Genetic dysfunction of \textit{MT-ATP6} causes axonal Charcot-Marie-Tooth disease

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Supplemental data: Supplemental Table 1, 2 and 3 – Table e-1, e-2 and e-3

Search terms: CMT2; MT-ATP6; neuropathy; complex V; mitochondrial disease
ABSTRACT

Objective: Charcot-Marie-Tooth (CMT) disease is the commonest inherited neuromuscular disorder affecting 1 in 2,500 individuals. Mitochondrial DNA (mtDNA) mutations are not generally considered within the differential diagnosis of patients with uncomplicated inherited neuropathy, despite the essential requirement of ATP for axonal function. We identified the mtDNA mutation m.9185T>C in *MT-ATP6*, encoding the ATP6 subunit of the mitochondrial ATP synthase (OXPHOS complex V), at homoplasmic levels in a family with mitochondrial disease in whom a severe motor axonal neuropathy was a striking feature. This led us to hypothesise that mutations in the two mtDNA complex V subunit encoding genes, *MT-ATP6* and *MT-ATP8*, might be an unrecognised cause of isolated axonal CMT and distal hereditary motor neuropathy (dHMN).

Methods: 442 probands with CMT2 (270) and dHMN (172) were screened for *MT-ATP6/8* mutations following exclusion of mutations in known CMT2/dHMN genes. Mutation load was quantified using restriction endonuclease analysis. Blue-native gel electrophoresis (BN-PAGE) was undertaken to analyse the effects of m.9185T>C on complex V structure and function.

Results: Three further probands with CMT2 harboured the m.9185T>C mutation. Some relatives had been classified as having dHMN. Patients could be separated into four groups according to their mutant m.9185T>C levels. BN-PAGE demonstrated both impaired assembly and reduced activity of the complex V holoenzyme.
Conclusions: We have shown that m.9185T>C in *MT-ATP6* causes CMT2 in 1.1% of genetically undefined cases. This has important implications for diagnosis and genetic counselling. Recognition that mutations in *MT-ATP6* cause CMT2 enhances current understanding of the pathogenic basis of axonal neuropathy.

Abbreviations: BN-PAGE = Blue-native polyacrylamide gel electrophoresis; CK = creatine kinase; CMCT = central motor conduction times; CMT = Charcot-Marie-Tooth; CMTES2 = Charcot-Marie-Tooth examination score 2; CMTNS2 = Charcot-Marie-Tooth neuropathy score 2; COX = cytochrome c oxidase; CSF = cerebrospinal fluid; EMG = electromyography; LS = Leigh syndrome; MELAS = mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MERRF = myoclonic epilepsy and ragged red fibres; MRI = magnetic resonance imaging; mtDNA = mitochondrial DNA; NARP = neurogenic muscle weakness, ataxia and neuropathy; NCS = nerve conduction studies; OXPHOS = oxidative phosphorylation; SANDO = sensory ataxic neuropathy, dysarthria, and ophthalmoparesis; SDH = succinate dehydrogenase; UMN = upper motor neuron; Pi = inorganic phosphate.
INTRODUCTION

Mitochondrial ATP generation by oxidative phosphorylation (OXPHOS) underpins key molecular processes that are essential for normal central and peripheral nervous system axonal function. Axonal peripheral neuropathies are a well-recognised complication of primary mitochondrial DNA (mtDNA) mutations; however, the neuropathy is rarely the presenting or predominant clinical manifestation of the disease.\textsuperscript{1–8} In contrast, mutations in the nuclear-encoded mitochondrial genes \textit{MFN2} and \textit{GDAP1},\textsuperscript{10} which encode outer mitochondrial membrane proteins, usually present with isolated peripheral neuropathy, and are now recognised to be important causes of both axonal and demyelinating forms of Charcot-Marie-Tooth (CMT) disease.

One of the major unresolved challenges in neuromuscular diseases is the determination of the genetic cause of inherited axonal neuropathy, such as CMT2 and distal hereditary motor neuropathy (dHMN). We investigated an extended family in which the index case presented with a pure motor neuropathy in childhood evolving into motor-predominant CMT2 in later life. The pathogenic missense mutation m.9185T>C in \textit{MT-ATP6}, encoding the ATP6 subunit of the mitochondrial ATP synthase (OXPHOS complex V), was identified and shown to segregate with disease in affected members of the pedigree. In view of the striking neuropathic features observed in this family, we screened a large cohort of 442 unrelated probands with genetically undefined CMT2 and dHMN for mutations in \textit{MT-ATP6} and \textit{MT-ATP8}, encoding two components of complex V. We show that mitochondrial mutations that impair the function and stability of complex V are an important but previously unreported cause of CMT2.
PATIENTS AND METHOD

Standard protocol approvals, registration and patient consents

The study was approved and performed under the ethical guidelines issued by our institutions for clinical studies, with written informed consent obtained from all subjects for genetic studies.

Patient cohort

We selected a cohort of patients from the Medical Research Council (MRC) Centre for Neuromuscular Diseases inherited neuropathy database. These were patients presenting with a clinical phenotype compatible with either CMT2 (n=270) or dHMN (n=172). Diagnosis was based on clinical and electrophysiological phenotypic evaluation. Patients were excluded from the study if inheritance was consistent with paternal transmission.

Clinical assessment

The authors performed detailed clinical assessments on all patients with pathogenic mutations. Data ascertained when possible included: age of symptom onset; clinical history and examination findings; CMT examination (CMTES2) and neuropathy (CMTNS2) scale, as an indicator of neuropathy severity; nerve conduction studies (NCS) and electromyography (EMG); plasma creatine kinase (CK) and lactate; cerebrospinal (CSF) lactate; central motor conduction times (CMCT); and magnetic resonance imaging (MRI) studies.
Muscle histology and histochemistry

Muscle biopsies were performed following informed consent. Standard histological and histochemical stains were used on cryostat sections as previously described.\textsuperscript{12}

Biochemical studies

Spectrophotometric enzyme assays of mitochondrial respiratory chain complex I (NADH: ubiquinone reductase), complex II+III (succinate: cytochrome c reductase) and complex IV (cytochrome c oxidase) activities were performed and corrected for citrate synthase activities, and Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was used on available muscle tissue as previously described.\textsuperscript{13,14}

Genetic analysis

Total genomic DNA was extracted from peripheral blood leucocytes, cultured skin fibroblasts, urinary tract epithelial cells and muscle tissue using standard extraction protocols. Amplification of fragments for sequencing was performed using specific overlapping primers, Amplitaq Gold 360 mastermix (Applied Biosystems) and BigDye Terminator v.1.1 cycle sequencing kit (Applied Biosystems). The samples were run on a 3730xl DNA Analyzer, assembled and analysed using Seqscape v.2.5 software (Applied Biosystems) and were compared to the rCRS reference sequence (NCBI accession number NC_012920). Full mitochondrial genome sequencing (primers available on request) was performed in one patient (family A, patient III-6). Targeted sequencing of \textit{MT-ATP6/8} was performed in all remaining patients (see Table e-1 for primer sequences). Restriction endonuclease analysis was used to quantitate mutant load in the m.9185T>C positive cases as previously described.\textsuperscript{15} The experiment was performed in triplicate and the mean mutant mtDNA level
calculated. All patients with CMT2 were negative for mutations in *MFN2*. The majority of patients with CMT2 were also screened for mutations in *MPZ, HSPB1, HSPB8, TRPV4* and *GJB1* where appropriate. Patients with dHMN were negative for mutations in *HSPB1, HSPB8, TRPV4*, and selected patients were negative for mutations in *BSCL2* and *GARS*.

**RESULTS**

Disease-causing m.9185T>C mutations were detected in three further unrelated probands (family B, C and D discussed below) from the 270 CMT2 index cases analysed, representing a specific mutation frequency for this variant in *MT-ATP6* of 1.1%. No pathogenic *MT-ATP6* variants were found in patients with dHMN (172), and no pathogenic mutations in *MT-ATP8* were found in either the CMT2 or dHMN groups. All synonymous and non-synonymous single nucleotide variants detected in *MT-ATP6* and *MT-ATP8* are outlined in Table e-2. The potential pathogenicity of each variant was evaluated using the following databases: mitowheel (http://mitowheel.org/mitowheel.html); mtDB (http://www.mtdb.igp.uu.se); and HmtDB (http://www.hmtdb.uniba.it:8080/hmdb). One patient with CMT2 had a novel variant m.8828A>G, causing a missense change from Asparagine with Serine at amino acid position 101 of the ATP6 protein (Asn101Ser). Unfortunately, there was no muscle tissue available from the patient for BN-PAGE, thus the potential pathogenic nature of this variant remains uncertain.
Clinical and electrophysiological characteristics of index family harbouring m.9185T>C in \textit{MT-ATP6}

The index case (Figure 1, Table 1 and Table e-3, family A, patient III-8) presented with recurrent falls and foot drop aged 6 years following normal early development. Clinical examination aged 21 years showed distal muscle wasting of the legs, pes cavus, and clawing of the toes (Figure 2). Formal manual muscle strength testing (MRC graded) was normal in the upper limbs with mild proximal lower limb weakness (hip flexion 4+ bilaterally) and moderate distal lower limb weakness (ankle dorsiflexion 3, plantarflexion 4+, inversion 5, and eversion 2 bilaterally). Ankle jerks were absent. Plantar responses were extensor. Pin prick sensation was normal but vibration detection was reduced to the knees. Mitochondrial DNA sequencing in an older sibling (Figure 1, family A, patient III-6) revealed m.9185T>C, a homoplasmic (i.e. mutant load 100%) pathogenic mutation in \textit{MT-ATP6}, causing a missense change from leucine to proline at amino acid position 220 of the ATP6 protein (Leu220Pro). Presence of the m.9185T>C mutation was confirmed in the index case and was shown to segregate with disease within the family. All affected individuals were homoplasmic, whilst the mutational load was present at much lower levels in unaffected relatives (Figure 1, family A and Table e-3). Clinical features in addition to a pure motor/motor-predominant axonal neuropathy included: learning difficulties (patients II-3, II-5, III-4, III-5, III-6, III-7, III-8, IV-1 and IV-2); sensorineural hearing loss (patient III-7); and retinal degeneration (patient III-8). Early proximal lower limb weakness was evident in three patients despite a relatively mild neuropathy (patients III-6, III-7 and III-8). Two patients suffered rapid decompensation following a febrile illness (patient III-5 and patient IV-1). The available clinical data suggests a sudden onset Leigh-like illness, with cortical blindness documented in one patient (patient III-
5). Electrophysiologically the neuropathy was a pure motor neuropathy/neuronopathy in three individuals (patients II-1, III-7 and III-8); however, sensory signs have since developed in one patient, making the diagnosis clinically compatible with motor-predominant CMT2 (patient III-8). The neuropathy was particularly severe in two adults with wheelchair dependence in the third decade (patient II-3 and patient III-4).

**Clinical and electrophysiological characteristics of the three further families harbouring m.9185T>C in MT-ATP6**

**Family B** (Figure 1, Table 1 and Table e-3): Patients with CMT2 (patients II-8, III-6, and IV-3, all with 100% mutant load) presented in their first and second decades with typical features of inherited neuropathy. Patient III-6 had a pure motor neuropathy/neuronopathy electrophysiologically aged 29; but repeat studies aged 45 revealed reduced sensory nerve action potentials compatible with motor-predominant CMT2. Despite a gradually progressive clinical course, two patients suffered a rapid decline in mobility in their fifth and sixth decades with wheelchair dependence from unaided walking over a 5-year period (patients II-8 and III-6). In these severe cases there was upper limb and proximal lower limb weakness without evidence of myopathy on EMG; however, early proximal lower limb weakness was also present in a mildly affected individual (patient IV-3) without upper limb involvement.

**Family C** (Figure 1, Table 1 and Table e-3): Symptom onset was in the first and second decades (patients III-13 and IV-2, both with 100% mutant load). Electrophysiologically patients with homoplasmic mutant loads had a pure motor
neuropathy/neuronopathy (patient IV-2) or sensorimotor axonal neuropathy (patient III-13). Those with lower mutant levels were asymptomatic (patient III-5 with 75% mutant load and patient IV-1 with 45% mutant load). Patient IV-2 had a severe neuropathic phenotype associated with learning difficulties, behavioural problems and wheelchair dependence by early adulthood. Patient III-13 had motor-predominant CMT2 with pyramidal tract signs and proximal muscle weakness which developed in later life.

**Family D** (Figure 1, Table 1 and Table e-3): Unlike family A, B and C, patient III-1 appeared to be a sporadic case of motor-predominant CMT2 (92% mutant load). He presented in the first decade with recurrent ankle sprains and falls and was diagnosed with CMT2 aged 11 years. NCS demonstrated a length-dependent sensorimotor axonal neuropathy and EMG showed proximal and distal lower limb denervation with no evidence of myopathy.

**Laboratory, muscle histological and biochemical findings in patients harbouring m.9185T>C in MT-ATP6**

Investigations for all four families are summarised in Table 1 and Table e-3. BN-PAGE (Figure 3) was performed on four muscle samples and revealed both impaired assembly (demonstrated by multiple bands indicative of abnormal assembly intermediates) and/or activity (demonstrated by reduced band intensity) of complex V when patients were compared with control muscle tissue (family A, patients III-5, III-6, IV-2 and family B, patient III-6).
Quantitation of m.9185T>C mutant load

Quantitation of the m.9185T>C mutant load showed segregation with disease severity and phenotype (Table 1 and Table e-3), and allowed patients to be broadly classified into four clinical groups according to m.9185T>C mutant load: (Group 1) unaffected individuals (<64% mutant load); (Group 2) asymptomatic individuals with upper motor neuron (UMN) signs detectable on examination only (64-79% mutant load); (Group 3) affected individuals with clinical symptoms and signs of UMN involvement without neuropathy (80-91% mutant load); (Group 4) affected individuals with symptoms and signs of motor-predominant CMT2 (92-100% mutant load).

DISCUSSION

In our genetically undefined CMT2 cohort we identified the disease-causing MT-ATP6 m.9185T>C variant in three unrelated probands in addition to the index family in which we originally found the mutation. Some affected family members were also classified as having dHMN. That 1.1% of our genetically undefined CMT2 cohort harboured the m.9185T>C mutation in MT-ATP6 is important given that molecular defects are currently only detected in approximately 35% of patients with CMT2 and in 15% of patients with dHMN. Patient m.9185T>C mutant load correlated with disease severity within each pedigree.

It is notable that all of the patients with m.9185T>C had previously been assessed and considered to have a CMT2 phenotype based on clinical and electrophysiological evaluation by experienced clinicians. All but one of these cases was homoplasmic for the m.9185T>C mutation. Although each of the pedigrees was compatible with matrilineal transmission, the underlying mitochondrial basis for
disease had not been considered because of the isolated peripheral nerve-specific phenotype in the absence of multisystem involvement.

We were able to broadly divide patients into four clinical severity-related groups according to their m.9185T>C mutant load: (Group 1) unaffected individuals (<64% mutant load); (Group 2) asymptomatic individuals with upper motor neuron (UMN) signs detectable on examination (64-79% mutant load); (Group 3) affected individuals with clinical symptoms and signs of UMN involvement without neuropathy (80-91% mutant load); (Group 4) affected individuals with symptoms and signs of motor-predominant CMT2 (92-100% mutant load).

The m.9185T>C variant was originally reported in a seven year-old patient who developed sudden onset ptosis, ophthalmoparesis and fatigue.\textsuperscript{20} Although isolated axonal neuropathy is documented at 85% m.9185T>C heteroplasmy, higher mutant loads have been reported to cause a more severe neurological phenotype, including neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) and early and late-onset Leigh syndrome.\textsuperscript{15,20–22} Families B, C and D exhibit surprising tissue-specificity of disease expression considering they had homoplasmic m.9185T>C mutant levels detected in blood, urine, skin and muscle.

BN-PAGE was used in muscle tissue to demonstrate the deleterious effects of the m.9185T>C; p.Leu220Pro mutation on complex V structure and function. The complex V assay measures the ATP synthase activity in the reverse direction. ATP is hydrolysed to produce ADP and inorganic phosphate (Pi). In the presence of Pi the lead ions used in the buffer (lead (II) nitrate) form a precipitate which correlates
with the degree of ATP hydrolysis. This technique has not been used to assess complex V in patients with m.9185T>C previously. It has been shown that the m.8993T>G mutation associated with LS and NARP not only impairs assembly and stability of complex V, but also reduces intrinsic activity of the ATP synthase holoenzyme.\textsuperscript{23} We confirmed impaired complex V assembly (Figure 1, family A, patients III-6 and IV-2, Table e-3 and Figure 3), reduced complex V activity (Figure 1, family B, patient III-6, Table e-3 and Figure 3) or both (Figure 1, family A, patient III-5, Table e-3 and Figure 3) in patients who were homoplasmic for m.9185T>C. These data support the pathogenicity of m.9185T>C and indicate that this mutation impairs both complex V assembly and activity. In view of the previously published data documenting impaired assembly and stability of complex V associated with the NARP m.8993T>G mutation,\textsuperscript{23} the m.9185T>C mutation may also be expected to result in a poorly assembled and unstable complex V holoenzyme. The variation in both complex V activity and assembly observed on BN-PAGE for patients with identical m.9185T>C mutation loads may, therefore, be attributable to holoenzyme disassembly occurring during sample preparation. In our patients with the m.9185T>C mutation, muscle histology and histochemistry were either normal or exhibited subtle non-specific mitochondrial abnormalities on electron microscopy only, as is frequently the case for mutations involving mtDNA protein encoding genes.

It is unclear why some patients harbouring homoplasmic levels of m.9185T>C should develop a tissue-specific peripheral neuropathic phenotype, whilst other reported patients and families with similar mutant loads develop a multi-system neurological syndrome such as LS. Variable clinical severity in patients with similar mutant loads has been reported with a number of known pathogenic mtDNA mutations. It is
possible this variability relates to subtle differences in tissue heteroplasmy; however, no correlation has been shown between the presence of neuropathy and muscle m.3243A>G mutant levels despite the disease phenotype being more severe in the patients with neuropathy.\textsuperscript{6} This finding suggests there may be additional factors that increase the susceptibility of certain patients to neuropathy, such as activation of nuclear encoded genes that enhance pathways which partially compensate for aberrant complex V function or reduced ATP production. This hypothesis requires further study.

The precise molecular mechanisms linking respiratory chain dysfunction to axonal degeneration are not determined. One possibility is that axonal degeneration relates to loss of the axon membrane potential as a consequence of failure of the energy-dependent Na\textsuperscript{+}/K\textsuperscript{+} ATPase pump, comparable to ischaemia. However, a recent study did not show detectable changes in nerve excitability using the TROND protocol in patients with mitochondrial disease, as would be expected if there was significant axonal depolarisation due to failure of energy-dependent systems.\textsuperscript{24}

The findings reported here add to increasing evidence that mitochondrial dysfunction is an important cause of CMT2. Mutations in \textit{MFN2}, a critical nuclear gene that regulates mitochondrial fusion, have been identified as a cause of CMT2. Evidence is emerging that \textit{MFN2} has a distinct role aside from mitochondrial fusion in mitochondrial axonal transport, and is an important component of the linker/adaptor complex between mitochondria and kinesin/microtubules.\textsuperscript{25} Disruption of this complex may explain the length-dependent nature of the neuropathy seen with \textit{MFN2} mutations. More recently, a novel \textit{MFN2} mutation causing optic atrophy,
Axonal neuropathy and mitochondrial myopathy was found to cause multiple mtDNA deletions in skeletal muscle. This finding was thought to result from mtDNA instability, caused by the variability of repair protein content across the mitochondrial population as a consequence of impaired mitochondrial fusion, and expands current knowledge of the pathogenic basis of MFN2-related neurological disease. It has also been shown that nerves expressing mutant forms of neurofilament (which causes CMT2E) can also alter mitochondrial dynamics, suggesting that dysfunctional axonal transport might precipitate axonal degeneration in a number of other CMT subtypes. These observations imply that impaired ATP dependant axonal transport is a candidate for axonal damage in other mitochondrial diseases.

We suggest that MT-ATP6 should be considered in the molecular diagnostic evaluation of patients with CMT2, together with MFN2, MPZ, RAB7, HSPB1, HSPB8, GDAP1, TRPV4, NEFL and GARS, especially in pedigrees where there appears to be no male-to-male transmission. These findings have important clinical implications, particularly for genetic counselling. Diagnosis can be made in DNA extracted from blood, using restriction fragment length polymorphism genetic analysis, since m.9185T>C mutant load appears to be high in peripheral blood leucocytes in all affected cases reported to date. An invasive muscle biopsy is not required for diagnostic purposes, and histology is usually unhelpful; however, if muscle tissue is available, BN-PAGE demonstrates both the structural and functional impairment caused by the effects of m.9185T>C; p.Leu220Pro on the ATP6 subunit.

The clinical and electrophysiological phenotype in our families was typical of CMT2/dHMN; however, the following features should be used to help prioritise MT-
$ATP6$ for mutation analysis in patients with neuropathy: (1) disease onset in the first or second decades; (2) variable clinical severity with wheelchair dependence as early as 19 years; (3) an initial slowly progressive neuropathy, which may accelerate in the fifth and sixth decades from unaided walking to wheelchair dependence within a relatively short time frame; (4) an evolving clinical phenotype from a pure motor to a motor-predominant axonal neuropathy in the third and fourth decades; (5) early proximal lower limb muscle involvement despite mild distal weakness; (6) multi-system involvement in the patient or relatives; (7) UMN signs in affected or asymptomatic relatives; (8) rapid clinical decompensation in affected individuals following viral or septic illness.

The data presented adds to the increasing evidence that mitochondrial dysfunction is an important cause of CMT2. We suggest that $MT-ATP6$ mutations should be considered early in the diagnostic evaluation of patients with inherited axonal neuropathies.
ACKNOWLEDGEMENTS

RDSP is funded by MRC grant number G0800674. MGH and MMR are supported by an MRC Centre grant G0601943. MMR is grateful to the Medical Research Council (MRC) and the Muscular Dystrophy Campaign and SMM and MMR are grateful to the NINDS/ORD (1U54NS065712-01) for their support. SR is supported by Great Ormond Street Hospital Children’s Charity. JH and MGH are supported by The Myositis Support Group and JH is supported by the Reta Lila Weston Institute for Neurological Studies. HH is grateful to the MRC and Wellcome Trust for funding.

This study was supported by the NIHR UCLH/UCL Comprehensive Biomedical Research Centre and 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.
REFERENCES


### Table 1: Clinical and electrophysiological findings in patients with the m.9185T>C mutation in \textit{MT-ATP6}

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Sex</th>
<th>Age onset (years)</th>
<th>Symptom at onset</th>
<th>Age last exam (years)</th>
<th>Pes Cavus</th>
<th>Motor weakness</th>
<th>Sensory loss</th>
<th>UMN signs</th>
<th>Other features</th>
<th>Mobility</th>
<th>CMTES2(28)/CMTNS2(36)</th>
<th>Summary of NCS/EMG</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>II-1</td>
<td>F</td>
<td>2\textsuperscript{nd} decade</td>
<td>Falls</td>
<td>42</td>
<td>Y</td>
<td>Distal LL</td>
<td>N</td>
<td>N</td>
<td>Blackouts</td>
<td>Unaided</td>
<td>ND</td>
<td>Pure motor neuropathy</td>
</tr>
<tr>
<td></td>
<td>II-3</td>
<td>F</td>
<td>2\textsuperscript{nd} decade</td>
<td>Unsteady</td>
<td>30</td>
<td>Y</td>
<td>Distal LL</td>
<td>N</td>
<td>N</td>
<td>Mild LD, ataxia</td>
<td>Wheelchair (3\textsuperscript{rd} decade)</td>
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<td>ND</td>
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<tr>
<td></td>
<td>II-4</td>
<td>F</td>
<td>Asymptomatic</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>II-5</td>
<td>M</td>
<td>1\textsuperscript{st} decade</td>
<td>Late walker, falls</td>
<td>31</td>
<td>N</td>
<td>Distal LL</td>
<td>N</td>
<td>N</td>
<td>Dyssmorphic, LD</td>
<td>Unaided</td>
<td>ND</td>
<td>Axonal neuropathy</td>
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<td>U</td>
<td>Unsteady, falls</td>
<td>32</td>
<td>N</td>
<td>Distal UL and LL</td>
<td>N</td>
<td>N</td>
<td>Mild LD, migraine, ataxia</td>
<td>Wheelchair (3\textsuperscript{rd} decade)</td>
<td>ND</td>
<td>Motor neuropathy/neuronopathy</td>
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<td></td>
<td>III-5</td>
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<td>16</td>
<td>Rapid decline following viral illness with Leigh-like syndrome and cortical blindness. Died aged 16 years.</td>
<td>16</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>LD</td>
<td>Unaided</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>III-6</td>
<td>M</td>
<td>11</td>
<td>Tripping</td>
<td>27</td>
<td>Y</td>
<td>Mild prox and mod distal LL</td>
<td>Knees</td>
<td>N</td>
<td>Mild LD, foot surgery</td>
<td>Unaided with ankle support</td>
<td>9(28)</td>
<td>Sensorimotor axonal neuropathy</td>
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<tr>
<td></td>
<td>III-7</td>
<td>M</td>
<td>9</td>
<td>Unsteadiness, poor concentration</td>
<td>15</td>
<td>Y</td>
<td>Mild prox LL</td>
<td>N</td>
<td>N</td>
<td>Mod LD, SNHL, kyphoscoliosis.</td>
<td>Unaided</td>
<td>ND</td>
<td>Pure motor axonal neuropathy</td>
</tr>
<tr>
<td></td>
<td>IV-1</td>
<td>M</td>
<td>6</td>
<td>Deterioration in gait. Sudden death following viral illness aged 9</td>
<td>8</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Hyper-reflexia</td>
<td>LD</td>
<td>Unaided</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IV-2</td>
<td>F</td>
<td>10 years</td>
<td>Painful legs, tripping</td>
<td>10</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Febrile seizure, mild LD</td>
<td>Unaided</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>B</td>
<td>II-3</td>
<td>F</td>
<td>Asymptomatic</td>
<td>54</td>
<td>N</td>
<td>None</td>
<td>None</td>
<td>N</td>
<td>None</td>
<td>Unaided</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>II-8</td>
<td>M</td>
<td>2nd decade</td>
<td>Slow running</td>
<td>60</td>
<td>N</td>
<td>Mild prox and distal UL, severe prox and distal LL</td>
<td>Distal UL and LL</td>
<td>N</td>
<td>Late-onset DM</td>
<td>Wheelchair (6th decade)</td>
<td>21(28)</td>
<td>Aged 31: Pure motor axonal neuropathy</td>
<td></td>
</tr>
<tr>
<td>III-1</td>
<td>F</td>
<td>Asymptomatic</td>
<td></td>
<td></td>
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<tr>
<td>III-2</td>
<td>M</td>
<td>1-2nd decade</td>
<td>Foot drop</td>
<td>U</td>
<td>Hammer toes</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>None</td>
<td>Unaided</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>III-3</td>
<td>F</td>
<td>5th decade</td>
<td>Leg cramps, reduced exercise tolerance, toes extend whilst walking</td>
<td>49</td>
<td>Y</td>
<td>Mild distal LL</td>
<td>N</td>
<td>Hyper-reflexia, clonus, extensor plantars</td>
<td>None</td>
<td>0(36)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-5</td>
<td>F</td>
<td>Asymptomatic</td>
<td></td>
<td></td>
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<tr>
<td>III-6</td>
<td>F</td>
<td>2nd decade</td>
<td>Slow walking and flapping feet</td>
<td>40</td>
<td>N</td>
<td>Mild distal LL</td>
<td>None</td>
<td>N</td>
<td>Migraine, late-onset DM</td>
<td>Wheelchair (5th decade)</td>
<td>20(28)/24(36)</td>
<td>Aged 29: Pure motor axonal neuropathy</td>
<td></td>
</tr>
<tr>
<td>IV-3</td>
<td>F</td>
<td>3</td>
<td>Falling, clumsy</td>
<td>19</td>
<td>Y</td>
<td>Mild prox and distal LL</td>
<td>Ankles</td>
<td>None</td>
<td>Kyphoscoliosis, focal seizures</td>
<td>Unaided</td>
<td>9(28)/11(36)</td>
<td>Sensorimotor axonal neuropathy</td>
<td></td>
</tr>
<tr>
<td>IV-4</td>
<td>F</td>
<td>Asymptomatic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>II-4</td>
<td>F</td>
<td>Asymptomatic</td>
<td>83</td>
<td>N</td>
<td>Mild distal LL</td>
<td>N</td>
<td>Y</td>
<td>Late onset SNHL</td>
<td>Rollator</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>III-5</td>
<td>F</td>
<td>Asymptomatic</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-13</td>
<td>M</td>
<td>17</td>
<td>Weak legs</td>
<td>43</td>
<td>Y</td>
<td>Mod prox and distal LL</td>
<td>N</td>
<td>Catch, hyper-reflexia</td>
<td>None</td>
<td>Walking stick</td>
<td>ND</td>
<td>Aged 43: Sensorimotor axonal neuropathy with proximal recruitment</td>
<td></td>
</tr>
<tr>
<td>IV-1</td>
<td>M</td>
<td>Asymptomatic</td>
<td>Falls, delayed motor skills, toe walking</td>
<td>19</td>
<td>Y</td>
<td>Distal LL</td>
<td>Distal LL</td>
<td>N</td>
<td>Mild learning difficulties, ADHD, OCD</td>
<td>Wheelchair</td>
<td>ND</td>
<td>Motor axonal neuropathy</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IV-2</td>
<td>M</td>
<td>4</td>
<td>Falls, delayed motor skills, toe walking</td>
<td>19</td>
<td>Y</td>
<td>Distal LL</td>
<td>Distal LL</td>
<td>N</td>
<td>Mild learning difficulties, ADHD, OCD</td>
<td>Wheelchair</td>
<td>ND</td>
<td>Motor axonal neuropathy</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>II-1</td>
<td>M</td>
<td>1st decade</td>
<td>Recurrent ankle sprains, falls</td>
<td>45</td>
<td>N</td>
<td>Mild distal UL, mild prox and mod distal LL</td>
<td>Knees</td>
<td>Pout reflex</td>
<td>Foot surgery</td>
<td>Two sticks</td>
<td>15(28)/17(36)</td>
<td>Sensorimotor axonal neuropathy, distal more than prox denervation in LL</td>
</tr>
</tbody>
</table>

4 ND = not done; U = unknown; Y = yes; N = no; M = male; F = female; BG = basal ganglia; UMN = upper motor neuron; UL = upper limbs; LL = lower limbs; mod = moderate; 
5 prox = proximal; ADF = ankle dorsiflexion; ADHD = attention deficit hyperactivity disorder; OCD = obsessive compulsive disorder; SNHL = sensorineural hearing loss; LD = learning difficulties; DM = diabetes mellitus; SNHL = sensorineural hearing loss; SAPs = sensory nerve action potentials; CMTES = Charcot-Marie-Tooth examination score 2 
6 (total score 28); CMTNS2 = Charcot-Marie-Tooth neuropathy score 2 (total score 36, mild neuropathy: 0-10; moderate neuropathy: 11-20; severe neuropathy: >20).
FIGURE LEGENDS

**Figure 1:** Pedigrees of four unrelated families harbouring the m.9185T>C mutation in *MT-ATP6*. Filled symbols indicate individuals with CMT2; dark grey shaded symbols indicate individuals with upper motor neuron signs only; light grey shaded symbols indicate individuals with unknown phenotype; square symbols indicate male gender; round symbols indicate female gender; diamond symbols indicate gender unknown; numbers in symbols indicate multiple individuals; symbols with slashes indicate deceased; a small square with a slash indicates still birth; a small triangle indicates miscarriage; asterisks indicate affected individuals not examined by authors. Abbreviations: (b) = blood; (u) = urine; (f) = fibroblasts; (m) = muscle; yo = year old; % = percentage m.9185T>C mutant load detected in each patient.

**Figure 2:** Photograph demonstrating distal lower limb muscle wasting and pes cavus in a patient with the m.9185T>C mutation in *MT-ATP6* (family A, patient III-8).

**Figure 3:** Blue-native polyacrylamide gel electrophoreses (BN-PAGE) was performed in muscle tissue from patients with the m.9185T>C mutation and compared with control muscle tissue. BN-PAGE was also performed in a patient with the m.8993T>G mutation as a positive control. Abbreviations: P = patient; C = control; V (F₀ + F₁) = complex V holoenzyme; F₁ = F₁ catalytic site of complex V only; * indicates reduced complex V activity in patient compared to control muscle tissue (demonstrated by reduced band density); ** indicates impaired complex V assembly in patient compared to control muscle tissue (demonstrated by multiple bands indicative of abnormal assembly intermediates); */** indicates both impaired complex V activity and assembly in patient compared to control muscle tissue (demonstrated
by co-existence of reduced band density and multiple bands indicative of abnormal assembly intermediates). (A) Patient with the pathogenic mutation m.8993T>G (positive control). (B)-(D) Family A, patients III-5, III-6 and IV-2 respectively. (E) Family B, patient III-6.