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(Article begins on next page)

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Abstract

Background: Inherited peripheral neuropathies (IPNs) represent a broad group of genetically and clinically heterogeneous disorders, including axonal Charcot-Marie-Tooth type 2 (CMT2) and hereditary motor neuropathy (HMN). Approximately 60%-70% of cases with HMN/CMT2 still remain without a genetic diagnosis. Interestingly, mutations in HMN/CMT2 genes may also be responsible for motor neuron disorders or other neuromuscular diseases, suggesting a broad phenotypic spectrum of clinically and genetically related conditions. Thus, it is of paramount importance to identify novel causative variants in HMN/CMT2 patients to better predict clinical outcome and progression.

Methods: We designed a collaborative study for the identification of variants responsible for HMN/CMT2. We collected 15 HMN/CMT2 families with evidence for autosomal recessive inheritance, who had tested negative for mutations in 94 known IPN genes, who underwent whole-exome sequencing (WES) analyses. Candidate genes identified by WES were sequenced in an additional cohort of 167 familial or sporadic HMN/CMT2 patients using next-generation sequencing (NGS) panel analysis.

Results: Bioinformatic analyses led to the identification of novel or very rare variants in genes, which have not been previously associated with HMN/CMT2 (*ARHGEF28*, *KBTBD13*, *AGRN* and *GNE*); in genes previously associated with HMN/CMT2 but in combination with different clinical phenotypes (*VRK1* and *PNKP*), and in the *SIGMAR1* gene, which has been linked to HMN/CMT2 in only a few cases. These findings were further validated by Sanger sequencing, segregation analyses and functional studies.

Conclusions: These results demonstrate the broad spectrum of clinical phenotypes that can be associated with a specific disease gene, as well as the complexity of the pathogenesis of neuromuscular disorders.

Introduction

Inherited peripheral neuropathies (IPNs) comprise a clinically and genetically heterogeneous group of disorders, including Charcot-Marie-Tooth (CMT) disease, distal hereditary motor neuropathy (HMN or distal spinal muscular atrophy), hereditary sensory autonomic neuropathy and hereditary neuropathy with liability to pressure palsy.[1 2] They commonly present with a characteristic phenotype of a length-dependent, isolated neuropathy progressing over decades.

The advent of next-generation sequencing (NGS) techniques has significantly expanded the number of genetic variants and genes identified. Thus, in the last few years, almost 100 genes responsible for IPN have been reported.[1 2]

However, among IPNs, approximately 60%–70% of axonal CMT type 2 (CMT2) and HMN cases still remain without a genetic diagnosis.[1] This could be due to the large number of responsible genes, many of which affect only a few families. Moreover, some HMN/CMT2 genes are also associated with motor neuron or muscle disorders.[3–9] These recent findings have changed the classical distinction criteria used for classification and diagnosis and suggested that a broad spectrum of clinical phenotypes can indeed share common pathogenetic mechanisms. In this context, the identification of mutations in genes responsible for HMN/CMT2 can not only contribute to genetically diagnose these disorders but also to better predict clinical outcomes.

In this study, we used whole-exome sequencing (WES) to identify candidate causative variants in HMN/CMT2 patients from families with predicted autosomal recessive inheritance. We detected 14 candidate causal variants in known disease genes, which have not been previously associated with HMN/CMT2 (*ARHGEF28* - Rho guanine nucleotide exchange factor 28; *KBTBD13* - Kelch repeat and BTB domain containing 13; *AGRN* - Agrin, and *GNE* - Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase); in genes previously associated with HMN/CMT2 but in combination with other clinical phenotypes (*VRK1* - VRK serine/threonine kinase 1 and *PNKP* - Polynucleotide kinase 3'-phosphatase), and in the *SIGMAR1* (Sigma non-opioid intracellular receptor 1) gene, which has been already linked to HMN/CMT2 in a few cases.[10–12] Thus, our findings contribute to further expanding the clinical and genetic association among these phenotypes.

Methods

Patients

All data were collected in an anonymised way. All subjects were interviewed for family and personal history and underwent a standard neurological examination, including evaluation for pyramidal or cerebellar signs, cranial nerve involvement, as well as the presence of bone abnormalities, joint retractions and skin lesions. Further inclusion criteria were based on pedigree structure, with at least two affected siblings born from healthy parents (consanguineous or not) and on the absence of mutations in 94 IPN genes, including the most frequently mutated HMN/CMT2 genes, which were screened by performing NGS panel analysis (listed in online supplementary material).

WES and NGS custom panel analysis

To perform WES analysis, libraries were captured using Agilent SureSelect-Human-All-Exon V.6 probes for 16 hours and run on NextSeq 500 platform (Illumina, San Diego, California, USA) (online supplementary material). To search for additional variants in the candidate disease genes identified by WES (*AGRN, ARHGEF28, GNE, KBTBD13, PNKP, SIGMAR1* and *VRK1*) in the 167 unrelated patient cohort, a custom-made panel of genes has been defined with capture system (Agilent Technologies, Santa Clara, California, USA) and sequenced using a MiSeq platform (online supplementary material).

Tissue sampling

When necessary for diagnostic procedures, patients underwent tissue sampling including muscle or sural nerve biopsy, performed and stained as previously described.[8] Images were obtained with light (Leica DM 2500, Leica Microsystems, Wetzlar, Germany) or electron (LEO 912AB TEM, Milano, Italy) microscopes.

Neurophysiology

Nerve conduction studies and electromyography (EMG) were performed according to standard techniques and evaluating either proximal or distal muscles. For single-fibre EMG (sf-EMG), the recordings were performed using a concentric coaxial facial needle, with thin recording surface; the band-pass filter was set at 2000–10 000 Hz.

Results

To identify gene variants responsible for HMN/CMT2, we collected 33 patients and 27 healthy relatives from 15 unrelated Italian families, with evidence for autosomal recessive inheritance. These families tested negative for mutations in 94 known IPN genes, including the most frequently mutated HMN/CMT2 genes, as assessed by NGS panel analysis.

Thus, a total of 40 individuals underwent WES and 11 candidate pathogenic variants were identified in 7 known motor neuron (*SIGMAR1, ARHGEF28, VRK1* and *PNKP*) or neuromuscular disease genes (*KBTBD13, GNE* and *AGRN*) (table 1). Interestingly, the *ARHGEF28, KBTBD13, AGRN* and *GNE* genes have not been previously associated with HMN or CMT2, whereas *VRK1* and *PNKP* have been associated with HMN or CMT2 but in combination with other clinical phenotypes (table 2).

Genes associated with motor neuron disorders

WES identified candidate causal variants in four genes previously associated with CNS (Central Nervous System) disorders. In family A (figure 1A,B; tables 1 and 3), two siblings born from first cousin healthy parents were homozygous for a novel missense variant (p.Glu138Lys) in *SIGMAR1*, a gene responsible for amyotrophic lateral sclerosis (ALS),[13] and recently found to be associated in a

few cases with motor neuropathy and Silver-like syndrome.[10–12] Of note, a different homozygous substitution (p.Glu138Gln) at the same residue was previously associated with HMN with brisk proximal reflexes.[12] The siblings reported here showed moderate-to-severe muscle weakness (the older is wheelchair bound) and atrophy, with split hand sign in the upper limbs, distal limb paresthesias but no sensory deficits. Mild pyramidal signs were present with brisk knee jerks and an extensor plantar on the left in the younger sister, but motor evoked potentials were within the normal range. Neurophysiology was consistent with distal motor neuropathy (table 4) and sural nerve biopsy performed in the younger patient was normal. We did not detect fasciculations, respiratory deficits or cranial nerve involvement. We also screened an additional cohort of 167 unrelated sporadic and familial HMN/CMT2 cases by performing NGS analysis using a custom-made panel, which included the seven disease genes identified by WES. We, thus, found another novel homozygous variant (c.352+1G>A) likely affecting splicing in a sporadic patient characterised by motor deficits distally in the four limbs, no sensory deficits, EMG typical of motor axonal polyneuropathy (table 4) and no pyramidal or other CNS sign (figure 1C). Interestingly, this change, which occurs at the splice-junction sequence signal between exons 2 and 3, is predicted to lead to the skipping of exon 3 and to the generation of a truncated SIGMAR1 isoform, hSIG1-A, lacking 31 amino acids. Previous studies suggest that this isoform does not bind the SIGMAR1 ligand, but competes with the full-length protein for the dimerization with receptors, such as the opioid receptor MOR-1 (Opioid receptor mu 1).[14]

In family B (figure 1A; tables 1 and 3), we identified two compound heterozygous variants (p.Arg475Thr and p.Cys1458Trp) in *ARHGEF28*, previously associated with autosomal dominant ALS.[15 16] The siblings showed distal muscle atrophy, weakness and areflexia of all four limbs, stepping gait, pes cavus, atrophic hands with flexor contractures and severe scoliosis. Neither displayed sensory abnormalities, pyramidal signs, fasciculations or respiratory deficits. Neurophysiology showed an axonal sensory (predominant) motor polyneuropathy (table 4). Screening for *ARHGEF28* mutations in the additional 167 unrelated HMN/CMT2 cases by NGS custom panel analysis revealed two compound heterozygous variants (p.Glu1549Glu and p.Leu1708Val) in a 54-year-old woman (Pt 2, tables 1 and 4), who reported frequent falls and progressive motor deficits distally in the lower limbs since adolescence, pes cavus, and moderate superficial and deep sensory deficits in the feet, without pyramidal signs. Of note, among the four variants, we identified in the *ARHGEF28* gene, p.Cys1458Trp and p.Glu1459Glu reside in the RNA-binding domain, whereas p.Leu1708Val in the microtubule-binding domain of RGNEF (Rho guanine nucleotide exchange factor), the protein encoded by the *ARHGEF28* gene.[17]

In family C (figure 1A; tables 1 and 3), the two siblings shared a rare homozygous variant (p.Arg321Cys) in *VRK1*, a gene previously associated with ALS and spinal muscular atrophy (SMA) with pontocerebellar hypoplasia.[18–20] The p.Arg321 residue resides at the border of the protein kinase domain, and this variant has been already reported together with p.His119Arg in a patient with probable ALS.[18] Parents were healthy second cousins. The two siblings developed progressive motor deficits in the lower limbs without sensory abnormalities, with disease duration of more than 40 years in the older and 20 years in the younger brother. Deep tendon reflexes were reduced distally in the lower limbs and increased elsewhere. Both showed Hoffman (but not Babinski) positive sign and delayed latencies in the four limbs in motor evoked potentials. EMG showed chronic neurogenic changes (table 4). Brain MRI was normal in the older brother and revealed an incidental benign tumour (dysembryoplastic neuroepithelial tumour) in the right temporal lobe of the younger brother.

In family D (figure 1A,D; tables 1 and 3), we identified two rare compound heterozygous variants in *PNKP* (c.1295_1298+6 del and p.Gln50Glu), which were shared by the three affected siblings of this family. The first change likely affects splicing of intron 14, whereas p.Gln50Glu lies within the FHA

(forkhead-associated) domain of the protein. Mutations in *PNKP* have been previously associated with ataxia with or without oculomotor apraxia.[21–23] One sibling (D:II,2) reported mild distal weakness and cramps in the lower limbs. All three siblings showed absent deep tendon reflexes and reduced distal deep sensation, and EMG showed diffuse chronic denervation and sensorimotor axonal neuropathy (table 4). Brain MRI in patient D:II,1 showed moderate cerebellar atrophy (figure 1E). We did not detect additional variants in the *VRK1* and *PNKP* genes in the 167 unrelated HMN/CMT2 patient cohort by performing NGS custom panel analysis.

Genes associated with neuromuscular disorders

The last three genes identified by WES were previously associated with neuromuscular disorders.

In family E (figure 2; tables 1 and 3), we found that the two siblings were homozygous for a novel missense variant (p.Ala55Gly) in *KBTBD13*, a gene previously associated with autosomal dominant Nemaline myopathy type 6 (NEM6).[24] Parents, deceased at an advanced age, were asymptomatic second cousins. Both siblings started to complain about gait difficulties in their thirties, with 10 years progression to foot-drop. They showed severe distal weakness in the lower limbs (moderate in the hands), pes cavus (figure 2B), increased tendon reflexes in the upper limbs, absent in the lower limbs, positive Hoffman and Rossolimo's signs and mute plantar reflexes. Neurophysiology showed chronic axonal motor polyneuropathy in all four limbs (table 4). At age 55, the female underwent sural nerve biopsy, showing mild axonal neuropathy (figure 2C). To exclude NEM, when she was 64-year-old, we also performed muscle biopsy, which did not show signs of muscle damage nor nemaline bodies (figure 2D–F). KBTBD13 belongs to the Kelch family proteins, involved in several cell functions, including ubiquitination.[24] KBTBD13 interacts with Cullin 3 through its BTB/POZ (Broad-Complex, Tramtrack and Bric a brac/POxvirus and Zinc finger) domain to form a RING ubiquitin ligase (Cul3-RL) complex capable of protein ubiquitination (figure 2G). To investigate the impact of the p.Ala55Gly variant on the KBTBD13/Cullin 3 interaction, we performed co-immunoprecipitation experiments in 293 T cells, endogenously expressing Cullin 3. We observed that mutated KBTBD13 had a reduced affinity for Cullin 3 as compared with wild-type KBTBD13 (figure 2H), suggesting that the p. Ala55Gly variant is pathogenic.

In family F (figure 3; tables 1 and 3), we identified compound heterozygous mutations (p.Tyr706His and p.Glu33Gly) in *GNE*, previously associated with Nonaka or hereditary inclusion body myopathy (hIBM).[25 26] Siblings dated the onset of the disease around their thirties, characterised by weakness and atrophy distally in the lower limbs and gait abnormality (figure 3G–I). All developed foot-drop and loss of deep tendon reflexes in the lower limbs, in the first two brothers associated with hypopallesthesia at the ankles. EMG performed in the first years of the disease showed chronic motor denervation (table 4). Only recently, the two older brothers developed proximal muscle weakness in the lower limbs with characteristic waddling plus stepping gait. Repeated EMG revealed myopathic changes whereas CK elevation was never reported (table 4). Patients F:II,1 and F:II,2 underwent muscle biopsy (vastus lateralis, due to complete fat substitution of leg and posterior thigh muscles at MRI examination) showing myopathic features including rimmed vacuoles (figure 3B,D). Muscle biopsy also revealed small angulated fibre, type grouping and nuclear clusters, usually interpreted as neurogenic features (figure 3C, E and F).

In family G (figure 4; tables 1 and 3), we found that the two siblings shared rare compound heterozygous (p.Leu991Phe; p.Gln1135Arg) mutations in *AGRN*, a gene previously associated with congenital myasthenia type 8.[27] The father died at age 55 due to cardiac infarction, and did not have muscle atrophy nor weakness. The

mother and paternal aunt, who carried the variants in heterozygosity, are both healthy. Brother and sister recalled clumsy gait and frequent falling since their childhood, with rapid progression to foot-drop and distal atrophy and weakness in the upper limbs (figure 4B–E). More recently, they developed proximal weakness in the lower limbs. Both showed marked distal muscle atrophy, absent tendon reflexes, positive Romberg's sign, severe reduction of deep sensation and reduced tactile sensation distally in the lower limbs. Several neurophysiological evaluations since childhood reported severe chronic sensorimotor axonal polyneuropathy (table 4). They also underwent sural nerve biopsy showing typical findings of chronic axonal neuropathy (figure 4F,G). To further assess the pathogenicity of the variants, patients underwent repetitive nerve stimulation (RNS) and sf-EMG. In patient G:III,1, the RNS gave a decremental pattern of 9%, obtained after facilitation by effort for 20 s; the sf-EMG analysis of 22 complexes of single fibres resulted in a mean jitter greatly increased (100 µs); the percentage of the complex with jitter higher than 55 µs was 66%; the blocking phenomenon was recorded in seven complexes (figure 4H). In patient G:III,2, the RNS gave a decremental pattern in basal condition of 25%; after facilitation by effort, the decremental pattern almost normalised and then 2 min later, a decremental pattern of 20% was confirmed; the test was then interrupted by patient request. The sf-EMG mean jitter of 20 complexes was greatly increased (103 µs); the percentage of complex with jitter greater than 55 µs was 60%; the blocking phenomenon was observed in eight complexes (figure 4H). Both patients had negative anti-acetylcholine receptor, muscle-specific kinase (MusK) and voltage-gated calcium channel antibodies. Finally, we did not detect additional variants in the *KBTBD13*, *GNE* and *AGRN* genes in the 167 unrelated HMN/CMT2 patient cohort by performing NGS custom panel analysis.

Discussion

Depending on the clinical series, approximately 60%–70% of axonal CMT2 and HMN remain genetically undiagnosed.[1] Moreover, among the known HMN/CMT2 genes, the same gene might also be responsible for motor neuron or muscle disorders, thus suggesting that these apparently distinct clinical entities share common pathogenetic mechanisms, which are still largely unknown.[3–9] In this study, using a combination of WES and NGS panel analyses, we further widened the spectrum of genes responsible for motor IPN with autosomal recessive inheritance. We report, here, novel or very rare variants in disease genes, which have not been previously associated with HMN or CMT2, or which cause HMN or CMT2 but in combination with other clinical phenotypes.

Heterozygous missense mutations in *ARHGEF28* have been previously reported in autosomal dominant familial ALS.[15 16] RGNEF expression, the protein encoded by *ARHGEF28*, has been detected in pathological inclusions of motor neurons in sporadic and familial ALS patients, consistent with the fact that in neurons this protein controls neurofilament light chain mRNA and protein stability. Here, we report novel compound heterozygous variants in the *ARHGEF28* gene in two unrelated CMT2/HMN cases with putative autosomal recessive inheritance. RGNEF also possess a Rho-A guanine nucleotide exchange factor activity and is involved in the regulation of actin and focal adhesion dynamics in cells.[28] Thus, different pathogenetic mechanisms of gain or loss of function may result in different phenotypes ranging from ALS to pure motor axonal neuropathies, respectively. Similarly, *SIGMAR1* is another gene previously associated with ALS[13 29 30] and in a few cases with distal motor neuropathy with[10 11 31] or without pyramidal signs.[32 33] In this study, we further confirmed *SIGMAR1* as a gene responsible for pure HMN, as in our cohort, we found two novel homozygous variants in two unrelated cases with motor neuropathy with no or subtle pyramidal features.

Mutations in V*RK1* have been reported in familial ALS, SMA with pontocerebellar hypoplasia and motor neuropathy without cerebellar anomalies.[18–20] Our case confirms *VRK1* as a gene responsible for motor neuropathy and supports the pathogenicity of the p.Arg321Cys mutation, which has been previously described in ALS as compound heterozygous allele together with

p.His119Arg.[18] The *VRK1* gene encodes a serine/threonine kinase closely interacting with several transcription factors including p53, and involved in the regulation of cell cycle and DNA repair.[34] How defective DNA repair results in axonal neuropathy is still not clear. However, DNA damage repair must be a key process in axon maintenance as other genes involved in this process have been associated with inherited neuropathies, such as *MORC2* (MORC family CW-type zinc finger 2), *HINT1* (Histidine triad nucleotide binding protein 1) and *PNKP* described in this study.[1 2]

PNKP has been found to be mutated in CNS disorders, as initially associated with microcephaly and epilepsy, and then with ataxia with oculomotor apraxia type 4.[35] Of note, human mutations should represent hypomorphic alleles, as complete loss of *PNKP* in mice is lethal consistent with the hypothesis that PNKP promotes cell cycle progression and survival.[36] Interestingly, one single patient (homozygous p.Thr408del) was also recently reported with early-onset axonal sensorimotor neuropathy (CMT2-like) followed years later by ataxia without oculomotor apraxia and cerebellar atrophy at brain MRI.[22] The case we describe here is consistent with this last report as clinically presenting with a restricted involvement of the peripheral nervous system but also displaying neuroradiological signs of cerebellar atrophy. The *VRK1* and *PNKP* cases further confirm the relevance of a defined genetic diagnosis to better predict the disease progression and outcome.

We also found novel variants in genes responsible for muscle disorders, which have not previously associated with motor neuropathies or IPNs in general.

Mutations in *KBTBD13* are responsible for autosomal dominant NEM6.[24 37] Here we report a novel homozygous change (p.Ala55Gly) in the *KBTBD13* gene in two siblings from family E with motor neuropathy and exclusion of muscular involvement on the basis of EMG, muscle biopsy and CK (Creatine kinase) levels. Of note, p.Ala55Gly resides within the conserved BTB domain, which mediates the formation of the functional Cullin E3 ubiquitin ligase complex.[38] Consistent with this, we found that the mutated KBTBD13 protein presents defective interaction with Cullin3, thus supporting pathogenicity of this mutation. Moreover, mutations in another Kelch protein, KLHL16 or gigaxonin, is known to be responsible for giant axonal neuropathy.[38] Gigaxonin mutations are thought to result in a generalised disorganisation of the intermediate filaments in the axonal cytoskeleton, as a consequence of defective ubiquitin-proteasome degradation and abnormal protein accumulation.[38] We did not observe abnormal microtubule or intermediate filaments organisation in a sural nerve biopsy, but our patients developed a predominantly motor neuropathy, thus limiting the significance of findings in a pure sensory nerve.

Mutations in *GNE* have been so far reported in hIBM.[25 26] Indeed, at least two out of the three siblings we report here have myopathic features of hIBM in their muscle biopsies. However, they have been considered for a long time to have HMN due to distal muscle atrophy, distal motor deficits and hypopallesthesia in the lower limbs, absent tendon reflexes and neurogenic features on EMG. Muscle biopsy also revealed neurogenic features, such as small angulated fibres, type grouping and clumped nuclei. Unfortunately, we could not investigate the histology of motor nerves as we did not find any nerve fibres in the muscle biopsy samples included in resin for ultrastructural study. Thus, we may only speculate that *GNE* variants detected in our patients may cause both hIBM and HMN. This relies on a number of lines of evidence: (1) although *GNE* myopathy is characterised by distal motor deficits, here we have clinical, histological and neurophysiological findings of lower motor neuron involvement; (2)

neurogenic features in the muscle biopsies have been described in other *GNE* patients[39]; (3) although we are aware that mutations in another gene may be responsible for the neurogenic features of our cases, we did not find by WES any other gene mutation segregating with the disease in this family and (4) homozygous missense mutation in *GNE* (p.His705Arg) has been recently reported in Turkish fALS (familial ALS)[40]; and p.His705 is just next to the p.Tyr706His described in our patient. *GNE* encodes a ubiquitously expressed enzyme involved in the first two rate limiting steps in sialic acid biosynthesis, which is an important component of cell surface glycoproteins, glycolipids, polysaccharides and gangliosides. Reduced enzymatic activity but not GNE expression has been considered the key pathomechanism of the disease.[39] Further potential mechanisms revealed by differentially expressed genes in *GNE* patients and models are related to protein ubiquitination, mitochondrial function, stress response and cytoskeleton organisation, which are common pathogenetic mechanisms also for neurogenic disorders.

Finally, we found variants in the *AGRN* gene in two siblings presenting with indisputable clinical, neurophysiological and histological evidence of CMT2 neuropathy. Mutations in the *AGRN* gene have been described so far in congenital myasthenia,[27] a neuromuscular junction disorder due to defective organisation of the postsynaptic membrane and maintenance of the acetylcholine receptors (AChRs). Agrin is an extracellular matrix heparan sulfate proteoglycan secreted by motor nerve terminals. The specific neuronal isoform (including two alternative spliced exons) induces the activation of the muscle-specific tyrosine kinase receptor (MusK) and its co-receptor lipoprotein-related protein 4 to stabilise the AChR clusters.[41] A short N-terminal form is also secreted into the neuromuscular junction, interacts with α-dystroglycan, but its exact function is unknown. Gene targeting deletion in mice results in complete failure to maintain neuromuscular junctions, and a hypomorphic model due to homozygous point mutation p.Phe1061Ser in the SEA domain of the protein shows muscle denervation resembling motor neuron disease models.[41] Of note, agrin is misplaced in motor neurons of SMA model mice, and its repletion mitigates the disease.[42] Interestingly, compound heterozygous variants in our siblings were also located in the SEA domain, a protein region that is still poorly understood, although associated with protein glycosylation, secretion and cleavage.[41] To confirm damaging prediction of *AGRN* variants in our patients, we performed a direct functional study by showing, as expected, neuromuscular junction dysfunction by sf-EMG consistent with congenital myasthenia. Thus, we believe that this family may represent a widening of phenotypes associated with *AGRN* mutations. Clinical overlapping due to distal weakness distribution between congenital myasthenia, distal myopathies and HMN/CMT2 might underestimate the co-existence of axonal neuropathy in previously reported case series.

In conclusion, in this paper, we identified novel or very rare candidate causal variants in HMN/CMT2 families in genes known to be associated with motor neuron or neuromuscular disorders. Further studies are necessary to confirm these novel associations and to estimate the frequency of mutations in additional HMN/CMT2 cohorts. Moreover, further analyses will be necessary to corroborate the pathogenicity of some variants, particularly in *SIGMAR1* and *ARHGEF28*, for which functional studies were not performed.

Overall, this manuscript increases the allelic variants responsible for HMN and suggest caution in the estimation of prognosis when clinicians are seeing patients with genetically undiagnosed HMN.

Finally, this manuscript confirms the concept that results from high-throughput sequencing techniques, such as WES and NGS panel analyses, must be carefully interpreted in the context of the clinical findings and whenever possible with the support of functional studies (figure 5).

Acknowledgments

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Table 1

Candidate variants identified in this study

Gene	Mutation cDNA	Protein	Patient	GnomAD NFE AF	CADD	GERP	Reference	ACMG class
SIGMAR1	c.412G>A	p.Glu138Lys	A: II,1; II,2	0	28.3	4.7	–	4
SIGMAR1	c.352+1G>A	Splicing, p.?	Pt 1	0	28.2	5.04	–	4
ARHGEF28	c.1424G>C	p.Arg475Thr	B: II,1; II,2	9.72E−06	22.7	6.07	–	3
ARHGEF28	c.4374T>G	p.Cys1458Trp	B: II,1; II,2	0	13.55	−7.95	–	3
ARHGEF28	c.4647G>A *last nucleotide of exon*	p.Glu1549Glu	Pt 2	0.0001599	4.691	4.19	–	3
ARHGEF28	c.5122C>G	p.Leu1708Val	Pt 2	0	17.84	2.97	–	3
VRK1	c.961C>T	p.Arg321Cys	C: II,1; II,2	4.49E−05	24.6	5.13	[20]	3
PNKP	c.1295_1298+6del	Splicing, p.?	D: II,1; II,2; II,3	5.68E−05	33	3.41	–	5
PNKP	c.148C>G	p.Gln50Glu	D: II,1; II,2; II,3	8.96E−06	26.1	5.43	–	4
KBTBD13	c.164C>G	p.Ala55Gly	E: II,2; II,3	0	12.96	4.6	–	4
GNE	c.2116T>C	p.Tyr706His	F: II,1; II,2; II,3	2.686E−05	27.5	5.57	[28]	4
GNE	c.98A>G	p.Glu33Gly	F: II,1; II,2; II,3	0	23.5	5.13	[29]	4
AGRN	c.2971C>T	p.Leu991Phe	G: III,1; III,2	0.0001373	9.644	1.43	–	4
AGRN	c.3404A>G	p.Gln1135Arg	G: III,1; III,2	0.008326	23.7	4.16	–	4

- CADD scores: scaled CADD scores (Phred like) for scoring deleteriousness.
- GERP scores: GERP conservation score of aminoacidic residues across different species. ACMG (American College of Medical Genetics) guidelines.[43]
- GnomAD, allele frequency aggregated database; NFE, Non-Finnish European.

Table 2

Previously reported and novel disease phenotypes

Gene	Previously reported phenotype	Phenotype in this manuscript
SIGMAR1	ALS/HMN	HMN
ARHGEF28	ALS	CMT2/HMN
VRK1	ALS/SMA with PCH	HMN
PNKP	Ataxia-CMT2/AOA	CMT2
KBTBD13	Myopathy (NEM)	HMN
GNE	Myopathy (hIBM)	hIBM/HMN?
AGRN	Congenital myastenia	CMT2

- ALS, amyotrophic lateral sclerosis; AOA, ataxia oculomotor apraxia; CMT2, Charcot-Marie-Tooth neuropathy type 2; hIBM, hereditary inclusion body myopathy;HMN, distal hereditary motor neuropathy; NEM, nemaline myopathy; PCH, pontocerebellar hypoplasia; SMA, spinal muscular atrophy.

Figure 1
Pedigrees and clinical features of families A–D. (A) Pedigrees of family A–D, where the genotype is reported. Individuals marked with an asterisk were subjected to WES. (B) Leg atrophy, pes cavus and first interosseous muscle atrophy of subject A:II,2. (C) Distal muscle atrophy of sporadic patient with homozygous variant (c.352+1G>A) in *SIGMAR1*. (D) Pes cavus and distal muscle atrophy of subject D:II,1. (E) Brain MRI showing mild cerebellar atrophy in subject D:II,1. WES, whole-exome sequencing.
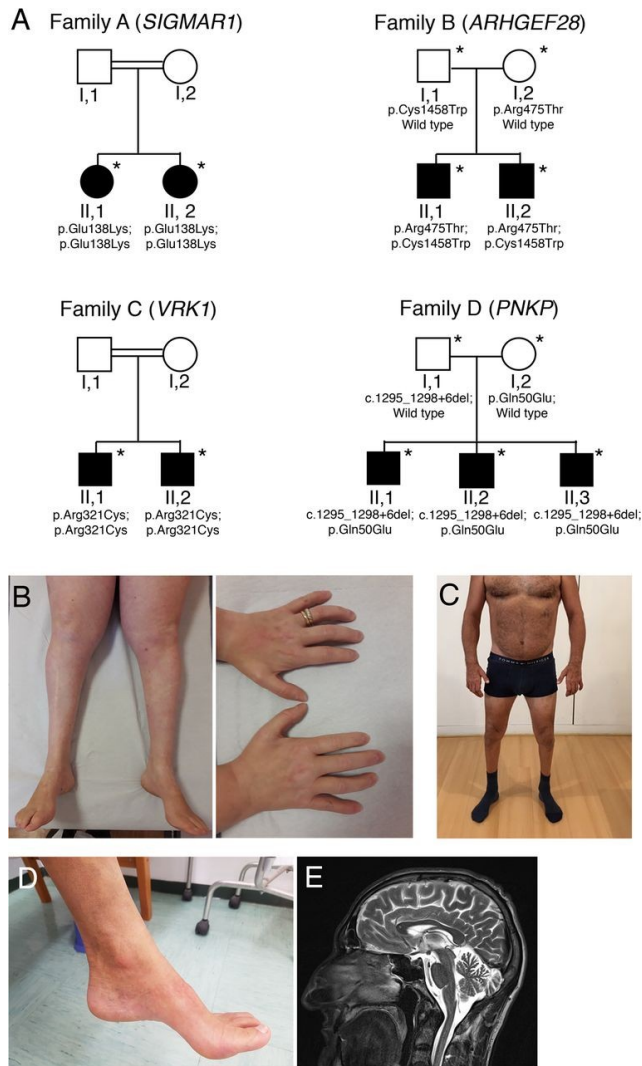
Table 3

Clinical findings and mutations in HMN/CMT2 patients

Pt	Age (years); sex	Onset age	Onset symptoms	Motor deficit	Deformities	Sensory deficit	CNS	Other	Gene	Variant
A:II,1	55;F	18	Gait	dUL and dLL	Pes cavus	Paresthesia	Subtle		SIGMAR1	p.Glu138Lys
A:II,2	61;F	10	Deformities	dUL; pLL and dLL	Pes cavus; clawhand and scoliosis	Paresthesia	No		SIGMAR1	p.Glu138Lys
B:II,1	32;M	2–5	Frequent falls	dLL	Pes equinus; clawhand and scoliosis	No	No	High CK (×3)	ARHGEF28	p.Arg475Thr and p.Cys1458Trp
B:II,2	29;M	2–5	Frequent falls	dLL	Pes cavus and scoliosis	No	No	High CK (×6)	ARHGEF28	p.Arg475Thr and p.Cys1458Trp
C:II,1	44;M	10	Weakness	dUL; pLL and dLL	Pes planum	No	Yes		VRK1	p.Arg321Cys
C:II,2	56;M	30	Weakness	dUL; pLL and dLL	Pes cavus	No	Yes	High CK (×3)	VRK1	p.Arg321Cys
D:II,1	40;M		Asymptomatic		Pes cavus	Hypopallesthesia	No	High CK (×3)	PNKP	c.1295_1298+6 del and p.Gln50Glu
D:II,2	39;M	30	Cramps	dLL	Pes cavus	Hypopallesthesia	No	High CK (×3)	PNKP	c.1295_1298+6 del and p.Gln50Glu
D:II,3	30;F		Asymptomatic		Pes cavus	Hypopallesthesia	No		PNKP	c.1295_1298+6 del and p.Gln50Glu
E:II,2	65;F	29	Gait	dLL	Pes cavus	No	Yes		KBTBD13	p.Ala55Gly
E:II,3	64;M	30	Gait	dLL	Pes cavus	No	Yes		KBTBD13	p.Ala55Gly
F:II,1	53;M	36	Weakness	dLL and pLL	Pes equinus	Hypopallesthesia	No		GNE	p.Glu33Gly and p.Tyr706His
F:II,2	51;M	s30	Weakness	pUL; dUL; pLL and dLL	Pes equinus	Hypopallesthesia and hypoesthesia	No		GNE	p.Glu33Gly and p.Tyr706His
F:II,3	49;M	30	Weakness	dLL	Pes cavus		No		GNE	p.Glu33Gly and p.Tyr706His
G:III,150	;F	2–5	Clumsy gait and falls	dLL; pLL and dUL	Pes cavus; clawhand and scoliosis	Hypopallesthesia and hypokinaesthesia	No	Nystagmus	AGRN	p.Leu991Phe and p.Gln1135Arg
G:III,234	;M	2–5	Clumsy gait and falls	dLL; pLL and dUL	Pes cavus and clawhand	Hypopallesthesia and hypokinaesthesia	Yes		AGRN	p.Leu991Phe and p.Gln1135Arg

- CMT2, Charcot-Marie-Tooth neuropathy type 2; HMN, distal hereditary motor neuropathy; Pt, patient; dLL, distal lower limbs; dUL, distal upper limbs; pLL, proximal lower limbs; pUL, proximal upper limbs.

Table 4

Neurophysiological findings

Patient		Sural		Peroneal		Median/ulnar		Median/ulnar		EMG
		SNAP (uV)	SNCV (m/s)	dCMAP (mV)	MNCV (m/s)	SNAP (uV)	SNCV (m/s)	dCMAP (mV)	MNCV (m/s)	
Fam A *SIGMAR1*	II,1	NR	NA	NR	NA	2.5 (Uln)	48	0.4 (Uln)	23	Neurogenic
	II,2	28	41.2	4.3	33.1	52 (Uln)	63.8	1.6 (Uln)	65	Neurogenic
Sporadic *SIGMAR1*	Pt 1	23	45	0.2	44	20 (Uln)	53	1.0 (Uln)	46	Neurogenic
Fam B *ARHGEF28*	II,1	ND	ND	NR	NA	0.51 (Med)	74.4	0.3 (Med)	42.1	Neurogenic
	II,2	ND	ND	NR	NA	0.89 (Med)	59.6	0.1 (Med)	45.4	Neurogenic
Sporadic *ARHGEF28*	Pt 2	ND	ND	0.4	36.6	12 (Uln)	44	3.5 (Uln)	39.6	Neurogenic
Fam C *VRK1*	II,1	44	47	7.2	39.4	27 (Uln)	47.1	14.6 (Uln)	54.3	Neurogenic
	II,2	ND	ND	7.7	41.2	ND	ND	13.3 (Uln)	52.7	Neurogenic
Fam D *PNKP*	II,1	NR	NA	3.3	40.3	NR (Med)	NA	12.4 (Med)	53.3	Neurogenic
	II,2	NR	NA	1.6	33.2	4.4(Med)	40.3	15.8 (Med)	52.7	Neurogenic
	II,3	2.3	41.3	9.3	46.9	22 (Med)	53.7	8.9 (Med)	57	Neurogenic
Fam E *KBTBD13*	II,2	7.2	48.3	0.9	40.5	31.4 (Med)	61.1	8.5 (Uln)	55.6	Neurogenic
	II,3	2.7	40	0.4	41	20.8 (Uln)	53.8	5.1 (Uln)	57.8	Neurogenic
Fam F *GNE*	II,1	2.7	44.1	1.2	45.2	45 (Med)	50	12.6 (Uln)	58.9	Neurogenic and myopathic
	II,2	6.4	50	0.4	46.3	26.9 (Med)	65.8	12.2 (Uln)	59	Neurogenic and myopathic
Fam G *AGRN*	III,1	NR	NA	NR	NA	NR (Uln)	NA	8.0 (Uln)	57	Neurogenic
	III,2	NR	NA	NR	NA	NR (Uln)	NA	3.5 (Uln)	56	Neurogenic

- EMG, electromyography; Fam, family; MNCV, motor nerve conduction velocity; NA, not applicable; ND, not determined; NR, no response; Pt, patient; SNAP, sensory action potential; SNCV, sensory nerve conduction velocity; dCMAP, distal compound muscular action potential.

Figure 2

Pedigree, morphology and functional studies in family E. (A) Pedigree of family E, where individuals marked with an asterisk were subjected to WES. (B) Pes cavus of patient E:II,2. (C) Sural nerve biopsy of patient E:II,2 showing minimal axonal neuropathy. (D and E) Light microscopy of muscle biopsy from patient E:II,2 showing normal histology at H&E and Gomori's trichrome staining. (F) Electron micrograph showing normal representation of sarcomeric structure and Z-line. (G) KBTBD13 structure and identified mutations; in red the mutation identified in family E. (H) Immunoprecipitation of wild type and mutated KBTBD13 transfected in 293 T cells. Lanes (a–c) represent immunoblot for cullin 3 and KBTBD13 (anti-FLAG antibody) in 293 T cell homogenate not transfected (NT) or transfected with wild type or mutated (mut) KBTBD13. Lanes (d–f) display lysates immunoprecipitated (IP) using anti-FLAG antibody showing reduced cullin 3 in the presence of mutated KBTBD13. Lanes (g–i) represent negative controls where lysates were immunoprecipitated using anti-IgG antibody. Bar=300 µm in C, 160 µm in D and E, and 1 µm in F. IgG, immunoglobulin G; WES, whole-exome sequencing.
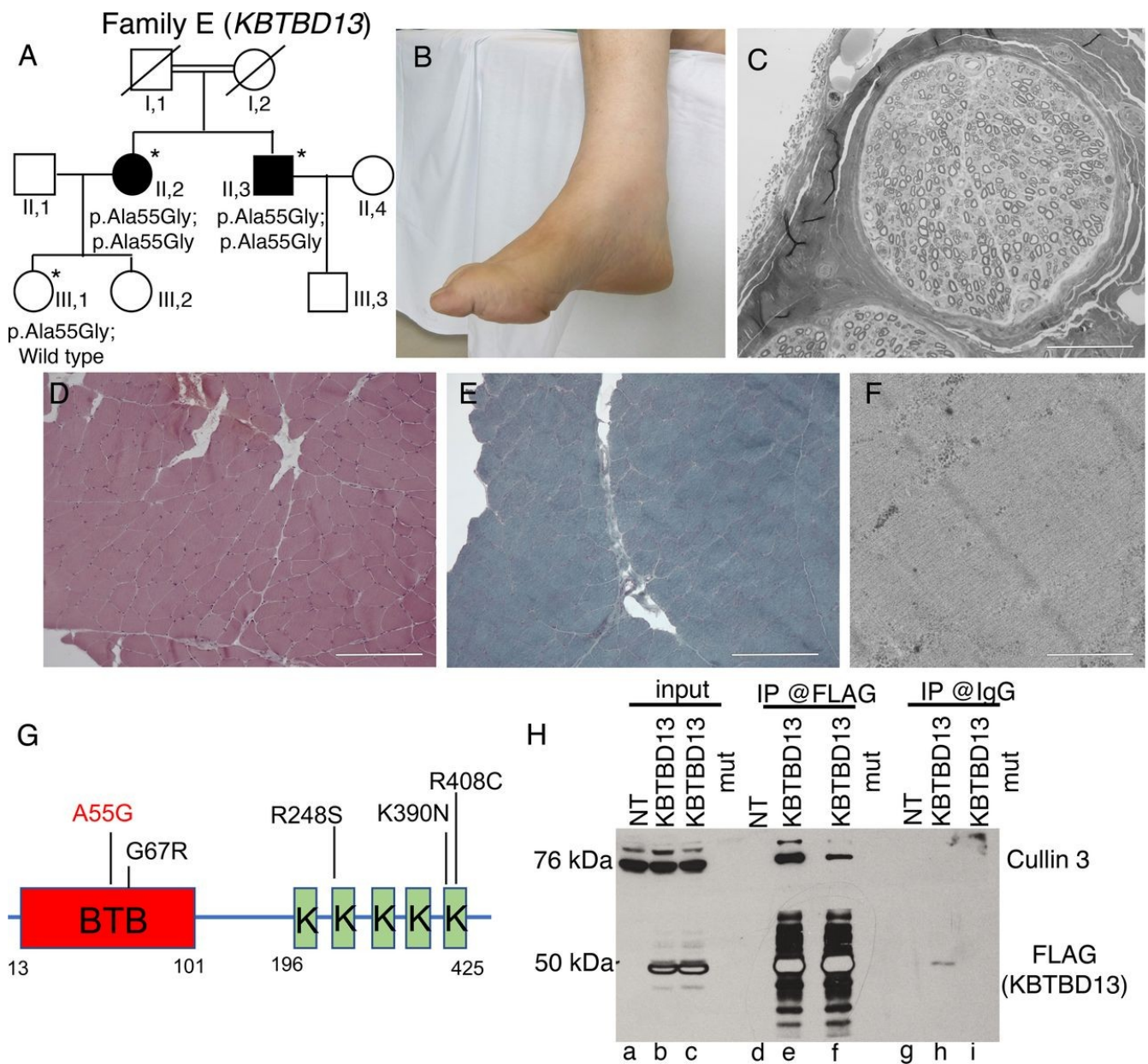
Figure 3

Pedigree and muscle biopsy in family F. (A) Pedigree of family F, where individuals marked with an asterisk were subjected to WES. (B–F) Muscle biopsy of patient F:II,2. (B) H&E showing fibres with centrally located nuclei, angulated atrophic fibres (asterisk), fibres with rimmed vacuoles (arrow) and nuclear clusters (arrowhead). (C) Magnification showing nuclear clusters (arrows). (D) Gomori staining showing rimmed cytoplasmic vacuoles (arrows). (E) ATPase pH 4.6 showing fibre-type grouping (asterisks). (F) Muscle biopsy of patient F:II,1 showing angulated atrophic fibres (arrows). (G and H) Distal muscle atrophy of patient F:II,2. (I) Distal muscle atrophy of patient F:II,1. Bar=50 µm in B; 25 µm in C; 7 µm in D; 25 µm in F and 250 µm in E. Pt, patient; WES, whole-exome sequencing.
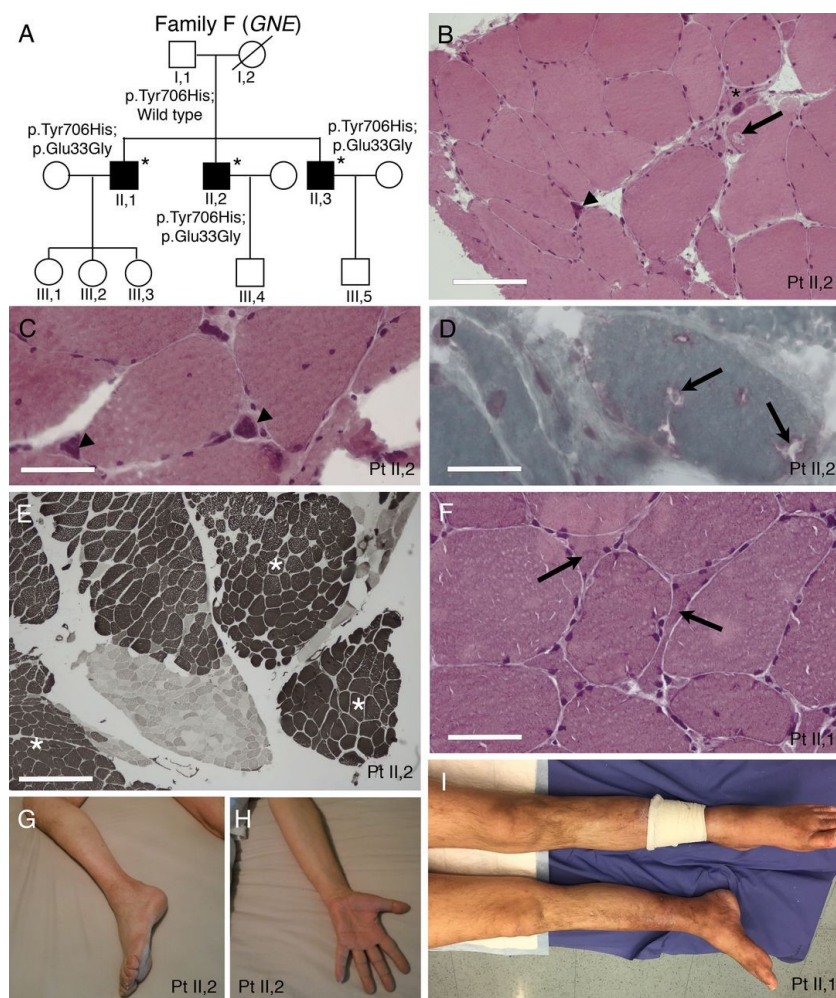
Figure 4
Pedigree, clinical aspect, nerve histology and neurophysiology in family G. (A) Pedigree of family G, where individuals marked with an asterisk were subjected to WES. (B–E) Distal muscle atrophy in patient G:III,1 (B and C) and G:III,2 (D and E). (F and G) Sural nerve biopsy of patient G:III,1 (F) and G:III,2 (G) showing signs of axonal neuropathy. (H) RNS and sf-EMG. The RNS (upper left) showed the typical decremental pattern on repetitive stimulation (3 Hz/10 pulses) observed in postsynaptic failure/defect. The sf-EMG recording in patient G:III,1 (a sample in the upper right panel) showed increase jitter and blocking; the sf-EMG recordings in patient G:III,2 (two samples in the lower left and right panel) showed increased jitter, fibre density and 'late' instable fibre (lower panel, right side). Bar=20 µm in F and G. Pt, patient; RNS, repetitive nerve stimulation; sf-EMG, single-fibre electromyography; WES, whole-exome sequencing.
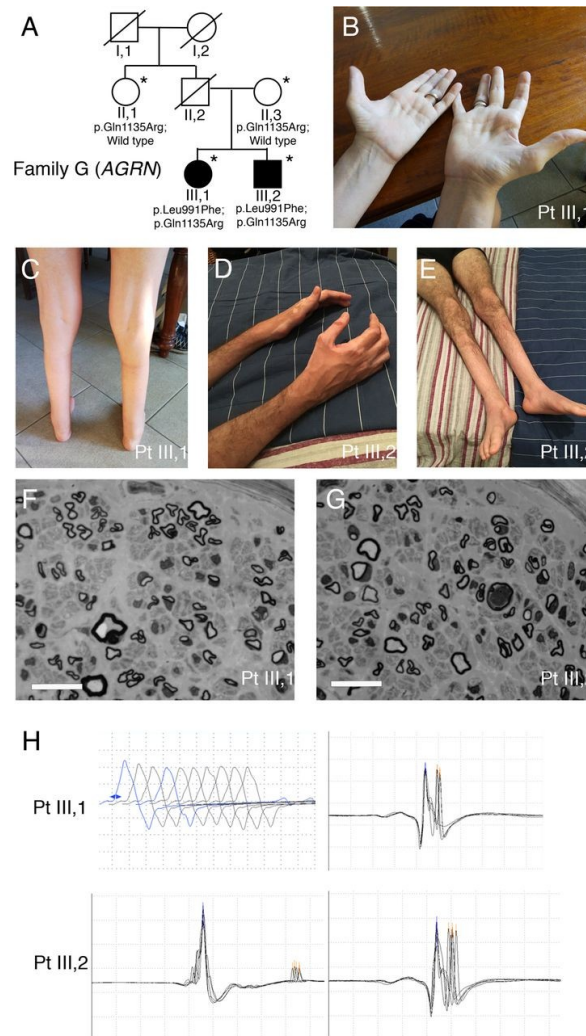
Figure 5
Suggested flowchart for IPN genetic diagnosis. ACMG, American College of Medical Genetics; EMG, electromyography; IPN, inherited peripheral neuropathy; NCV, nerve conduction velocity; NGS, next-generation sequencing.