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Vocal cord paresis and diaphragmatic dysfunction are severe and frequent symptoms of *GDAP1*-associated neuropathy

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Cranial nerve involvement in Charcot-Marie-Tooth disease (CMT) is rare, though there are a number of CMT syndromes in which vocal cord paralysis is a characteristic feature. CMT disease due to mutations in the *ganglioside-induced differentiation-associated protein 1 gene (GDAP1)* has been reported to be associated with vocal cord and diaphragmatic palsy. In order to address the prevalence of these complications in patients with *GDAP1* mutations we evaluated vocal cord and respiratory function in nine patients from eight unrelated families with this disorder. Hoarseness of the voice and inability to speak loudly were reported by eight patients and one had associated symptoms of respiratory insufficiency. Patients were investigated by means of peripheral and phrenic nerve conduction studies, flexible laryngoscopy, pulmonary function studies and polysomnography. Nerve conduction velocities and pathological studies were compatible with axonal CMT (CMT2). Flexible laryngoscopy showed left vocal cord palsy in four cases, bilateral cord palsies in four cases and was normal in one case. Restrictive respiratory dysfunction was seen in the eight patients with vocal cord paresis who were all chair-bound. These eight had confirmed phrenic nerve dysfunction on neurophysiology evaluation. The patient with normal vocal cord and pulmonary function had a less severe clinical course.This study shows that CMT patients with *GDAP1* mutations develop severe disability due to weakness of limb muscles and that laryngeal and respiratory muscle involvement occurs late in the disease process when significant proximal upper limb weakness has developed. The early and predominant involvement of the left vocal cord innervated by the longer left recurrent laryngeal nerve suggests a length dependent pattern of nerve degeneration. In *GDAP1* neuropathy, respiratory function should be thoroughly investigated because life expectancy can be compromised due to respiratory failure.

Keywords: Charcot-Marie-Tooth disease; CMT 2K; ARCMT; Vocal cord paralysis; GDAP1 mutations

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Introduction

The peroneal muscular atrophy syndrome or Charcot-Marie-Tooth disease (CMT) is divided into several groups according to clinical, electrophysiological and nerve biopsy findings: (i) CMT1 showing a median nerve motor conduction velocity (MCV) of <38 m/s and nerve fibre demyelination with proliferation of Schwann cells forming onion bulbs; (ii) CMT2 with normal or near normal conduction velocities and pathological signs of axonal degeneration and regeneration; (iii) an intermediate type (CMT-I) is now accepted in which MCV lies between 30 and 40 m/s and nerve pathology shows axonal and demyelinating features or it is undefined; and (iv) distal hereditary motor neuronopathy (DHMN), also known as distal spinal muscular atrophy or spinal CMT, in which motor and sensory nerve conduction velocities are normal, and electromyography shows features of neurogenic atrophy with sparing of sensory nerves (Dyck and Lambert, 1968*a*, *b*; Harding and Thomas, 1980; Houlden and Reilly, 2006). Inheritance can be autosomal dominant (AD), autosomal

recessive (AR) and X-linked. Molecular studies have shown extensive genetic heterogeneity in CMT neuropathies, so each of the main types are subdivided according to the correspondent genes or loci, being nominated either in alphabetical tags (A/Z) or genes names. In this way, AD CMT1 contains five gene-related subgroups (*PMP22*, *MPZ*, *LITAF*, *EGR2* and *NEFL*), and AD CMT2 integrates at least six genes (*MFN2*, *RAB7*, *GARS*, *NEFL*, *HSP27* and *HSP22*). AR demyelinating CMT, more often known as CMT4, contains 10 gene-subgroups (*EGR2*, *GDAP1*, *SH3TC2*, *MTMR2*, *MTMR13*, *NDRG1*, *PRX*, *FGD4*, *FIG4* and *CTDP1*), and AR CMT2 has two recognized genes (*LMNA* and *GDAP1*). The intermediate group is caused by a limited number of distinct gene mutations (*DNM2*, *YARS* and *NEFL*). The best known X-linked form (CMTX) is due to mutations of *GJB1* gene. DHMN is divided by clinical criteria into seven types, four of them having autosomal dominant inheritance (DHMN types I, II, V and VII); all types, except DHMN-I, have mapped genetic loci and some of them like DHMSN VII is also subdivided (Irobi *et al.*, 2006). The same gene can manifest different phenotypes thus increasing complexity in CMT classification.

Vocal cord palsy and diaphragmatic dysfunction are infrequent and are not specific to any one type of CMT. It is a feature of axonal CMT2C (MIM 606071), linked to chromosome 12q23-24 (Klein *et al.*, 2003), of DHMN type VII mapping to chromosome 2q14 (DHMN VIIA; MIM 158580; McEntagart *et al.*, 2001), of DHMN type VIIB (MIM 607641) associated with dynactin 1 (*DCTN1)* mutation (Puls *et al.*, 2003), and of early onset ARCMT2 or CMT4A (MIM 214400) owing to mutations in the *ganglioside differentiating associated protein 1 gene* (*GDAP1*) (Sevilla *et al.*, 2003).

GDAP1 related to CMT has been reported in families affected with either demyelinating CMT (CMT4A, MIM 214400) (Baxter *et al.*, 2002) or axonal CMT (ARCMT2, MIM 606598) diseases (Cuesta *et al.*, 2002). In both types onset occurs in early infancy with distal limb weakness, progressing proximally and causing severe disability. Peripheral motor nerve conduction velocity (MNCV) cannot be measured in many cases because of the absence of muscle response due to distal atrophy, but latencies to proximal muscles are within the normal range. Inherited neuropathies associated with mutations in the *GDAP1* gene show a complex phenotypic spectrum. Although CMT4A usually refers to demyelinating ARCMT forms, it has also been used on several occasions in which the primary phenotype was found to be axonal. According to the OMIM database axonal forms of *GDAP1* associated disease are referred to either as CMT2K (MIM 607831) or CMT2 plus vocal cord paresis (MIM 607706). Most *GDAP1* mutations show autosomal recessive inheritance but in some families the disease segregates as an autosomal dominant trait (Claramunt *et al.*, 2005; Chung *et al.*, 2008).

Vocal cord palsy and diaphragmatic dysfunction can cause airway compromise and respiratory failure. We have studied the frequency and characteristics of vocal cord and respiratory function in patients with CMT and mutations in *GDAP1* gene.

Patients and Methods

Families

We performed a systematic search for *GDAP1* mutations in all index cases of our CMT series in which mutations for *PMP22*, *MPZ* or *GJB1* genes had been excluded, independently of the observed phenotype. There were 11 patients from 8 families with *GDAP1* mutations to whom it was offered to participate in the study, but two of them refused. The pedigrees are displayed in Fig. 1. All parents were unaffected and there was only one consanguineous union. All the patients came from Valencian Community and have been submitted to regular follow-up for a mean of 15.7 years (range 2–37 years) except one patient who came from other geographic region and was specifically investigated for this study. The neuropathic symptoms were evaluated by two of the authors (T.S. and J.J.V.) and the electrophysiological studies were all performed by the same person (M.J.C.).

The patients were classified using the Harding and Thomas criteria (1980) as CMT1 if the conduction velocity of the median nerve was below 38 m/s or as CMT2 if the velocity was higher. Patients with CMAP of very low amplitude or absent were classified according to the motor conduction velocity from the axillary nerve or, when possible, by sural nerve biopsy.

Clinical study

All probands and individuals at-risk of having inherited the disease living in Valencia were examined. In the family living outside Valencia only the proband was examined. Evaluation of mutation carriers revealed no clinical manifestations. Assessment included: strength, muscle atrophy, sensory loss, reflexes, foot deformities, scoliosis, changes in the voice and alterations of other cranial nerves. Muscle strength was assessed using the standard Medical Research Council (MRC) scale. CMT neuropathy score (CMTNS) was used to determine neurological impairment (Shy *et al.*, 2005). Patients could be divided into three categories: mild (CMTNS $\leqslant$10 points), moderate (CMTNS 11–20) and severe (CMTNS 21–36). A screen for voice disturbances, stridor, dysphagia, dyspnoea at rest or after exercise and the presence of snoring during sleep was carried out by questionnaire.

Electrophysiological study

Electrophysiological studies were performed in all the patients and at-risk members of seven families. In family LF250 the study was only performed on the proband (II-5). Nerve conduction studies (NCS) were tested with surface electrodes. Amplitudes of compound muscle action potentials (CMAPs), distal latency (DL) and conduction velocity from median, ulnar, peroneal, tibial and axillary nerves were recorded using conventional methods. Futhermore, motor nerve conduction studies of more proximal upper limb muscles like palmaris longus muscle for the median nerve and flexor carpi ulnaris for the ulnar nerve were also tested. CMAP and DL from the diaphragm muscle were recorded by phrenic nerve stimulation in the neck (Bolton, 1993).

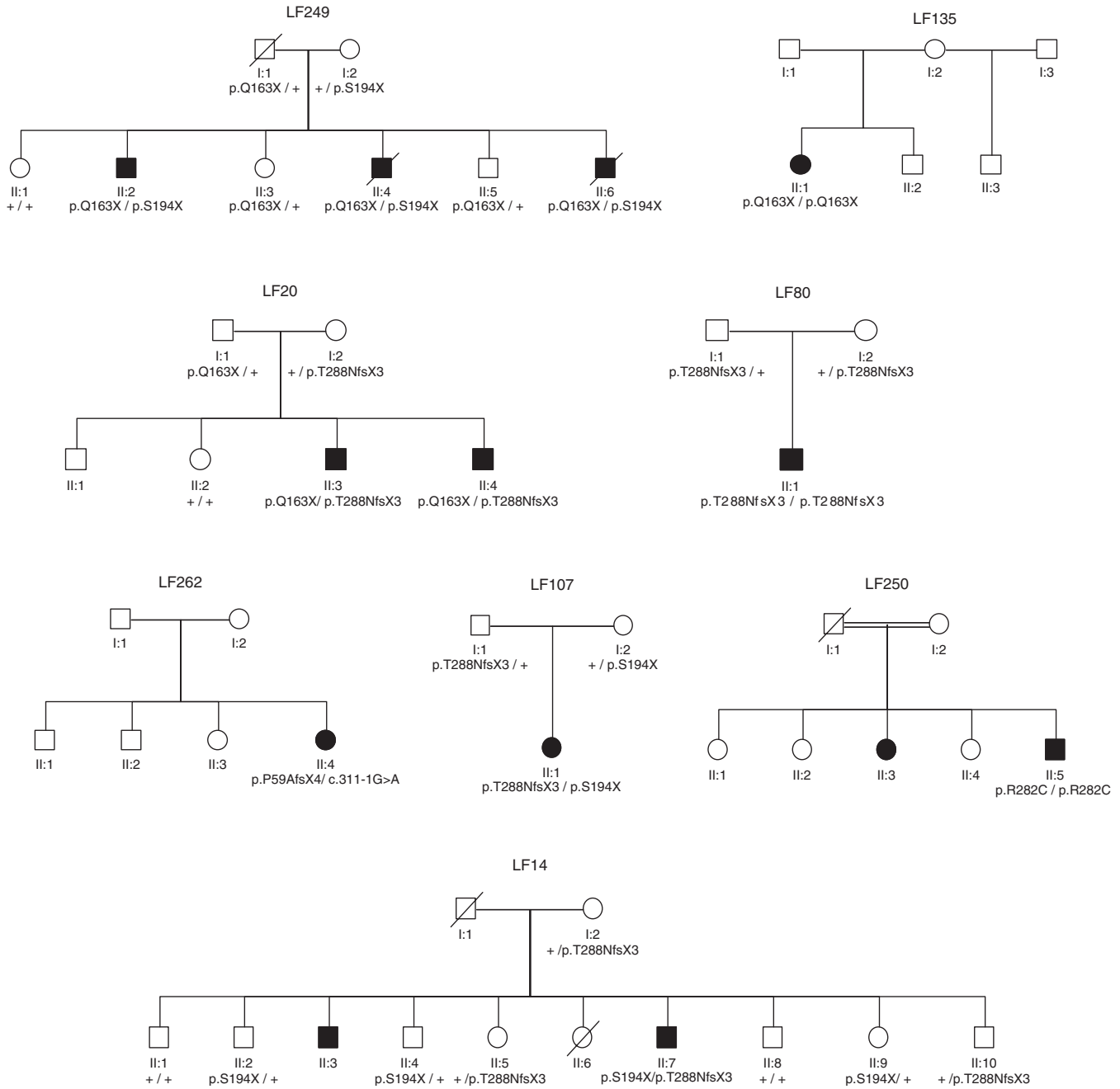

Fig. 1 Pedigrees of the families reported in the present study.

Recordings of sensory nerve action potentials (SNAPs) from median and ulnar nerves were performed orthodromically while sural nerve was tested antidromically. Concentric needle electromyography was performed in the proximal and distal muscles of the upper and lower limbs.

Flexible laryngoscopy and pulmonary function tests

The laryngeal study was performed by an ENT specialist using a flexible fibreoptic laryngoscope. The position of the cords was evaluated during inspiration and phonation. The results were filmed and reviewed. The normal separation of the vocal cords during respiration is of ~13.5 mm.

Pulmonary function tests including spirometry, static lung volumes, maximum inspiratory and expiratory pressures and arterial blood gases breathing air, postero–anterior chest X-ray and respiratory polygraphy were performed in all patients.

Spirometry was performed using Collins® G II Plus spirometer (Collins, MA, USA) following the recommendations of the American Thoracic Society and using their references values (Gardner *et al.*, 1987). The lung volumes were measured using a Collins® BO-XII plethysmograph, using the reference values recommended in the official guideline of the European

Community (Quanjer Ph, 1983). The maximum inspiratory pressure was measured from the residual volume and the maximum expiratory volume from the total lung capacity using a Siebelmed 163® electromanometer (Siebel, Barcelona, Spain) connected to and x-y Servogor 731® recorder (Goetz Metrawatt, Nuremberg, Germany). The reference values used were those of the Spanish Society of Pneumology and Thoracic Surgery (SEPAR) (Casan *et al.*, 1989). All values were given as the absolute figure and as a percentage of the predicted value. The arterial blood gas sample was taken by puncture of the radial artery and introduction of the blood sample into the ABL700® gas analyser (Radiometer, Copenhagen, Denmark). Respiratory polygraphy was performed at the patient's home during the hours of sleep, recording the heart rate, oxygen saturation and number of apnoeas and hypopnoeas per hour, also enabling us to differentiate between central and obstructive apnoea. Episodes of apnoea were defined as the complete cessation of airflow for >10 s. Hypopnoea refers to a 50% reduction in the airflow or a 30–50% reduction of the flow associated with microarousals or desaturation ⩾3%, with a duration of >10 s in both cases. The instrument used was the Somte® Polygraph (Compumedics, Abbotsford, Australia).

Pathological study

Biopsy of the sural nerve was performed in four patients and was studied by light and electron microscopy using standard methods as previously reported in detail (Sevilla *et al.*, 2003).

Mutation analysis

Blood samples were drawn from the patients and relatives after informed consent and in accordance with the Helsinki declaration. DNA was extracted using conventional procedures. All the probands who had been shown to be negative for CMT1A duplication (17p11.2) and point mutations in *PMP22*, *MPZ* and *GJB1* genes were screened for mutations in the *GDAP1* gene (NM_018972). PCR amplification of the six exons of the *GDAP1* gene was performed using primers previously described (Cuesta *et al.*, 2002; Claramunt *et al.*, 2005). PCR products were screened for sequence variants by dHPLC on a WAVE DNA Fragment Analysis System, Model 3500HT (Transgenomic). The running conditions for each amplicon were determined by the Navigator™ Software version 1.6.4. based on the DNA sequence. In order to detect homozygous variants, PCR products from patients were mixed with the corresponding PCR fragment from a normal control. Elution peaks were analysed with the Navigator™ Software and fragments displaying abnormal elution peaks were analysed by direct sequencing on an automated sequencer (ABI PRISM-3130XL, Applied Biosystems).

Results

Clinical characteristics and electrophysiological studies

The study included nine patients (6 male and 3 female) with ages ranging from 16 to 49 years (median 34). Clinical data are summarized in Table 1. Most of the patients had difficulty walking from early childhood. Additionally, five patients presented with congenital hypotonia and four had delay in their motor development. The most consistent clinical abnormality was distal muscle wasting and weakness of upper and lower limbs. The upper limbs were never involved to a greater extent than the lower. All the patients, except LF250 II-5, presented with proximal upper limb weakness. Sensory abnormalities and areflexia was found in all the patients. Seven patients were chairbound in the first and second decade. Two patients (LF80 II-1 and LF262 II-4), presented with joint laxity and were obese. One patient presented bilateral facial weakness (LF80 II-1).

The affected members of family LF249 had been followed up in our hospital since 1971 and the clinical findings have been reported previously (Sevilla *et al.*, 2003). A description of the evolution of their symptoms is given in order to emphasize the severity of the disease. Patient LF249 II-4 had severe proximal upper limb weakness, needing help for feeding; he suffered from HIV acquired immunodeficiency syndrome and died of pneumonia at 41 years old. Patient LF249 II-6 had a sudden death while asleep at 40 years without warning. Patient LF249 II-2 complained of defecatory urgency and intermittent episodes of faecal incontinence over the past 5 years; coloproctologic examination demonstrated a significant reduction in anal pressure during voluntary contractions with preservation of the excitatory rectoanal reflex. Internal and external anal sphincters atrophy was detected by ultrasounds, and pudendal nerves EMG study revealed abnormal latencies.

The electrophysiological data are shown in Table 2. All patients had been subject to serial electrophysiological studies and three of them (LF135 II-1, LF80 II-1 and LF107 II-1) to an initial testing at a very early age. In five patients (LF249 II-2, LF20 II-4, LF80 II-1, LF262 II-4 and LF14 II-7) it was not possible to obtain a distal motor response. The study on patient LF135 II-1 was performed at the age of two showing a conduction velocity of 35 m/s. The case was classified as axonal CMT taking into account the fact that the myelination at this age is not fully mature and also the results of the sural nerve biopsy. The latency of the axillary nerve was normal in all patients. The studies performed before 3 years of age showed that the amplitude of the CMAPs was very low in two cases and absent in one. The subsequent serial studies revealed a fall in the potential amplitude or an absent response a few years later. Peroneal MNCV can still be detected in patient LF250 II-5 at 45 years of age, with almost normal values in the upper limbs. This patient presented a much milder clinical course. The SNAP, although severely abnormal in the majority of the cases, persisted for longer than the CMAP. These findings are compatible with an axonal form of CMT. Phrenic nerve responses were abnormal in all patients except in LF250 II-5 (Table 2).

Evaluation of laryngeal and respiratory function

Laryngeal symptoms and indirect laryngoscopy

The symptoms related to laryngeal and respiratory function are listed in Table 3. Six patients reported changes in their

Table 1 Clinical findings

	Family/case								
	LF249 (II-2)	LF20 (II-3)	LF20 (II-4)	LFI35 (II-1)	LF80 (II-1)	LF262 (II-4)	LFI07 (II-1)	LFI4 (II-7)	LF250 (II-5)
Age (years)/sex	49/M	42/M	36/M	22/F	16/M	38/F	16/F	47/M	45/M
Age of walking	12 months	Delayed	18 months	12 months	18 months	18 months	12 months	12 months	12 months
Age of onset	18 months	<1 year	<1 year	18 months	<1 year	<1 year	7 months	12 months	8 years
Hypotonia at birth	No	Yes	Yes	Yes	Yes	Yes	No	No	No
Proximal UL weakness	+	+	+	+	++	+	+	+	No
Distal UL weakness	+++	+++	+++	+++	+++	+++	+++	+++	++
Proximal LL weakness	++	++	++	++	++	++	++	++	No
Distal LL weakness	+++	+++	+++	+++	+++	+++	+++	+++	+++
Sensory loss in UL	P, V, T	P, V, T	P, V, T	P, V, T	P, V, T	P, V, T	P, V, T	P, V, T	P
Sensory loss in LL	All	All	All	All	All	All	All	All	P, V
Reflexes	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Scoliosis	Mild	No	No	No	No	No	No	No	No
Functional disability UL	Claw-hand	Claw-hand	Claw-hand	Claw-hand	Claw-hand	Claw-hand	Claw-hand	Claw-hand	Moderate
Functional disability LL	W-B, 13 years	W-B, 9 years	W-B, 10 years	W-B, 12 years	W-B, 14 years	W-B, 18 years	W-B, 12 years	W-B, 38 years	AFO
CMTNS	31	31	31	30	30	30	29	30	19
Mutation	p.QI63X/ p.SI94X	p.QI63X/ p.T288NfsX3	p.QI63X/ p.T288NfsX3	p.QI63X/ p.QI63X	p.T288NfsX3/ p.T288NfsX3	p.P59AfsX4/ c.3II-IG>A	p.SI94X/ p.T288NfsX3	p.SI94X/ p.T288NfsX3	p.R282C/ p.R282C
Biopsy/age	22 years	19 years	ND	30 months	ND	32 years	ND	ND	ND

Muscle weakness in upper limbs (UL) or lower limbs (LL): + = strength 4/5 on MCR scale; + + = strength <4/5 on Medical Research Council scale; +++ = complete paralysis. Sensory changes: P, V, T = decreases pinprick, vibration and touch; all = absent pinprick, vibration, touch and position sense. ND = not done; CMTNS = CMT neuropathy score, AFO = ankle-foot orthosis, W-B = wheelchair bound.

Table 2 Electrophysiological data

Patient/age at study	Axillary		Phrenic		Median					Ulnar					Peroneal		
	CMAP	DL	CMAP	DL	CMAP	DL	MCV	SCV	CSNAP	CMAP	DL	MCV	SCV	CSNAP	CMAP	DL	MCV
LF249 (II-2)/39 years	11.7	3.2	0.1	6.5	NR			53[a]	1.3	NR			50[a]	1.9			
LF20 (II-3)/27 years	4.5	3.2	0.1	4.8	0.2	5.3	37	NR		NR			NR				
LF20 (II-4)/26 years	4.8	2.7	0.1	5.1	NR			NR		NR			NR				
LFI35 (II-1)/2 years	NP		NP		3	2.5	35	NP							NR		
LFI35 (II-1)/5 years	NP		NP		0.7	3.7	26	33	1	2	3.1	32	NP		NR		
LFI35 (II-1)/17 years	11.4	4.6	0.3	7.2	NR			NR		NR			NR		NR		
LF80 (II-1)/2 years	NP		NP		NR			NP		NR			NP		NR		
LF80 (II-1)/8 years	11.2	2.5	NR[b]		0.6[c]	4.2	70	42	1.8	NR			54[a]	2.1	NR		
F262 (II-4)/31 years	10.4	4.8	0.1	6.8	0.3[c]	5	53	36	1.1	NR			53[a]	1.5	NR		
LFI07 (II-1)/3 years	NP		NP		0.4	3	46	41	3.9	2.2	2.4	54	NP		0.2	4.6	
LFI07 (II-1)/11 years	7.8	4	0.2	6.5	0.6[c]	4	48	NR		NR			52[a]	1.7	NR		
LFI4 (II-7)/45 years	6.3	3.1	0.1	7.5	NR			NR		NR			NR		NR		
LF250 (II-5)/45 years	14.4	3.4	1	7.2	6.3	3.5	50	34	1.3	8.1	2.7	58	34	1.6	0.3	6	40

[a]Mixed; [b]Study was done at 16 years old; [c]Flex.carp.radialis; (axilla-elbow) distal potential not recorded; Normal values = axillary CMAP > 7 mv, DL < 5.3; Phrenic = CMAP >0.3 mV, DL < 7.9 ms; MCVs = median and ulnar nerves > 51m/s, peroneal > 44 m/s; CMAP = median >9 mv, ulnar > 7.7 mv, peroneal > 5.9 mv; DL = median < 4.1, ulnar < 3.3, peroneal < 5.5; SCVs = median and ulnar > 43 m/s; CSNAP = median > 16.5 μv; ulnar > 6.8 μv. CMAP = compound muscle action potential; DL = distal motor latency; MCV = motor nerve conduction velocity; SCV = sensory conduction velocity; CSNAP = compound sensory nerve action potential; NP = not performed; NR = no response.

Table 3 Laryngeal and pulmonary function test findings

	Family/case								
	LF249 (II-2)	LF20 (II-3)	LF20 (II-4)	LF135 (II-1)	LF80 (II-1)	LF262 (II-4)	LF107 (II-1)	LF14 (II-7)	LF250 (II-5)
Age	49	42	36	22	16	38	16	47	45
Sex	M	M	M	F	M	F	F	M	M
Hoarseness	Yes/14	Yes/14	Yes/15	Yes/15	Yes/13	Yes/20	Yes/12	Yes/45	No
Dyspnoea	No	No	No	No	No	Yes	No	No	No
Exercise intolerance	Yes	No	No	No	No	Yes	No	Yes	No
Dysphagia/choking	Fluids	No	No	No	No	No	No	No	No
Shortness of breath	No	No	No	No	No	Yes	No	No	No
Snoring	Yes	?	No	No	Yes	Yes	No	No	No
VC palsy	Both	Both	Both	Left	Left	Left	Left	Both	No
VC position inspiration	Median both	PM both	PM both	PM both	PM left	PM left	PM left	PM both	Abducted
FVC (l/m) predicted	2.5 (71%)	2 (40%)	2.8 (55%)	2.4 (63%)	2.6 (50%)	1.2 (38%)	2.5 (75%)	2.8 (61%)	4.5 (95%)
TLC (l/m)	3.6 (62%)	2.8 (42%)	4.2 (63%)	3 (59%)	3.4 (51%)	1.9 (45%)	3.3 (73%)	5 (70%)	6.4 (91%)
FRC (l/m)	1.8 (60%)	1 (33%)	2.3 (70%)	1.5 (55%)	1.5 (45%)	1.9 (45%)	1.5 (71%)	3.2 (95%)	3.5 (102%)
RV (l/m)	0.8 (44%)	0.7 (41%)	1.3 (74%)	0.6 (43%)	0.7 (48%)	0.6 (45%)	0.6 (60%)	2 (100%)	1.8 (85%)
MIP	72.7 (66%)	65 (52%)	122 (111%)	100 (87%)	80 (75%)	61 (53%)	92 (87%)	116 (93%)	61 (56%)
MEP	72 (93%)	107 (51%)	114 (79%)	98 (103%)	80 (90%)	66 (78%)	82 (62%)	120 (59%)	175 (124%)
PSG	Abnormal	Normal	Normal	Normal	Normal	Abnormal	Normal	Normal	ND
Chest X-ray/ elevation diaphragm	Left side	Both sides	Left side	Left side	Both sides	Both sides	Right side	Left side	Normal

M = male; F = female; VC = vocal cord; PM = paramedian; TLC = total lung capacity; FRC = functional residual capacity; RV = residual volume; l/m = litres/minute; MEP = maximal expiratory pressure; MIP = maximal inspiratory pressure; PSG = polysomnography; ND = not done.

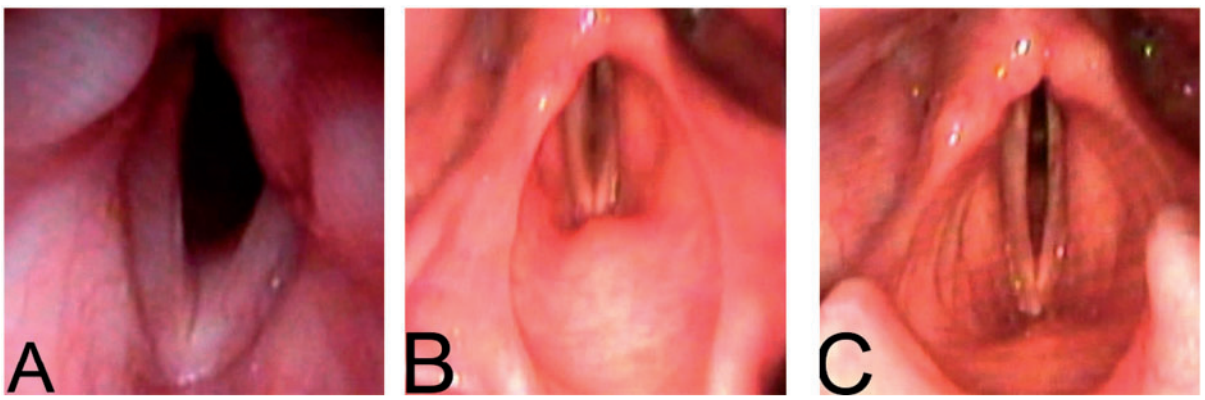

Fig. 2 *GDAP1* neuropathy with vocal cord paralysis demonstrated by flexible indirect laryngoscopy. (**A**) A 16-year-old man (LF80 II-1) with paralysed left vocal cords. (**B** and **C**) A 49-year-old man (LF249 II-2) with bilateral vocal cord paralysis and atrophy. Note lack of complete glottis closure during vocal cord adduction (**B**); vocal cords lie near the midline during abduction (**C**) due to fibrotic contracture and atrophy.

voice since adolescence, being particularly noticeable on singing or shouting. Initially, the dysphonia was fluctuating in two cases (LF249 II-2 and LF107 II-1), becoming persistent in one of them (LF249 II-2) and improving subjectively in the other during the past year (LF107 II-1). One patient (LF262 II-4) has not noticed changes in her voice, although she admits always having a shrill voice and being unable to sing or shout since adolescence. Another patient (LF14 II-7) states that his voice problems began at 45 years of age with episodes of hoarseness that lasted for about 3 days and persisted for 2 months. None of the patients reported stridor, difficulty expectorating secretions or problems when sleeping. One patient (LF249 II-2) has had occasional episodes of choking when drinking in recent years, and has to pause frequently to catch his breath when speaking. Two patients suffer occasional aspiration (LF249 II-2 and LF262 II-4).

The glottis opens to permit respiration and is closed during phonation. Flexible laryngoscopy showed paresis of both vocal cords in four patients and paresis of the left vocal cord in other four (Table 3 and Fig. 2). The paralysed cords were in a paramedian position (partial abduction) during inspiration. In patient LF249 II-2 only minimal movements of adduction were observed during phonation (Fig. 2B). During phonation, vocal cords in patients with bilateral paralysis were not fully adducted leaving a gap,

which was not observed in three of the patients with a unilateral alteration and was minimal in the other. Vocal cords were atrophic in those patients with a longer history of dysphonia. Flexible laryngoscopy had been performed previously in two of the patients with bilateral paralysis (LF20 II-3 and LF14 II-7), and revealed paresis of left vocal cord. Pharyngeal movement was also very poor in patients with bilateral vocal cord paralysis.

Symptoms and pulmonary function tests

None of the patients spontaneously complained of respiratory symptoms, except for one patient (LF262 II-4) who is on non-invasive ventilation since 2005 for this reason. On direct questioning, three patients reported dyspnoea on exertion, two on speaking and one when practising yoga (Table 3); the remainder of the patients did not perform exercise except for one who regularly went swimming. Three patients snored (LF262 II-4, LF249 II-2 and LF14 II-7). None of the patients presented significant malformations of the thoracic cage; one had a mild degree of scoliosis. Chest X-ray revealed elevation of the diaphragm in eight patients and was normal in one patient (Table 3); this elevation was unilateral in five cases and bilateral in three. Table 3 shows the spirometry values, volumes and maximum inspiratory and expiratory pressures. All the patients except one presented a restrictive functional alteration that varied from mild (75% of the predicted value) to very severe (38% of the predicted value). Serial pulmonary function studies have been performed in three patients (LF20 II-3, LF14 II-7 and LF262 II-4) since 2005, finding a progressive decrease in the forced vital capacity (FVC). The maximum inspiratory pressure (MIP), as an index of muscle strength, was moderately reduced (<75% of the predicted value) in four cases and was normal in the other patients. Arterial blood gases showed gas exchange within the normal range in all patients. Respiratory polygraphy was abnormal in two patients (Table 3), who presented an apnoea/hypopnoea index (number of episodes of apnoea plus hypopnoea per hour of sleep), >15 (normal <16).

Pathological results

Sural nerve samples were obtained from several patients at differing ages (LF135 II-1: 30 months; LF20 II-3: 19 years; LF249 II-2: 22 years; LF262 II-4: 32 years) thus showing the pathological progression of the neuropathy over time. The most outstanding finding is a progressive depletion of myelinated fibres (myelinated fibre density in LF135 II-1: 8700/mm^2; LF20 II-3: 998; LF249 II-2:1211; and LF262 II-4: 600). The size histograms of the remaining myelinated fibre were shifted to the left; there was no fibre >8 μm of diameter in any nerve. There were clusters of small myelinated fibres that were abundant in LF135 II-1. Hypomyelinated fibres with g-ratio >0.7 were present in all nerves, especially in LF135 nerve; their proportion was related to the amount of regenerating fibres. No alteration in myelin sheath compaction was observed. There were concentric Schwann cell formations in crescent shape, usually around cluster of small myelinated fibres. We consider these structures 'pseudobulbs' to distinguish them from the typical onion bulb associated to the well characterized demyelinating neuropathies (see Sevilla *et al.*, 2003 for details). The pathological picture was summarized as a progressive axonal degeneration with very active non-efficient axonal regeneration process.

Genetic analysis

Six different mutations in the *GDAP1* gene have been found in these families, including one missense (p.R282C), two nonsense (p.Q163X and p.S194X), two frameshift mutations (p.T288NfsX3 and p.P59AfsX4) and one splice-site variant (c.311-1G>A). Pedigrees of families and associated mutations are described in Fig. 1. All mutations had previously been reported except a frameshift mutation, produced by a deletion followed with an insertion in exon 2 of the *GDAP1* gene (c.172_173delCTinsTTA, p.P59AfsX4) creating a premature stop codon. Five families were found to be compound heterozygous and the remaining three families homozygous.

Discussion

All except one of the families with *GDAP1* mutations included in this series have been the subject of an extended observation enabling us to keep an accurate record of the clinical course of the disease. Two patients have died during follow-up, one due to respiratory insufficiency and the other to sudden death during sleep; both patients were members of family LF249 and their clinical data have been published previously (Sevilla *et al.*, 2003). This event was the main reason that encouraged us to examine the vocal cords by means of flexible laryngoscopy and to plan a study to evaluate respiratory function in these patients.

We found laryngeal and respiratory dysfunction in all except one of our patients. Alterations of the voice started to appear when the patients had a significant functional disability, usually developing during adolescence. Four patients presented bilateral paralysis of the vocal cords and another four paralysis of the left vocal cord. None of the patients presented isolated right vocal cord paralysis and in two of the patients with bilateral paralysis we have evidence that left cord paresis was detected in a previous examination. The course of the disease was longer in patients with bilateral vocal cord paralysis. All but one patient (LF250 II-5) showed proximal weakness of upper limbs, the only case with normal strength showed normal mobility of vocal cords at examination, their neuropathy started later and he is still able to walk at forty five.

Despite that vocal cord paresis is a prominent feature in some forms of CMT, few studies have paid detailed attention to vocal cord paralysis in these diseases. In DHMN-VIIA (Young and Harper, 1980), symptoms started in second

decade of life, and atrophy of hand muscles preceded involvement of the lower limbs. All but one affected subjects developed unilateral or bilateral vocal cord paralysis, the onset of dysphonia was variable and in some cases preceded atrophy of the hand muscles. Although dyspnoea on effort was present in two patients, screening of diaphragm movement was normal. Pridmore *et al.* (1992) found another family with similar characteristics which was related to the former as proved by subsequent molecular studies and genealogy investigations (McEntagart *et al.*, 2001).

In DHMN-VIIB (Puls *et al.*, 2005), clinical phenotype was characterized by bilateral vocal cord paralysis appearing in adulthood followed by facial, hand, and finally distal leg weakness. Laryngoscopy showed either a symmetrical deficit reduction in vocal cord abduction or an abduction deficit greater on the left side. However, phrenic nerve responses were normal in all patients. The severity of the disease manifestations in patients of the same age was similar. Clinical findings of this family resembles our patients in that vocal cord deficit was greater on the left than the right side but differs in the progression of muscle involvement. DHMN-VIIB follows a craneo–caudal pattern (laryngeal, facial, upper and lower limbs), whereas *GDAP1* neuropathy follows the opposite pattern (lower limbs, upper limbs, diaphragm and laryngeal muscles). In DHMN-VII types, phrenic involvement has not been reported.

CMT2C is an autosomal dominant axonal form of peroneal muscular atrophy with progressive muscle weakness and atrophy of limbs, diaphragm, vocal cord, and intercostals muscles with variable degrees of acral sensory loss (Dyck *et al.*, 1994; Lacy *et al.*, 2001; Santoro *et al.*, 2002; McEntagart *et al.*, 2005). Among affected persons, the age at onset and clinical severity were variable. Voice change was the initial symptom in several cases, being atrophy of the hand muscles the second most reported symptom. The clinical features of CMT2C and DHMN-VIIA and B overlap considerably but are distinguished by the presence of sensory involvement in CMT2C. Contrary to our cases, vocal cord involvement in these three types sometimes precedes the neuropathy and is not necessarily related to its severity.

Vocal cord palsies and diaphragmatic weakness have also been described in other varieties of CMT; three cases of diaphragmatic weakness, and one of vocal cord palsy were reported in the most severely affected patients of a large CMT1A series (Thomas *et al.*, 1997). Another case was reported in one member of a CMT1 family associated with a dominant heterozygous *EGR2* mutation, developing vocal cord palsy and dyspnoea several years after becoming chairbound (Pareyson *et al.*, 2000). In both types, the clinical picture, like in our patients, was of a length related neuropathy affecting the lower limbs to a greater extent than the upper limbs. Other cases of CMT with vocal cord paresis have been reported in patients who have not been genetically typified. In these cases the vocal cord palsy was usually associated to diaphragmatic weakness and appeared in the latter stages of the disease (Johnson *et al.*, 1981; Tyson *et al.*, 1997). Sulica *et al.* (2001) reported a patient with CMT and episodic paralysis of both vocal cords who was asymptomatic at the time of examination but had presented episodes of dysphonia during the previous year. They performed a review of the literature and concluded that, in general, vocal cord paresis is well tolerated in patients with CMT as only two cases had required tracheotomy. This good tolerance was thought to be due to the insidious course of the neuropathy, permitting reinnervation and recovery of part of the function.

Table 4 shows a summary of the characteristics of published cases with *GDAP1* mutations and vocal cord paresis (Azzedine *et al.*, 2003; Boerkoel *et al.*, 2003; Stojkovic *et al.*, 2004; Bouhouche *et al.*, 2007a; Kabzinska *et al.*, 2007). Two siblings (Azzedine *et al.*, 2003) developed intermittent dysphonia, becoming permanent in one of them after 10 years; in this patient, laryngoscopy revealed paresis of the left vocal cord. In these reports, as well as in our series, the features associated with vocal cord paresis were: proximal muscle weakness, long history of the disease and frequently diaphragmatic involvement. These patients were of different ethnic origins and had different mutations and phenotypes. As we can see in Tables 1 and 4, p.Q163X and p.S194X are prevalent mutations in patients with vocal cord palsy and *GDAP1* neuropathy. In our series all mutations except p.R282C were associated to vocal cord involvement. The patient harbouring this mutation progressed with a less severe phenotype and without vocal cord and diaphragm involvement. The p.R282C mutation has been reported in two other families, one of Turkish and one of Croatian origin (Ammar *et al.*, 2003; Nelis *et al.*, 2002) and the clinical course in these patients was less severe than usually seen in cases of *GDAP1* neuropathy.

All the laryngeal muscles except for the cricothyroid muscle are innervated by the recurrent laryngeal nerve, a branch of the vagus. The path of the left recurrent laryngeal nerve is longer than the right, leading us to suspect that the vulnerability of this nerve in patients with CMT and *GDAP1* mutations is due to its length and not to genetic or environmental factors. Paralysis in adduction is probably due to the tensor action of the cricothyroid muscle which is innervated by the superior laryngeal nerve, also a branch of the vagus but shorter than the recurrent nerve, and it is usually spared or less affected. In *GDAP1* neuropathy, paresis of the vocal cords probably represents a stage in the neuropathy and may be an indicative parameter of the severity of the disease. Dysphagia has been reported in a patient with vocal cord paresis and severe weakness (Boerkoel *et al.*, 2003), this finding is probably due to dysfunction of the pharyngeal branches of the vagus nerve, whose path is shorter than laryngeal recurrent nerves. Other cranial nerves apart from the vagus nerve may be affected, such as the facial nerve: one of our patients presented

Table 4 Vocal cord paralysis in reported CMT patients with *GDAP1* gene mutations

Authors	Onset	Age/sex	Ethnicity/ Mutation	CMT type	Hoarseness (years)/ Laryngoscopy	Respiratory impairment	Functional disability
Boerkoel *et al*. (2003)	2 years	34/F	Japanese/ p.RI20Q/ p.RI20Q	Demyelinating	Yes	NR	Proximal weakness
Boerkoel *et al*. (2003)	0.5 year	61/M	Costa Rican/ p.QI63X/ p.QI63X	Demyelinating	Yes	NR	Proximal weakness
Azzedine *et al*. (2003)	3 years	30/M	Morocco p.SI94X/ p.R310Q	Axonal	Yes, (20–30 years intermittent)/ Left VC palsy	Yes Diaphragm palsy FVC 49%	W-B from 15 years
Azzedine *et al*. (2003)	3 years	26/F	Morocco p.SI94X/ p.R310Q	Axonal	Yes, episodic 2nd decade	No FVC 99%	Crutch from 16 years
Stojkovic *et al*.(2004)	DW 20 months	32/F	French p.II86fsX205/ p.II86fsX205	Axonal	Early in life/ VC paresis	Dyspnoea MIP ↓ Diaphragm palsy	W-B from 25 years
Stojkovic *et al*. (2004)	DW 20 months	33/F	French p.II86fsX205/ p.II86fsX205	Axonal	Early in life/ VC paresis	Dyspnoea MIP ↓ Diaphragm palsy	W-B from 25 years
Bouhouche *et al*. (2007a)	1.5 years	19/F	Morocco p.P78L/ p.P78L	Mixed	Yes	NR	W-B from 15 years
Bouhouche *et al*. (2007a)	<1 year	15/M	Morocco p.P78L/ p.P78L	Mixed	Yes	NR	Walked with cane
Kabzinska *et al*. (2007)	3.5 years	32/M	Polish p.PI53L/ p.PI53L	Axonal	Yes (29 years)	NR	W-B from 27 years

F = female; M = male; DW = delayed walking; W-B = wheelchair bound; NR = not reported.

bilateral facial weakness, a finding reported in other cases (Boerkoel *et al*., 2003).

The majority of our patients presented a severe form of CMT, with a delay in the acquisition of motor functions or with an onset of the disease around one year of age and marked weakness of the proximal muscles during the first decade of life. All the patients presented sensory loss on physical examination, although neither subjective sensory complaints nor sensory ataxia were a feature. The clinical profile was quite similar to the description of the Moroccan families reported by Bouhouche *et al*. (2007b) who were homozygous for the *GDAP1* p.S194X mutation, but the course in our cases was somewhat more severe. In fact, in the second decade of their lives the majority of the Moroccan patients were still able to walk with the aid of a walking-stick whereas our cases were already in wheelchairs. Dysphonia was not reported in any of the Moroccan patients. All of them were examined before the age of 20 with the exception of a man of 38 years old, and flexible laryngoscopy was not reported in any of them. Moreover, vocal cord paresis may be overlooked during a routine neurological evaluation. Three of our families are compound heterozygotes for the Moroccan mutation p.S194X and another mutation. As observed in the Moroccan families, all our cases showed also an axonal form of CMT.

Despite allelic heterogeneity, the natural history of the disease and clinical phenotype of our patients were fairly homogenous, except for LF250 II-5 proband. The molecular pathology of this patient is different to the others. While LF250 II-5 is homozygous for the missense p.R282C mutation, the other patients were homozygous or compound heterozygous for nonsense or frameshift mutations that predict a truncated protein. GDAP1 is a protein located in the outer mitochondrial membrane (Niemann *et al*., 2005) that contains two GST domains and transmembrane domains. Cell expression of truncated GDAP1 proteins with no transmembrane and C-terminal domains (amino acids 320–358) are misallocated in the cell cytoplasm and nucleus, suggesting that these forms may be non functional (Pedrola *et al*., 2005). By contrast, the p.R282C GDAP1 mutant protein is correctly located in the mitochondria and induces mitochondrial fission as GDAP1 wild-type (Pedrola *et al*., 2005; Pedrola *et al*., 2008). It could be argued that the presence of a non functional mutation may have a more severe deleterious effect than the cell expression of a missense mutation in the GDAP1 protein. Thus, in the case of LF250 II-5 proband, the deleterious effect of p.R282C mutations on the nerve physiology could induce a milder clinical phenotype.

All our patients except one presented a restrictive alteration of respiratory function, elevation of the diaphragm on the

chest X-ray, and a reduction of phrenic nerve CMAP, demonstrating that the respiratory dysfunction was due to muscle weakness. Global respiratory muscle strength is measured using the MIP and MEP. The MIP was clearly altered in three patients, two of whom presented a severe reduction of the FVC and the other a mild reduction (71% of the predicted value) but with a very significant paralysis of the vocal cords with marked limitation of abduction that could impede air entry. Four patients had normal or only slightly altered MIP and MEP values despite a moderate reduction of the FVC (50–75% of the predicted value). Preservation of intercostals muscle function may explain this lack of correlation between the FVC values and those of the MIP and MEP. Similar findings have been reported in other patients with weakness of the diaphragm and CMT (Laroche *et al.*, 1988; Hardie *et al.*, 1990). Blood gases were normal in all patients, demonstrating that there was no hypoventilation in any of them. Four factors were associated with respiratory dysfunction in our series. They were: proximal upper extremity weakness, bilateral diaphragmatic alteration, advanced age and obesity. The patient affected by all four factors (LF262 II-4) had the most severe respiratory failure, those patients affected by three of the four factors showed moderate to severe respiratory dysfunction (LF20 II-3 and LF80 II-1), and those affected by two of the factors had only moderate dysfunction (LF249 II-2, LF20 II-4, LF135 II-1, LF107 II-1 and LF14 II-7). Patient LF250 II-5 showed no evidence of either respiratory dysfunction or upper limb proximal weakness. Upper limb proximal weakness and bilateral diaphragmatic paresis are the factors most clearly predictive of respiratory involvement in our series; obesity clearly played a role in determining the severity. Our findings are in keeping with other studies that have shown that proximal upper limb weakness and age are predictive of abnormal pulmonary function (Nathanson *et al.*, 1989; Aboussouan *et al.*, 2007). Sleep apnoea syndrome was present in two patients. It was of the obstructive type in case LF249 II-2 due to severity the pharyngolaryngeal neuropathy while in the other case it was of the central type associated with obesity-hypoventilation syndrome. Although others studies have found a correlation between sleep apnoea syndrome and neuropathy severity in patients with CMT1 (Dematteis *et al.*, 2001; Dziewas *et al.*, 2008), in our series it is not easy to establish such a correlation because in all but one patient functional disability is quite homogeneous.

Two members of a single family (LF249) died in their fifth decade of life; both presented vocal cord paresis and phrenic nerve dysfunction. One of them died due to respiratory insufficiency secondary to recurrent infections that could have been related to an acquired immunodeficiency syndrome, although the weakness of the respiratory muscles was probably a compounding factor. The sibling died of sudden death, and it cannot be determined whether vocal cord paresis could have precipitated the event. The only living member of this family with the disease has presented signs and symptoms of pudendal nerve involvement in recent years, demonstrating that shorter nerves are affected as the disease progresses. Weakness of pelvic floor muscles has also been reported in two severe CMT1A cases (Thomas *et al.*, 1997).

In conclusion, patients with CMT and mutations of *GDAP1* have a length dependent neuropathy that causes progressive muscle weakness, affecting the more distal muscles of the limbs initially and finally involving the cranial nerves and respiratory muscles. The earlier involvement of the left recurrent laryngeal nerve compared to the nerve on the right side supports this conclusion. Vocal cord paralysis and respiratory insufficiency can lead to aspiration and bronchopneumonia, decreasing the life expectancy of these patients. It is therefore important to specifically investigate patients with CMT due to *GDAP1* mutations for evidence of laryngeal and respiratory dysfunction.

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