VARIABLE PHENOTYPES ARE ASSOCIATED WITH PMP22 MISSENSE MUTATIONS
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ABSTRACT

Charcot-Marie-Tooth disease (CMT) is the commonest hereditary neuropathy encompassing a large group of clinically and genetically heterogeneous disorders. The commonest form of CMT, CMT1A, is usually caused by a 1.4 megabase duplication of chromosome 17 containing the PMP22 gene. Mutations of PMP22 are a less common cause of CMT. We describe clinical, electrophysiological and molecular findings of 10 patients carrying PMP22 missense mutations. The phenotype varied from mild hereditary neuropathy with liability to pressure palsies (HNPP) to severe CMT1. We identified six different point mutations, including two novel mutations. Three families were also found to harbour a Thr118Met mutation. Although PMP22 point mutations are not common, our findings highlight the importance of sequencing the PMP22 gene in patients with variable CMT phenotypes and also confirm that the PMP22 Thr118Met mutation is associated with a neuropathy albeit with reduced penetrance.
**Introduction**

Charcot-Marie-Tooth (CMT) disease is the commonest hereditary neuropathy with a prevalence of 1:2500 [1]. CMT is a clinically and genetically heterogeneous disorder characterized by distal muscle wasting and weakness, skeletal deformities and distal sensory loss [2]. CMT is classified on the basis of neurophysiology into CMT type 1 (demyelinating; upper limb motor conduction velocity <38 m/s) and type 2 (axonal; upper limb motor conduction velocity > 38 m/s) [2]. The commonest cause of CMT1 is a 1.4 Mb duplication of chromosome 17p11.2 containing the gene encoding Peripheral Myelin Protein 22 (PMP22) (CMT1A) [3,4]. A deletion of the same region causes hereditary neuropathy with liability to pressure palsies (HNPP) [5]. Point mutations in PMP22 gene are less common (1-5% of all CMT) [6-10] and they may cause either CMT1 or HNPP [11].

So far 44 single base substitutions (37 missense, four nonsense, three silent), 14 deletions and two insertions have been described within the coding region of PMP22 (exons 2-5); furthermore one reciprocal translocation [12] as well as a few splice-site mutations and several single base substitutions in the non coding exon 1A and 3’ UTR have been reported (online supplementary table) ([http://www.molgen.ua.ac.be/CMTMutations/](http://www.molgen.ua.ac.be/CMTMutations/)).

The CMT phenotypes described with missense mutations are often more severe than classical CMT1A [6, 13-18]. Motor nerve conduction is usually slower and occasionally cranial nerve abnormalities are observed [15,19]. The majority of PMP22 missense mutations are transmitted in an autosomal dominant fashion except for the Arg157Trp variation that has been identified in a homozygous state [20], the Arg157Gly variation that has been identified in a hemizygous state [10] and a Thr118Met mutation that has been found in heterozygous unaffected individuals [21] as well as in heterozygous affected patients with a mild form of a HNPP-like neuropathy [22]. Homozygosity for Thr118Met has also been found in a patient causing severe motor axonal neuropathy [22].
Herein we describe the clinical, electrophysiological and molecular findings of eight families affected by various CMT phenotypes carrying six different point mutations in the \textit{PMP22} gene, two of which are novel. Among these families three were found to harbour a Thr118Met mutation.

\textbf{Patients and methods}

\textit{Patients}

Ethical approval was obtained from the joint medical and ethics committee at The National Hospital for Neurology and Neurosurgery to perform this study (UCLH 99N103).

In this study, we performed genotype/phenotype correlations in 10 patients from eight kindreds with \textit{PMP22} missense mutations. Clinical, neurophysiological and molecular examinations were performed in all patients. Patients were examined by Drs MMR, MPTL and DLHB.

Nerve conduction studies were performed using standard techniques. Sensory conduction in the upper limbs was performed orthodromically and in the lower limbs antidromically. A muscle belly to tendon arrangement of recording electrodes was used for motor response recordings with the motor nerve being stimulated at two sites, one distal and one proximal. A sural nerve biopsy and a vastus lateralis muscle biopsy were performed in one patient.

\textit{Molecular genetics}

DNA was extracted from peripheral blood lymphocytes for sequencing of the 4 coding exons of \textit{PMP22} (exons 2-5; primer sequences available on request). Following PCR amplification,
DNA was sequenced with the BigDye Terminator kit v1.1 (Applied Biosystems). Capillary electrophoresis was performed on an Applied Biosystems 3730XL genetic analyser.

352 control chromosomes were screened for *PMP22* mutations. The chromosome 17 duplication/deletion was excluded in all families by performing quantitative fluorescent microsatellite marker analysis, which detects copy number changes within the commonly duplicated or deleted region of chromosome 17p11.2. PMP22 duplication/deletion was excluded in the index case of family 7 by multiplex ligation-dependent probe amplification (MLPA), which is sensitive to detect single exon rearrangements of PMP22 [23].

DNA was not available from the parents of the sporadic patients to determine if the mutations were de novo.

**Results**

*Clinical and neurophysiological findings*

The detailed clinical features of 10 affected patients belonging to eight families are summarized in Table 1. Early onset was observed in all but two patients with the Thr118Met substitution, whose symptoms started at the age of 55 and 68. The majority of patients presented with either delayed walking or difficulties in running. Two patients were floppy at birth. The phenotype was variable among the families and within the same kinship and varied from severe weakness and sensory loss involving distal and proximal muscles to mild distal weakness and sensory loss. Four out of 10 had a typical HNPP phenotype (Table 1). Charcot-Marie-Tooth Neuropathy Score (CMTNS) [24] varied from 8 to 32 in patients with CMT1 (not relevant in HNPP patients). Three patients were wheelchair dependent.

Neurophysiological results are detailed in Table 2.
Detailed phenotypes of the patients carrying the two novel mutations and the Thr118Met mutation are described below (Figure 1, Tables 1 and 2).

Novel mutations

Family 1

A 36 year old man (IV-4) developed symptoms in the first decade with high foot arches and clumsy gait. By the age of 16 he noticed pain in the soles of his feet while walking and he developed slowly progressive numbness in his hands. These symptoms remained stable during the last two decades. Examination at the age of 35 showed mild distal wasting of upper and lower limbs, pes cavus and clawed toes. He had mild weakness in distal muscles of all four limbs (MRC grade 4/5). Reflexes were present except for the ankle reflexes. Sensory examination showed reduction of pinprick sensation below the knees and vibration below ankle.

Nerve conduction studies (NCS) were compatible with a demyelinating neuropathy, showing slowing of motor conduction velocities in the upper limbs (ranging from 31 to 41 m/s) and mild reduction of compound muscle action potentials (CMAPs). Sensory nerve action potentials (SAPs) were absent. (Table 2)

Because of the family history and the nerve conduction results (Fig. 1, Family 1) the clinical diagnosis was dominant intermediate CMT. Sequencing of GJB1 and MPZ genes were negative. Sequencing of the PMP22 gene revealed a novel mutation Ser131Cys (c.392C>G).

His mother (III-3) had a similar phenotype (Table 1) and carried the same PMP22 mutation. A maternal first cousin (IV-2), presented with a phenotype compatible with HNPP. She is a 40 year-old woman whose presenting symptom was difficulty running in the first decade of life. Over several years she developed progressive walking difficulties especially on uneven surfaces and transient tingling accompanied by occasional positional weakness in her feet or hands (eg. crossing her legs, driving). She also has a diagnosis of Ehlers Danlos syndrome.
type III, resulting in lax joints. At the age of 38, she had pes cavus with clawed toes, minimal wasting of first dorsal interossei (FDIO) and abductor pollicis brevis (APB) in both hands and minimal wasting of extensor digitorum brevis (EDB) and tibialis anterior (TA) in the legs. She was not able to stand on her heels. She had mild weakness (MRC grade 4) in FDIO on both sides, APB on the left and ankle dorsiflexion bilaterally. Pinprick sensation was reduced to a level just above the ankle.

NCS showed a patchy demyelinating motor peripheral neuropathy with normal sensory conduction compatible with the diagnosis of HNPP (Table 2). She also carried the same PMP22 point mutation Ser131Cys (c.392C>G). This mutation was not found in 352 control chromosomes.

Family 2

This 33 year old woman (II-2) had delayed motor milestones. She developed progressive lower limb weakness in the first decade that led to loss of ambulation by the age of 14 years. She also had progressive difficulties in fine movements of her hands. She had had long standing moderate deafness since early childhood but this had not been progressive. There was no family history. The parents were not consanguineous and they were apparently unaffected (Fig.1).

On examination at the age of 25, she was wheelchair bound. She had severe kyphoscoliosis with truncal instability. Cranial nerve examination showed horizontal nystagmus and jerky pursuit movements. She had distal wasting in the upper limbs, proximal and distal wasting in the lower limbs with proximal and distal weakness in all four limbs, more pronounced in the lower limbs with absence of movement in almost all muscles examined (Table 1). She was areflexic. Sensory examination showed globally abnormal pinprick sensation. Vibration sense was reduced distally to the elbows and to the anterior superior iliac spine (ASIS). Joint position sense was reduced to wrists and ankle. Brain MRI was normal except for the third
division of the trigeminal nerve that was thickened bilaterally. NCS were compatible with a severe demyelinating motor and sensory neuropathy with a motor response only obtainable in the ulnar nerve (Table 2) demonstrating an extremely prolonged distal motor latency (21 ms) and a very slow motor conduction velocity (3 m/s).

The clinical diagnosis was severe CMT1. Sequencing of GJB1 and MPZ genes were negative. Sequencing of the PMP22 gene revealed a novel mutation Met69Arg (c.206T>G). This mutation was not found in 352 control chromosomes.

Th118Met mutation

Family 6

A 63 year old gentleman (III-1) noticed progressive wasting of the small muscles of the hands at the age of 55 years. Four years later he developed acute onset pain in his neck radiating to the right arm for which he was treated by a chiropractor with sustained pressure applied to his brachial plexus. This relieved his pain but immediately after the treatment he developed right hand weakness and difficulty lifting his right arm. A diagnosis of diabetes type II was also made during this period. At the age of 61, he had severe wasting and weakness of the right deltoid, infraspinatus and supraspinatus, wasting, and asymmetric weakness of FDIO bilaterally that was more pronounced on the right. Reflexes were reduced in the right upper limb and were absent in the lower limbs. Pin prick was reduced in the ulnar distribution on the right and on the toes of the right foot. MRI of the cervical spine and brachial plexus showed signs of cervical spondylosis and thickened cervical roots and brachial plexus. NCS showed reduced SAPs in upper and lower limbs. Ulnar MAPs were absent on the right. Distal motor latencies in the upper limbs were prolonged with mild slowing of the motor conduction velocities (Table 2).

The clinical diagnosis was HNPP. Sequencing of GJB1 and MPZ genes were negative. Sequencing of the PMP22 gene revealed the Thr118Met (c.353C>T) mutation.
His father died at the age of 91 and had wasting of the small muscles of the right hand for which he had never been investigated. An unaffected sister was tested for the same mutation and found negative.

Family 7
A 41 year-old woman (F8 III-1) presented at the age of 37 years with a severe early onset predominantly sensory neuropathy. She was reported to have frequent falls at an early age. During her first decade she was unsteady and developed foot drop. These symptoms slowly progressed with worsening of the unsteadiness, especially in the dark, and progressive weakness of the hands. In her family her parents and sibling were unaffected. On examination she had pes cavus and clawed toes, she could not stand on her heels, she had an ataxic gait and a positive Romberg’s sign. She had bilateral ptosis, minimal facial weakness and lip and chin myokymia. She had also pale discs, but she had normal visual acuity, normal visual field by confrontation and normal ocular movements. She had moderate distal wasting and weakness in the upper limbs. In the lower limbs she had weakness of knee flexion (MRC grade 4) and ankle dorsiflexion (MRC grade 3) but all the other muscles were spared. She had pseudoathetosis. Pinprick and vibration sensation were reduced throughout the body, joint position sense was reduced to the wrists and knees. She was areflexic. NCS showed absent sensory responses in upper and lower limbs. CMAPs and motor nerve conduction velocities in the upper and lower limbs were markedly reduced, although the peroneal nerve conduction velocity when recorded from the proximal muscles was preserved (43 m/s). These features were suggestive of generalized demyelinating sensorimotor neuropathy, although the non-uniform slowing of the conduction velocities was rather atypical for classical CMT1 and initially raised the possibility of an inflammatory component.
Visual evoked potentials (VEPs) and brain MRI were normal. A CSF examination showed slightly raised protein at 0.74 g/L with no cells and no oligoclonal bands. Because of the rather atypical features, muscle and nerve biopsies were performed to rule out an inflammatory component or a mitochondrial disease. The muscle biopsy showed features of previous denervation with reinnervation and no features to support a mitochondrial disease. Respiratory chain enzyme complex activities were normal in the muscle. The sural nerve biopsy showed almost complete loss of myelinated fibres with only very occasional thinly myelinated fibres (Fig.2).

Sequencing of the *PMP22* gene revealed the mutation Thr118Met (c.353C>T). The severity of her presentation and the marked sensory involvement was very atypical for the *PMP22* Thr118Met mutation and a second mutation in another gene was suspected. Subsequent sequencing of GJB1, MPZ, GDAP1, LITAF SPTLC1, RAB7, HSN2, NGFB, Periaxin, EGR2 and NEFL genes were negative. She was also negative for common point mutations and large scale rearrangements of mitochondrial DNA (blood and muscle respectively), and for common mutations in the nuclear encoded DNA polymerase gamma (POLG) gene.

Family 8

The patient is a 72 year-old man with a history of recurrent radial nerve palsy. He first complained of a right wrist drop, after waking up, at the age of 68 years. Symptoms resolved completely after few weeks. After one month he had another episode of left wrist drop after waking up. There was no preceding trauma and no sensory features and the symptoms resolved completely after one month. At the age of 72 years, he noticed numbness on the dorsum of the left hand followed by left wrist and finger drop. It was not preceded by injuries or abnormal postures. The symptoms resolved over few weeks. There was no family history. Neurological examination was normal, except for absent ankle reflexes. Nerve conduction studies and EMG, performed at the age of 68 years, showed a moderately severe left radial
nerve palsy with evidence of axonal degeneration and partial re-innervation. The clinical diagnosis was of HNPP. Sequencing of PMP22 gene detected the mutation Thr118Met (c.353C>T).

The Thr118Met mutation was also found in 2 out 352 control chromosomes, a similar frequency to other reports suggesting reduced penetrance of this mutation [21,25].

Discussion

We describe 10 patients belonging to eight families who carry six missense mutations in the PMP22 gene. Five patients are sporadic and in five patients the neuropathy is transmitted in an autosomal dominant fashion. The phenotypes observed varied from mild HNPP to severe CMT1. Among the mutations, two mutations are novel (Ser131Cys, Met69Arg), three have been previously reported [11,15,17] and one is the Thr118Met mutation [21,22,25].

The Ser131Cys mutation is present in heterozygous state and has not been previously reported. It has been identified in all three affected patients and has not been found in one unaffected family member (Fig. 1, F1 III-4). This segregation of the mutation with the disease in the family suggests that the mutation is pathogenic and we have not seen it in 352 control chromosomes. The phenotype observed varied within this kinship: one patient had a HNPP-like neuropathy whereas two other family members had mild form of CMT with intermediate nerve conduction velocities best classified as intermediate CMT and no symptoms suggestive of HNPP. Intermediate CMT associated with a PMP22 mutation has been reported in only one patient with motor NCV of 39 m/s [26] although conduction velocities in the intermediate range have been described with HNPP secondary to PMP22 point mutations [22]. The patient with the HNPP phenotype in our kindred also had Ehlers Danlos type III and we speculate that her joint laxity combined with this PMP22 mutation may have made her more likely to get pressure palsies. The second novel mutation
Met69Arg was identified in a sporadic patient (F2 II-2) and causes a severe CMT1 phenotype. No other family members are affected to allow for segregation analysis and this mutation may be a de-novo change, we have not detected this mutation in 352 control chromosomes and it has not been reported in the past. This substitution also involves a change from a hydrophobic aminoacid (Met) to a positive charged polar aminoacid (Arg) and it occurs in codon 69 which is conserved among species making it more likely to be pathogenic. A different aminoacid substitution involving the same codon has been reported in a patient with severe CMT1 by Roa et al [27] supporting the pathogenicity of our novel mutation.

Three patients in the present series (F3, F4 and F5) have previously described mutations. In the first we identified the Ser22Phe mutation in an autosomal dominant family with a clinical diagnosis of HNPP. A similar phenotype has already been reported by Kleopa et al in a family with the same mutation [11]. The other two mutations we identified (Ser76Ile and Gly100Glu mutations) were previously reported from our laboratory by Tyson et al (1997) and Marques et al (1998) [15,17]. They are both sporadic and clinically characterized by early onset CMT1 with markedly reduced conduction velocities, a severe disease course and cranial nerve involvement [15,17].

In our series three patients (F6 III-1, F7 III-1, F8 II-1) were found to have the Thr118Met substitution, two patients with HNPP and one with an atypical demyelinating neuropathy. The patients with HNPP have a similar phenotype to that described by Shy et al [22] supporting the pathogenetic role of the mutation although the mutation may be more of a risk factor for disease rather than a fully penetrant mutation. The finding of the Thr118Met mutation in 2 out 352 of our control chromosomes can be interpreted as further evidence for reduced penetrance of this mutation. The patient with the atypical phenotype is unusual. She has not been found to have another cause for her neuropathy to date (including no evidence
for inflammation). It remains likely in our opinion that she will harbour another pathogenic mutation in another gene which we have yet to identify, despite testing a number of likely candidates to date. A more subtle variation in modifying genes might contribute to the atypical phenotype as well.

The phenotypic variability seen with PMP22 missense mutations is of interest and has been commented upon by other authors [17,28]. Various factors may contribute to determine the various phenotypes, such as the type and the site of the aminoacid substitution. The majority of PMP22 mutations previously reported are located in the four transmembrane domains of the protein, although very few mutations are described in the first extracellular loop [16]. To our knowledge, the Ser131Cys mutation reported in this study is the first mutation located in the second extracellular loop [13,29].

In conclusion the phenotype variability described in our series of patients highlights the importance of considering PMP22 mutations in patients with a broad spectrum of phenotypes including HNPP, CMT1 of all severity and intermediate CMT.

Acknowledgements

MMR is grateful to the Medical Research Council (MRC) and the Muscular Dystrophy Campaign for their support. This work was undertaken at University College London Hospitals/University College London, which received a proportion of funding from the Department of Health's National Institute for Health Research Biomedical Research Centres funding scheme. DLHB is a Wellcome Clinical Scientist.
References


**Table 1 Clinical features**

F = family; Pt = patient; I.P. = index patient; mths = months; yrs = years; UL = upper limbs; LL = Lower limbs; Weakness: - normal, + ≥4 in distal muscles, ++ <4 in distal muscles, +++ proximal weakness (knee flexion and extension, elbow flexion and extension, and/or above); Pinprick and vibration sense: - normal, + reduced below wrist/ankle, ++ reduced below elbow/knee, +++ reduced at or above elbow/knee; CNS= central nervous system; CMTNS= Charcot-Marie-Tooth Neuropathy Score; N/AV = Not available; * CMTNS not suitable to be done in patients with HNPP; § also had a diagnosis of Ehlers Danlos syndrome.

**Table 2 Nerve conduction studies**

Age = Age when the test was performed; Motor Amp = amplitude (mV); Sensory Amp = amplitude (µV); DML = distal motor latency (ms); CV = conduction velocity (m/s); abs = absent; NR = not recorded; NA = not available; I.C.= index case; *in a previous study performed at the age of 3 the CV was reported to be 3 m/s; §L radial SAP = 4.8 µV

Normative Values: Median nerve: SAP (Finger III) ≥ 8µV (orthodromic), CV ≥ 50m/s, DML ≤ 4.0 ms, Motor conduction velocity ≥ 50m/s, Compound motor action potential (CMAP) amplitude ≥ 5; Ulnar nerve: Sensory Action Potential (SAP) (Finger V) ≥ 5µV (orthodromic), CV ≥ 50m/s, DML ≤ 3.5ms, Motor conduction velocity ≥ 50m/s, CMAP amplitude ≥ 8mV; Peroneal nerve: DML upper limit of normal ≤ 5.5ms, Motor conduction velocity ≥ 40m/s, CMAP amplitude ≥ 2.5 mV; Sural nerve: SAP ≥ 5 µV, CV ≥ 40m/s. Abnormal values are indicated in bold.
Figure 1 Pedigrees of families identified with novel mutations (families 1 and 2 including sequencing electropherograms) and the Thr118Met substitution (families 6, 7, 8)

An arrow indicates the index case, square a male, circle a female, filled symbol indicates affected, half filled symbol indicates patient reported as having symptoms but not examined or investigated, a line through a symbol indicates the individual is deceased. Where family members have been sequenced, the PMP22 genotype is indicated (/- = normal genotype).

Figure 2 Sural nerve biopsy – Histology and ultrastructure

A, B; Semithin resin section (MBA-BF staining, collagen red, myelinated fibres dark blue). The overview (A) shows a multifascicular nerve with an almost complete, homogenous loss of myelinated fibres. High power magnification of one of the fascicles (B) confirms the subtotal loss of large and small myelinated fibres. Only very rare myelinated axons (1-3 / fascicle) remain. There is no endoneurial oedema.

C, D: Immunohistochemical labelling for the CD8 lymphocyte antigen reveals very few endoneurial T cells (1-3 per fascicle) and minimal presence of CD68 positive macrophages.

E, F: Electron microscopy showing one small myelinated fibre (E) and a demyelinated fibre (F), both surrounded by concentric formation of Schwann cell processes, which contain unmyelinated axons. These structures are commonly referred to as “pseudo-onion bulb” structures, thought to be associated with de and regeneration rather than being characteristic for de and remyelination. Red arrowhead points to the central axon and blue arrow points to the axon that is part of the concentric formation. Green arrow: collagen pouch, another sign of regeneration.

Scale bar: 700μm (A); 50μm (B, C, D), 12μm (E, F)