* missense variants. Among probands without a family history, the detection rate was still 12.3%, suggesting that WES is a reliable tool in the diagnosis of neuropathies in the middle-aged and elderly, even in the absence of familial aggregation. In a previous study including a small subgroup of patients with disease onset after age 40 years, the detection rate was 9% (13% in familial and 5% in nonfamilial cases).[26] This study was performed before identification of *MME* variants as a cause of late-onset neuropathies, which probably largely explains the lower rate of mutation-positive cases. On a more general note, our findings cannot be necessarily extrapolated to patient series with other genealogical background or differing clinical presentations, for example, less progressive and purely sensory neuropathies.

Pathogenic or probably pathogenic variants were detected in 12 genes known to be associated with peripheral neuropathies. Notably, genetic causes were not restricted to CMT genes. Detection of the p.Val50Met *TTR* variant highlights the importance of screening patients with unexplained axonal neuropathies for familial transthyretin amyloidosis, also to secure monitoring for cardiac involvement[19] and to make treatment options accessible.[27] In 1 patient, the neuropathy was linked to an *HMBS* variant known to cause acute intermittent porphyria, which may also lead to chronic neurologic deficits[28] and should be recognized because adequate treatment can reduce the risk of porphyric attacks.[29] Finally, another patient had a known variant in *VCP*, a gene associated with a degenerative myopathy but also neurodegenerative disorders,[21] including CMT2.[30]

Consistent with previous reports,[10–12] we show that biallelic *MME* variants result in autosomal-recessive CMT2, while heterozygous variants appear to confer risk to autosomal-dominantly inherited neuropathies (figure S5, doi.org/10.5061/dryad.66t1g1jzv). The high proportion of truncating variants among patients with both inheritance modes suggests that *MME* variants may generally represent loss-of-function or hypomorphic alleles. The differences in age at disease onset and clinical severity may then relate to a gene-dosage effect. A dual mode of inheritance (dominant and recessive), whereby a protein is either lost by 2 variants or decreased by a single heterozygous variant, has been shown for several other human mendelian conditions. Examples are *MFN2*, *HSPB1*, and *LRSAM1* variants causing hereditary neuropathy[7,31–36] or *STUB1* variants in spinocerebellar ataxia.[37,38] In line with these observations, simultaneous association of loss-of-function *MME* variants with both inheritance patterns was documented in 2 particular families in our cohort.

For many of the 61 probands with heterozygous *MME* variants, limited availability or usability of samples from additional

family members made results of family studies inconclusive. However, there were several observations in favor of an association of heterozygous variants and peripheral neuropathy. First, patients with late-onset neuropathy with *MME* variants typically have severe and rapidly progressive disease with significant motor involvement during disease course, although the neuropathy might be purely sensory at the beginning. This uniform and recognizable phenotype across patients with *MME* variants was in agreement with a common genetic cause. Second, WES largely excluded convincing variants in other neuropathy genes in the majority of index patients with heterozygous *MME* variants. Third, on the basis of allele frequencies from gnomAD, the likelihood of observing 9 cases with heterozygous loss-of-function variants among 230 individuals simply by chance not only is extremely low but borders on impossible. Notably, even after a Bonferroni correction (to account for the fact that *MME* is only 1 of ≈20,000 genes screened), the enrichment among cases was still significant. The trend toward an enrichment of rare heterozygous missense variants among cases is further consistent with a relevant effect of heterozygous *MME* variants. At the same time, it is obvious that only a particular subset of rare missense variants might be causative: nonsegregation was demonstrated for the *MME* variant p.Glu441Lys found in 1 family and for other unconvincing variants, which were mild or relatively frequent in databases.[25] Generally, variant classification is not static, and we anticipate reclassification of a subset of *MME* variants in the future because of wider application of *MME* mutation screening, improved population frequency data, and refined bioinformatic algorithms and functional tests.

The occurrence of rare *MME* truncating and missense variants in control datasets, together with the observation of elderly unaffected carriers or obligate heterozygotes in this and earlier reports, points to incomplete penetrance of heterozygous *MME* variants. Future studies will be necessary to unravel the possible synergistic interaction of heterozygous *MME* variants with additional genetic or exogenous factors that will ultimately precipitate or prevent the disease. Obvious candidates for such disease modifiers could be low-frequency *MME* variants. We indeed noted an excess of variants p.Met8Val and p.Val345Ile among cases. Notably, in patients from 8 families, these variants were observed in combination with truncating or possibly pathogenic missense variants. The potential of additional, presumedly benign variants has recently been demonstrated in individuals with hereditary spastic paraplegia carrying the variant p.Ala510Val *in trans* with a known pathogenic *SPG7* variant.[39] Beyond coding variants, deep intronic variants not detected by WES could contribute, as has been shown recently for a recurrent de novo intronic variant in *COL6A1* muscular dystrophy.[40]

While heterozygous *MME* variants were penetrant in a considerable proportion of confirmed or suspected carriers mainly from central Europe (this series), nonpenetrance of heterozygous *MME* variants was reported in families from Spain and Japan.[10,25] Significant variability of penetrance among families with distinct geographic origin has been observed in other genetic diseases such as familial amyloid polyneuropathy in which the penetrance of *TTR* variants is highly population specific.[41,42] Without implicating a causative link, we noted that the low-frequency *MME* variants p.Met8Val and p.Val345Ile, which could represent disease modifiers, are at least 150 times (p.Met8Val) or 35 times (p.Val345Ile) less frequent in the East Asian gnomAD subpopulation compared to the global population. MAFs in the Latino subpopulation are also 1.5 to 3 times lower than in the global gnomAD population.

Most patients recruited from WES databases and clinical testing laboratories also had late-onset but severe and rapidly progressive axonal sensorimotor neuropathy. However, in 3 cases, disease onset was before age 35 years. Moreover, we ascertained 5 patients with predominant motor involvement or pyramidal tract signs, leading to a diagnosis of distal hereditary motor neuropathy in 1 family (similar to previously reported cases[43]) and ALS in 3 families. Phenotypic and genetic overlaps of peripheral neuropathies and ALS are not unusual, as has been demonstrated recently for variants in the *KIF5A* gene.[44,45] In 2 other patients, sensory ataxia was the leading clinical manifestation, similar to cases from a large family with autosomal-dominant inheritance.[11]

The extended number of different potentially disease-related *MME* variants indicates the need for a convenient assay to discriminate pathogenic from neutral variants. We found that a significant decrease of neprilysin levels in blood plasma samples distinguishes patients with late-onset CMT with loss-of-function and relevant missense *MME* variants from controls, including individuals carrying only the likely harmless p.Met8Val variant. Neprilysin substrates in the peripheral nervous system are unknown, and the mechanism by which neprilysin deficiency causes peripheral neuropathy has remained elusive. On the other hand, neprilysin is known to degrade β-amyloid, and a role of neprilysin deficiency in Alzheimer disease has been concluded from mouse models.[46,47] Data from our patient cohort (tables S1, S3, and S4, doi.org/10.5061/dryad.66t1g1jzv) and previous studies[10–12,25] do not support an increased prevalence of cognitive impairment among patients with pathogenic or likely pathogenic *MME* variants, but further clinical follow-up is required to fully explore a potential link between neprilysin and Alzheimer disease.[48] At the same time, because neprilysin is able to degrade endogenous cardioprotective peptides, pharmacologic inhibition of neprilysin activity has recently been introduced as a new treatment for heat failure with reduced ejection fraction.[49] The established genetic link between neprilysin deficiency and peripheral neuropathy in humans and the association with Alzheimer disease in animal studies indicate a need for vigilance in the use of neprilysin inhibitor therapy.[50]

In essence, our study demonstrates that a detectable proportion of sensorimotor axonal neuropathies in the middle-aged and elderly is genetically determined. It also highlights the utility of WES as a diagnostic tool for these conditions and

confirms *MME* as a relevant disease gene. Biallelic *MME* variants show age-dependent complete penetrance, while heterozygous loss-of-function and particular missense *MME* variants appear to confer risk for developing axonal neuropathy with advanced age.

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Disclosure

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Continued

Appendix *(continued)*

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Appendix *(continued)*

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