

Clinical, electrophysiological and molecular genetic characteristics of 93 patients with X-linked Charcot–Marie–Tooth disease

O. Dubourg,¹ S. Tardieu,¹ N. Birouk,² R. Gouider,² J. M. Léger,² T. Maisonobe,² A. Brice,^{1,3} P. Bouche² and E. LeGuern^{1,3}

¹Inserm U289, ²Service d'Explorations Fonctionnelles Neurologiques, ³Département de Génétique, Cytogénétique et Embryologie, Hôpital de la Salpêtrière, Paris, France

Correspondence to: O. Dubourg, Inserm U289, Hôpital de la Salpêtrière, 47 boulevard de l'Hôpital, 75651 Paris Cedex 13, France
E-mail: dubourg@ccr.jussieu.fr

Summary

X-linked dominant Charcot–Marie–Tooth (CMTX) disease is a motor and sensory neuropathy caused by mutations in the connexin 32 (CX32) gene. In this study we report the clinical, electrophysiological and genetic features of 93 patients (41 males, 52 females) from 37 unrelated families with CMTX. Age at onset was 15.4 ± 9.6 years in males (range 1–40 years) and 18.7 ± 13.1 years in females (range 1–56 years) ($P = 0.22$) and the duration of disease at the time of examination was 18.3 ± 14.6 years in males and 23.9 ± 13.7 years in females ($P = 0.11$). Males were more severely affected than females, with significantly more frequent muscle weakness, amyotrophy, proprioception loss, upper limb areflexia and pes cavus. Females were more frequently asymptomatic, whereas high functional disability scores were more frequently encountered in males. The electrophysiological studies showed that motor nerve conduction velocities in CMTX females, but not males, were heterogeneous between nerves compared with Charcot–Marie–Tooth type 1A (CMT1A) patients and

controls. The terminal latency index (TLI) for the median nerve was 0.37 ± 0.08 ; it was similar in men and in women and a little higher than those observed in CMT1A and controls. The range of values for median TLI was wider in both male and female CMTX patients than in controls, but was similar to that of CMT1A patients, suggesting that motor conduction was relatively homogeneous within a given nerve. Twenty-seven different CX32 mutations, including missense ($n = 23$), nonsense ($n = 2$) and frameshift mutations ($n = 1$) and one entire deletion of the CX32 coding sequence, were observed in the 37 families. Four of these mutations are described for the first time. The phenotype of the patients, especially age at onset, is discussed in relation to the functional consequences of CX32 mutations, analysed *in vitro* in *Xenopus* oocytes and mammalian cells. CMTX patients with age at onset in the first decade mostly presented non-functional mutations, suggesting that the physiological consequences of the mutations affect age at onset in CMTX.

Keywords: X-linked CMT; connexin 32; demyelination; mutation; phenotype–genotype correlation

Abbreviations: CMT = Charcot–Marie–Tooth disease; CMT1A = Charcot–Marie–Tooth disease type 1A; CMTX = X-linked Charcot–Marie–Tooth disease; CMAP = compound muscle action potential; Cx32 = connexin 32; DML = distal motor latency; FDS = functional disability score; MNCV = motor nerve conduction velocity; TLI = terminal latency index

Introduction

Charcot–Marie–Tooth disease (CMT) is a genetically heterogeneous group of hereditary motor and sensory neuropathies characterized by slowly progressive weakness and atrophy, primarily in the distal leg muscles. The classification used currently is based on the type of neuropathy, which may be axonal or demyelinating, rarely purely motor, and on the mode of inheritance (Dyck, 1975). Electrophysiological studies are necessary to determine the

type of the neuropathy. Median motor nerve conduction velocity (MNCV) is usually used to distinguish axonal from demyelinating forms, the cut-off value varying from 30 (Bouche *et al.*, 1983) to 38 m/s (Harding and Thomas, 1980). There are several modes of inheritance: autosomal dominant, X-linked dominant and autosomal recessive. Dominant forms are preponderant in Europe. Advances in molecular genetics have confirmed the great genetic heterogeneity of CMT.

Table 1 Clinical characteristics of the 93 CMTX patients according to sex

	CMTX males	CMTX females	P
Number of patients	41	52	
Age at onset (years)	15.4 ± 9.6	18.7 ± 13.1	n.s.
Disease duration (years)	18.3 ± 14.5	23.9 ± 13.7	n.s.
Muscle weakness	33 (87%)	25 (52%)	P < 0.0001
Muscle wasting	37 (97%)	34 (71%)	P < 0.01
Sensory loss			
Pain and touch	23 (62%)	19 (41%)	n.s.
Proprioception	30 (83%)	28 (59%)	P < 0.05
Areflexia			
Upper limbs	25 (66%)	14 (29%)	P < 0.001
Lower limbs	34 (89%)	37 (77%)	n.s.
Foot deformities	37 (97%)	38 (79%)	P < 0.05
Kyphoscoliosis	12 (33%)	8 (19%)	n.s.
Functional disability score (0–8)			
0	4 (11%)	14 (29%)	P < 0.05
1	8 (22%)	9 (19%)	n.s.
2	2 (5%)	11 (23%)	P < 0.05
3	22 (59%)	9 (19%)	P < 0.001
4	1 (3%)	3 (6%)	n.s.
5	0	1 (2%)	n.s.

n.s. = not significant.

More than 20 loci and eight genes have been identified so far (Reilly, 2000; Thomas, 2000). The most frequent form of the disease, Charcot–Marie–Tooth Type 1A (CMT1A), is due in most cases to a duplication of the 17p11.2 region (Nelis *et al.*, 1996; Dubourg *et al.*, 2001) containing the peripheral myelin protein 22 gene (*PMP22*) (Lupski *et al.*, 1991; Raeymaekers *et al.*, 1991).

The X-linked form of CMT (CMTX) is the second most frequent form of CMT (Nelis *et al.*, 1996; Dubourg *et al.*, 2001) and is caused by mutations in the gene for connexin 32 (*Cx32*), which maps to chromosome Xq13 (Bergoffen *et al.*, 1993; Bone *et al.*, 1995). *Cx32* is thought to function as a gap junction protein in myelinating Schwann cells. It is located in the paranodal loops of non-compact myelin and in the Schmidt-Lanterman incisures. The MNCV is often reduced in CMTX males (<40 m/s), but ranges from slightly reduced to normal in females (Nicholson and Nash, 1993; Birouk *et al.*, 1998). The nature of the neuropathy in CMTX (primary axonal or demyelinating with secondary axonal degeneration) is still subject to controversy (Hahn *et al.*, 1990; Tan *et al.*, 1996; Birouk *et al.*, 1998; Scherer *et al.*, 1999; Senderek *et al.*, 1999). Recent electrophysiological studies in a single family suggest that CMTX is probably a demyelinating neuropathy in which motor conduction velocities vary between and within nerves, accompanied in some patients by temporal variation (Gutierrez *et al.*, 2000).

We present here a retrospective review of the clinical, electrophysiological and genetic features of 93 patients with mutations in the *CX32* gene. Attention was focused on electrophysiological features, in particular to assess whether motor conduction in CMTX neuropathy is heterogeneous

between and within nerves. Phenotype–genotype correlations in patients with onset in the first decade are discussed in the light of functional tests performed in *Xenopus* oocytes or mammalian cells *in vitro*.

Methods

Patients

Clinical and electrophysiological data were analysed in 93 patients (41 males, 52 females) from 37 families with 27 *CX32* mutations. Forty-eight patients were described in a previous publication (Birouk *et al.*, 1998). All participants gave written informed consent according to the protocol approved by the Ethics Committee of the Hôpital de la Salpêtrière, Paris. Clinical criteria for the diagnosis and clinical assessment of CMTX patients were identical to those previously reported. Disease severity was assessed according to a nine-point functional disability score (FDS): 0 = normal; 1 = normal, but with cramps and fatigability; 2 = inability to run; 3 = walking difficult but still possible unaided; 4 = able to walk with a cane; 5 = able to walk with crutches; 6 = able to walk with a walker; 7 = wheelchair-bound; 8 = bedridden.

Electrophysiological study

The electrophysiological study was performed with a Nicolet Viking IV (Nicolet Biomedical, Madison, Wis., USA). Nerve conduction studies were performed in most patients in sural sensory, peroneal motor, and median and ulnar sensory and

Table 2 Electrophysiological characteristics of the 93 CMTX patients according to sex

	Normal value	CMTX males	CMTX females	P
Median nerve		<i>n</i> = 34	<i>n</i> = 44	
MNCV (m/s)	48	34.5 ± 6.8 (20–60)	44.4 ± 7.1 (28–60)	<10 ⁻⁷
CMAP (mV)	5	2.2 ± 2.1 (0–8.6)	4.6 ± 2.5 (0.6–10.4)	<10 ⁻⁴
DML (ms)	3.6	4.9 ± 1.2 (2.3–8.8)	3.8 ± 0.8 (2.5–6.2)	<10 ⁻⁴
TLI	0.34	0.37 ± 0.08 (0.22–0.64)	0.37 ± 0.08 (0.22–0.59)	n.s.
Not recorded		5	0	
Ulnar nerve		<i>n</i> = 27	<i>n</i> = 36	
MNCV (m/s)	48	36.1 ± 4.9 (27–48.5)	46.6 ± 7.9 (29–67.2)	<10 ⁻⁸
CMAP (mV)	6	3.4 ± 2.2 (0–7.8)	6.4 ± 3 (0.9–11.6)	<10 ⁻⁴
DML (ms)	3	3.8 ± 0.5 (2.8–5.1)	2.8 ± 0.5 (1.9–4.1)	<10 ⁻⁹
Not recorded		1	0	
Peroneal nerve		<i>n</i> = 22	<i>n</i> = 33	
MNCV (m/s)	42	32 ± 8.9 (17–51.5)	38.6 ± 7.1 (14.2–52)	<0.05
CMAP (mV)	2	0.7 ± 1.6 (0–7.4)	1.7 ± 1.7 (0–5.4)	<0.05
DML (ms)	5.5	6.3 ± 2.2 (2.7–9.9)	5 ± 1.5 (2.3–9.8)	<0.05
Not recorded		21	8	

n.s. = not significant.

motor nerve fibres in at least one limb. Distal motor latencies (DMLs), compound muscle action potentials (CMAPs) and MNCVs were recorded as described previously (Birouk *et al.*, 1998). A terminal latency index [TLI = terminal distance (mm)/MNCV (ms) × DML (ms)] was calculated for the median nerve. Terminal distance was fixed at 60 mm. Motor nerve conduction velocity, reflecting proximal conduction, was determined across the forearm. F waves were not studied systematically, nor was the presence of temporal dispersion or conduction blocks, so that the few available data could not be analysed reliably. In addition to data on CMTX patients, electrophysiological data for 119 CMT1A patients (Birouk *et al.*, 1997) and 100 control subjects, examined under the same conditions, were used for comparison with CMTX.

Mutation analysis

In the 37 families, mutation screening was performed by non-radioactive SSCP (single-strand conformation polymorphism) and variants were characterized by sequencing both the 5'–3' and the 3'–5' strand of the entire coding region of the CX32 gene (Applied Biosystems automatic sequencer 377; Perkin-Elmer, Branchbury, NJ, USA). For each mutation, restriction sites that distinguished the normal from the mutated sequence were found by computer analysis (DNA Strider 1.2 software; Perkin-Elmer), permitting rapid characterization of the genotypes of relatives by restriction endonuclease digestion of the appropriate PCR (polymerase chain reaction) fragment. The W133R mutation, which did not affect a restriction site, was analysed in relatives by SSCP.

Statistical analysis

Clinical and electrophysiological data for CMTX males, CMTX females and CMT1A patients were compared using

the χ^2 -test for percentages and Student's *t*-test for means. Differences were considered significant with a probability $P < 0.05$.

Results

Patients

The clinical characteristics of the 93 patients (41 males, 52 females) from the 37 families are shown in Table 1. Age at onset was 15.4 ± 9.6 years in males (range 1–40 years) and 18.7 ± 13.1 years in females (range 1–56 years) and did not differ significantly between the sexes. Age at onset was before age 10 years in 11 males and eight females. Duration of disease at the time of examination was 18.3 ± 14.5 years in males and 23.9 ± 13.7 years in females; the difference was not significant. Clinical findings confirmed that males were more severely affected than females. The difference was statistically significant for more items than previously reported, *viz.* frequency of muscle weakness ($P < 0.0001$), muscle wasting ($P < 0.01$), proprioception loss ($P < 0.05$), upper limb areflexia ($P < 0.001$) and the presence of pes cavus ($P < 0.05$). Associated signs were rarely observed; when present, they were encountered exclusively in males and consisted of distal upper limb tremor (7.3%, $n = 3$) and auditory loss (2.4%, $n = 1$). Functional disability was mild or moderate in all patients. Females (29%, $n = 15$) were significantly more frequently asymptomatic (FDS = 0) than males (11%, $n = 4$) ($P < 0.05$). High FDS (score 3) was significantly more frequent in males (59%, $n = 22$) than in females (19%, $n = 9$) ($P < 0.001$). The rate of disease progression in males and females could not be compared because FDS was assigned at the time of examination, but the date at which each FDS began could not be determined. As CMT progresses slowly, the duration of each FDS may

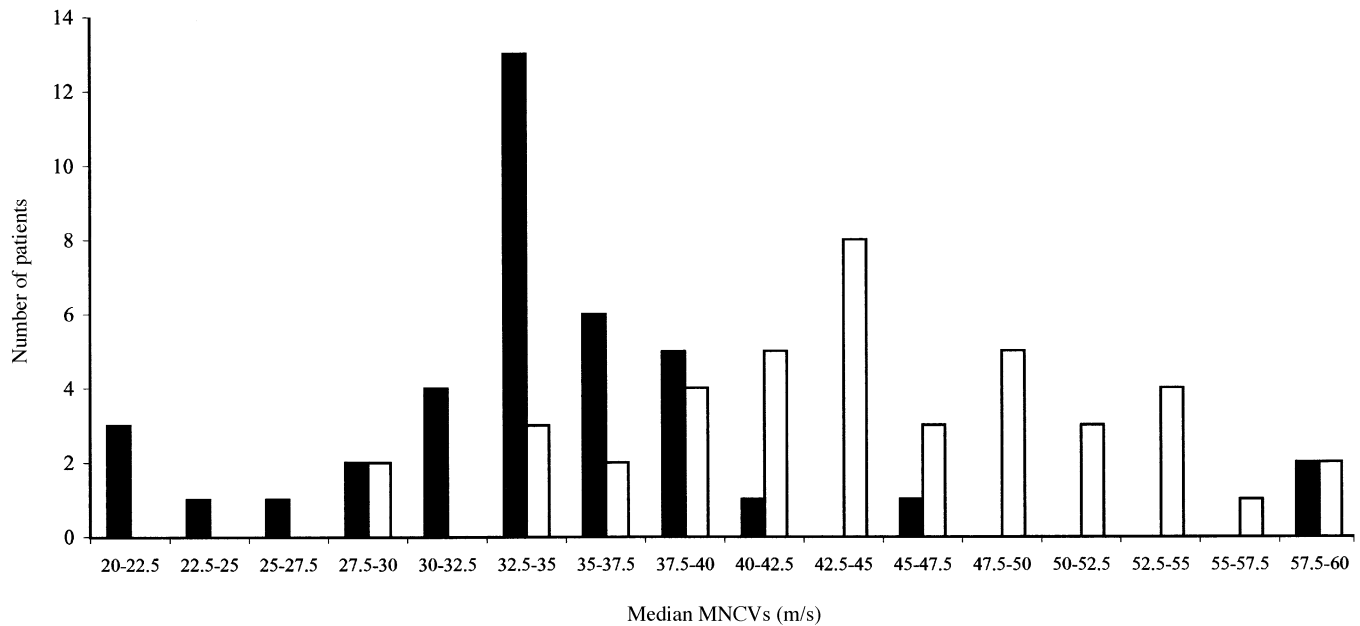


Fig. 1 Range of median MNCVs in the 93 CMTX patients. Males and females are represented by black and white bars, respectively.

last several decades. Such data cannot be used to estimate disease progression.

Electrophysiological findings

General findings

Motor nerve conduction data are shown in Table 2. This study, which included a large number of CMTX patients, confirmed the electrophysiological characteristics that have been described already. MNCVs were significantly more reduced in males than in females. Mean values ranged between 30 and 40 m/s for the median (34.5 ± 6.8 m/s), ulnar (36.1 ± 4.9 m/s) and peroneal nerves (32 ± 8.9 m/s) for males but were only slightly below normal in females. In both sexes, however, median MNCVs below 30 m/s were observed, with lowest values of 20 m/s in a man and 28 m/s in a woman. MNCVs below 30 m/s were found in 18% (7/39) of men but only 5% (2/42) of women (Fig. 1). Mean DML was prolonged in all nerves for males, whereas it remained in the normal range for females, except for the median nerve DML, which was slightly prolonged to a mean of 3.8 ms. Mean distal CMAP was reduced in all nerves for males, particularly in the peroneal nerve, but only in the peroneal and median nerves for females, the peroneal nerve being the most affected.

Axonal loss in upper limbs in CMTX and CMT1A patients

The χ^2 -test was performed to compare the percentages of normal CMAP values in the median and ulnar nerves. Median CMAPs (62%) were significantly more frequently reduced than ulnar CMAPs (29%) in CMTX females ($P < 0.05$).

This difference was not observed in CMTX males (92 versus 81%) or in CMT1A patients (91 versus 93%).

Comparison of motor conduction in upper limbs in CMTX versus CMT1A patients and controls

To test whether motor conduction was heterogeneous in CMTX patients, we compared it with that of CMT1A patients, in whom it is uniformly reduced in all nerves, and with that of controls. The electrophysiological data from a previously published series of 119 CMT1A patients and 100 controls studied in our department with the same protocol were used. We calculated the mean difference between median and ulnar MNCVs of the same limb in CMTX males, CMTX females, CMT1A patients and controls. The peroneal nerve was not chosen for comparison with upper limb nerves because it is the first nerve to be affected in CMT and is frequently unrecordable, which could bias conclusions. The absolute values of the mean differences were compared by Student's *t*-test among the above-mentioned groups. As expected, the mean difference for CMT1A patients did not differ significantly from that for controls (Table 3). The difference between median and ulnar MNCVs in CMTX females was significantly different from that in both CMT1A patients ($P < 0.0001$) and controls ($P < 0.01$). CMTX males did not differ significantly from CMT1A patients or controls. The mean difference in CMTX females also differed significantly from that in CMTX males ($P < 0.05$). These results suggest that MNCVs between the median and ulnar nerves vary more in CMTX females than in CMTX males, CMT1A patients and controls. MNCV was significantly more frequently reduced in the median than in the ulnar nerve in CMTX women ($P < 0.05$). This difference was not observed in CMTX males or

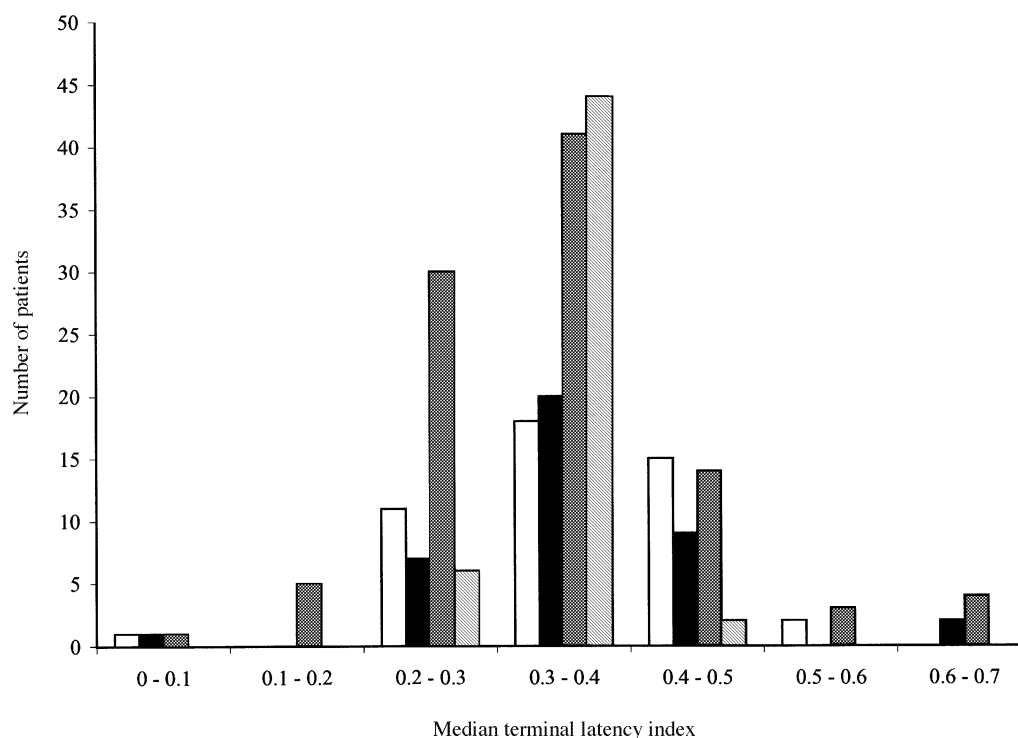


Fig. 2 Histogram of median nerve terminal latency index. CMTX males, CMTX females, CMT1A and controls are represented by black, white, grey and hatched bars, respectively.

Table 3 Mean difference between pairs of median and ulnar nerve MNCVs in CMTX males, CMTX females, CMT1A patients and controls

	CMTX males	CMTX females	CMT1A	Controls
Mean difference (m/s)	$3.7 \pm 2.9^*$	$6.1 \pm 4.8^\dagger$	2.6 ± 2.4	3.3 ± 2.8

*Significant difference between CMTX males and females ($P < 0.05$); † significant difference between CMTX females and CMT1A patients ($P < 0.0001$) and controls ($P < 0.01$).

in CMT1A patients. The median MNCVs in CMTX patients were not significantly related to age or disease duration.

Median nerve TLI

The mean median nerve TLI was 0.37 ± 0.08 in both sexes. In CMT1A patients it was 0.34 ± 0.1 , similar to that of the 100 controls (0.34 ± 0.04). The difference between CMTX males ($P = 0.08$) or females ($P = 0.07$) and CMT1A patients was also not significant. Furthermore, the TLI in the majority of patients in each group was distributed over a narrow range of values. This was expected in CMT1A but was also observed in CMTX patients of both sexes, supporting the hypothesis that demyelination in CMTX patients is uniform throughout the nerve (Fig. 2).

Molecular findings and phenotype-genotype correlation

Twenty-seven different mutations were identified in the 37 families (Table 4). Their distribution throughout the Cx32 protein is shown in Fig. 3. Fourteen were localized in one of the transmembrane domains, seven in the intracellular domains and five in the extracellular loops. No mutations were found in the N-terminal intracellular domain. The vast majority (23 out of 27) of the mutations corresponded to amino acid substitutions. Most of them modified the charge (12) or polarity (four) of the residue, but seven mutations of apolar residues did not change their status (Table 4). Three mutations corresponded to stop codons R22X and R220X or a frameshift insertion (InsC115frameshift) and resulted in truncated proteins. In one case, the entire coding sequence was deleted, resulting in the loss of the CX32 band on an *MspI* Southern blot hybridized with a CX32 exon 2 PCR product.

To our knowledge, four mutations (V91M, InsCframeshift, T130I and G159D) are reported for the first time. None of the three substitutions were detected in 50 normal subjects, which supports the hypothesis that they were responsible for the CMTX phenotype. Some mutations were recurrent, especially R22X, R22G and N205S, which were identified in three apparently unrelated families, confirming the relative frequency of mutations of the R22 residue.

Ages at onset are shown for each mutation in Fig. 4. Ages at onset were distributed more widely in females than in

Table 4 Cx32 mutations

Mutation	Nucleotide change	Amino acid change	Charge change	<i>In vitro</i> test	Family code	First reference
1	64 C → T	R22X		NF	276, 597, 1048	Ionasescu <i>et al.</i> , 1996
2	64 G → A	R22G	+ → A	NF	151, 481, 713	Ionasescu <i>et al.</i> , 1996
3	101 T → C	M34T	A → P	F	108	Tan <i>et al.</i> , 1996
4	100 A → G	M34V	A → A	n.t.	657	Bone <i>et al.</i> , 1997
5	166 C → T	L56F	A → A	F	524	Latour <i>et al.</i> , 1997
	InsC115 frameshift			n.t.	418	This paper
6	250	V84I	A → A	n.t.	273	Rouger <i>et al.</i> , 1997
7	271 G → A	V91M	A → A	n.t.	593	This paper
8	280 C → G	H94D	+ → -	n.t.	1097	This paper
9	282 C → G	H94Q	+ → P	NF	519	Bone <i>et al.</i> , 1997
10	283 G → A	V95M	A → A	NF	51, 157	Bone <i>et al.</i> , 1995
11	319 C → T	R107W	+ → A	n.t.	38, 579	Tan <i>et al.</i> , 1996
12	391 C → T	T130I	P → A	n.t.	753	This paper
13	399 T → C	W133R	A → +	n.t.	288	Bone <i>et al.</i> , 1995
14	423 C → G	F141L	+ → A	n.t.	373, 1061	Rouger <i>et al.</i> , 1997
15	425 G → A	R142Q	A → A	n.t.	1007	Bone <i>et al.</i> , 1997
16	472 C → G	P158A	A → A	n.t.	99	Cherryson <i>et al.</i> , 1994
17	476 G → A	G159D	A → -	n.t.	415	Dubourg <i>et al.</i> , 2001
18	490 C → T	R164W	+ → A	n.t.	341	Ionasescu <i>et al.</i> , 1996
19	491 G → A	R164Q	+ → P	n.t.	715	Bort <i>et al.</i> , 1997
20	556 G → A	E186K	- → +	NF	260	Bergoffen <i>et al.</i> , 1993
21	595 G → C	G199R	A → +	n.t.	623	Janssen <i>et al.</i> , 1997
22	608 T → A	I203N	A → P	n.t.	1023	Rouger <i>et al.</i> , 1997
23	614 A → G	N205S	P → A	n.t.	278, 464, 1020	Bone <i>et al.</i> , 1997
24		I213L/I214Del		n.t.	113	Rouger <i>et al.</i> , 1997
25	643 C → T	R215W	+ → A	NF	347, 812	Fairweather <i>et al.</i> , 1994
26	658 C → T	R220X		F	21	Fairweather <i>et al.</i> , 1994
27		Detection of coding region	+ → A		129	Ainsworth <i>et al.</i> , 1998

Column 4 shows the electric charge of the amino acids: + = positive charge; - = negative charge; P = polar. A = apolar. Column 5 shows the functionality of mutant channels determined *in vitro*: NF = non-functional channel; F = functional channel; n.t. = not tested.

males. Furthermore, there was less variation among males with the same mutation than in the total population of affected males, strongly suggesting an effect of mutation on age at onset. The mutations can be classified into two groups: a group of nine mutations (1, 2, 5, 7, 10, 14, 20, 21 and 25) associated with early age at onset (≤ 10 years) in at least one patient; and the remaining 18 mutations in patients with late onset (> 10 years).

Discussion

We present the clinical, electrophysiological and molecular characteristics of 93 CMTX patients from 37 independent families with 27 CX32 mutations. This is the largest series of CMT patients with identified CX32 mutations reported so far. Our clinical findings confirm the already well-documented characteristics of CMTX (Nicholson and Nash, 1993; Birouk *et al.*, 1998; Hahn *et al.*, 1999). Because of the large number of patients, more differences between males and females were found than in previous studies. Men are more severely affected than women, with significantly more frequent muscle weakness, muscle wasting, loss of proprioception, upper limb areflexia and pes cavus. Interestingly, onset before age 10 years was encountered in 27% of males and 15% of

females, although it has been thought that the onset of CMTX occurs mostly in the second decade (Birouk *et al.*, 1998). Asymptomatic females were significantly more frequent than asymptomatic males (29 and 11%, respectively). High FDS was more frequent in males (59%) than in females (19%). Associated signs were present exclusively in males, and were limited to 7% with upper limb tremor and 2% with auditory loss. However, infraclinical involvement of central visual, acoustic and motor pathways has been demonstrated in an X-linked family with an A205S mutation (Bahr *et al.*, 1999). Nicholson and colleagues (Nicholson *et al.*, 1998) considered that abnormal brainstem evoked auditory potentials, together with intermediate nerve conduction velocities, were efficient in identifying patients with CX32 mutations in families without male-to-male inheritance. Finally, deafness (Stojkovic *et al.*, 1999) and other CNS involvement (Panas *et al.*, 1998) have been reported in some CMTX families. No systematic study of CNS pathways was performed in our patients.

Some electrophysiological findings are also in line with previous reports (Nicholson and Nash, 1993; Birouk *et al.*, 1998). Men and women could be distinguished on the basis of MNCVs, DMLs and CMAPs that were slower, longer and lower, respectively, in men in all nerves. Interestingly, median

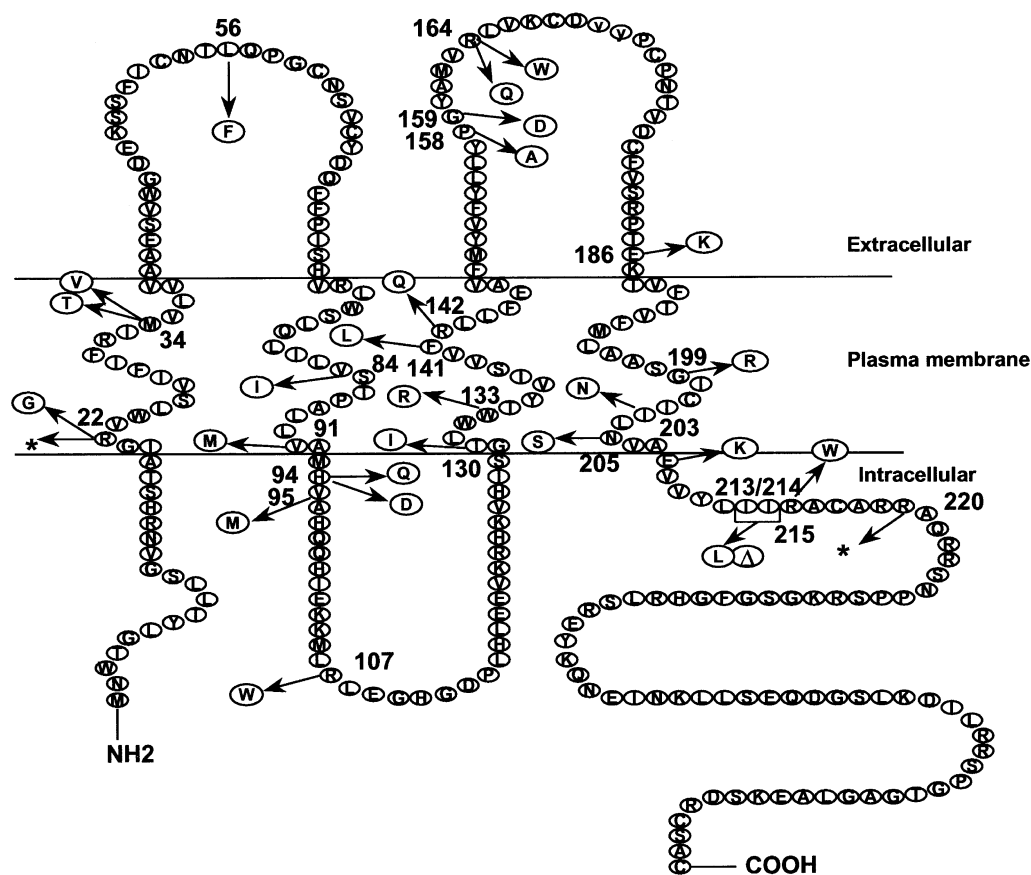


Fig. 3 Model of the structure of the Cx32 protein, showing the mutations reported here. *Nonsense mutation; Δ = deletion. Numbers in bold characters correspond to the positions of the mutations.

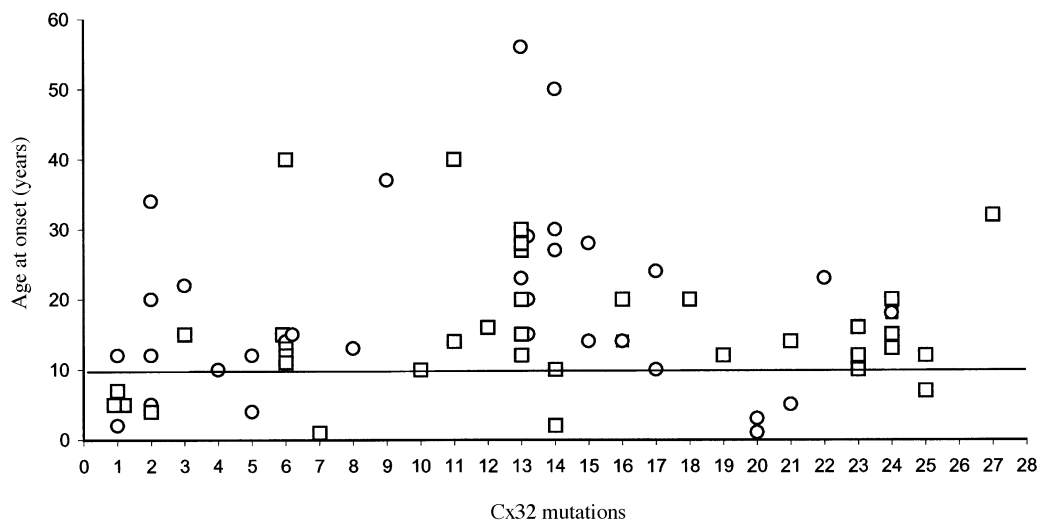


Fig. 4 Age at onset of the patients with CX32 mutations (numbered from 1 to 27). The corresponding mutations are described in Table 4. Males are represented by white squares, females by white circles.

MNCVs were <30 m/s in 18% (seven out of 39) of men but only 5% (two out of 42) of women. Thus, median MNCVs in the CMT1 range remain anecdotal in women but concern one-fifth of males with a CX32 mutation. It is important to

take this into account because CX32 is the second most frequent gene affected in CMT1, after the 17p11.2 duplication (Nelis *et al.*, 1996; Dubourg *et al.*, 2001). The nature of the neuropathy in CMTX—primarily axonal

or demyelinating—has been debated but no consensus has yet emerged. Axonal and demyelinating features are undoubtedly mixed and closely related in CMTX. We chose to focus the electrophysiological study on motor conduction in CMTX and, particularly, to estimate whether motor conduction in the upper limbs was heterogeneous among and within nerves, as has been suggested recently (Tabaraud *et al.*, 1999; Gutierrez *et al.*, 2000). CMT1A, in which demyelination is uniform, was selected for comparison. Since uniform demyelination results in similar decreases in MNCVs, the difference between two nerves should be small, particularly when upper limb nerves are compared. Lower limb nerves are too severely affected in CMT for comparison. To test whether conduction was uniform in CMTX, we compared the mean differences between MNCVs in the median and ulnar nerves in CMTX males, CMTX females, CMT1A patients and controls. As expected, the mean difference was low in CMT1A patients, as it was also in controls. The mean difference was greater in CMTX patients, particularly in females, and differed significantly from that in CMT1A patients. Motor conduction is, therefore, not uniform in CMTX females; there is marked variation of velocities within motor nerves of the same limb. In contrast, similarity between CMTX males and CMT1A patients supports the hypothesis that demyelination in CMTX males is rather homogeneous. These results are similar to those of the electrophysiological study of Gutierrez and colleagues in five patients (two females and three males) of a family with an R15W CX32 mutation, in which the range of conduction velocities among nerves was wide, particularly in women (Gutierrez *et al.*, 2000).

The inter-nerve heterogeneity of MNCVs in CMTX females can be partly explained by Lyonization or X-inactivation. Each nerve is colonized by a very small number of Schwann cell precursors, in which X-inactivation varies among nerves. When most of the Schwann cells in a nerve have the normal X chromosome inactivated, demyelination is severe and MNCVs are greatly decreased. However, when the mutated X chromosome is inactivated, MNCV remain normal or subnormal. For ethical reasons, it is difficult to confirm this hypothesis in CMTX patients as it requires the study of nerves at the cellular level.

The TLI is usually used to show differential slowing of motor conduction between distal and proximal segments of a nerve. In this study, the median nerve TLI was the same in males and females with CMTX, but greater, although not significantly so, than in CMT1A patients and controls. Median nerve TLI varied very little in controls. In CMT1A patients the range of values was wider but remained concentrated around the mean. A similar distribution was observed in males and females with CMTX, suggesting that demyelination occurring in CMTX is rather homogeneous throughout a given nerve. Previous studies have suggested that temporal dispersion might be specific to CMTX neuropathy (Tabaraud *et al.*, 1999; Gutierrez *et al.*, 2000; Sumner *et al.*, 2000). It was demonstrated in only a few patients. We also observed temporal dispersion in at least one female patient in this

study (Family 524), as well as conduction blocks outside the usual nerve entrapment sites in two patients (Families 151 and 657) (data not shown). However, data concerning the presence of conduction blocks or temporal dispersion were not available for all patients in our study. A systematic analysis of motor conduction on different segments of the nerves would undoubtedly be the best way to determine whether demyelination is heterogeneous in CMTX. Nevertheless, the similarity between median nerve TLI between CMTX and CMT1A patients may answer the question partially, in that it suggests that differential demyelination in different segments of a given nerve is not more common in CMTX than in any other inherited neuropathy.

Axonal loss differed between the median and ulnar nerves in CMTX females. Indeed, median nerve CMAPs were more frequently reduced than ulnar nerve CMAPs in CMTX females. This was not observed in CMTX males or in CMT1A patients. The vulnerability of the median nerve in CMTX women may be related to the frequency of carpal tunnel syndrome in women rather than to a property of this nerve.

The functional consequences of several CX32 mutations have been studied previously in *Xenopus* oocytes and mammalian cells (Castro *et al.*, 1999; Abrams *et al.*, 2000; Ressot *et al.*, 2000). Some mutations have been classified as 'functional', when the affected connexons conserve channel activity, or 'non-functional', when the connexons are inactivated. Some of the mutations reported in the present paper have already undergone functional analyses (Table 4). Correlations were found between biological data and age at onset, which was used as a criterion of severity in previous studies (Birouk *et al.*, 1997, 1998). The R22X(1), R22G(2), L56F(5), V91M(7), V95M(10), F141L(14), E186K(20), G199R(21) and R215W(25) mutations were associated with onset in the first decade in at least one patient (Fig. 4). Four of the five mutations tested for function, R22G(2), V95M(10), E186K(20) and R215W(25), were found to be non-functional, and only L56F(5) was functional. In addition, R22X leads to a very short protein and undoubtedly forms inactive channels. A female with a V91M (7) substitution was also affected at the age of 1 year. The electrophysiological consequences of this mutation have not been tested. However, we can hypothesize that the X chromosome with the normal gene was preferentially inactivated in her Schwann cell progenitors and that the mutation had drastic consequences at the protein level, since it is close to the membrane-cytoplasm interface at the internal pore gate (Rabadan-Diehl *et al.*, 1994; Castro *et al.*, 1999; Martin *et al.*, 2000). For the 18 remaining mutations, onset was always after age 10. Only two of these mutations, M34T(3) and R220X(26), were analysed in cellular models. In both cases, the channel was active. The W133R(13) is associated with a relatively late age at onset in males (ranging from 12 to 30 years, mean = 20 years), demonstrating that changes in electric charges ($\Delta = +1$) in the pore did not change channel activity

dramatically. It should be noted that two males with mutations in the second extracellular loop also had a relatively late age at onset (20 years). Most striking was the late age at onset of the male in whom the complete coding sequence of the gene was deleted. A deletion of the entire coding sequence of *CX32* has been reported previously, but it led to a severe phenotype with onset in the first decade (Ainsworth *et al.*, 1998; Hahn *et al.*, 2000). The discrepancy between the two observations may be explained by compensation during development, which has been postulated to explain the absence of a phenotype in knockout mice. We can therefore hypothesize that genes encoding other connexins are derepressed in Schwann cells in this patient because of the lack of expression of the *CX32* gene. This case also contrasts with CMTX patients with a R22X mutation, which leads to a very short protein and a more severe phenotype. Mean median nerve MNCVs with non-functional mutations ranged from 30 to 38.7 m/s (mean = 34.3 m/s) in males and from 35.6 to 57 (mean = 42.3 m/s) in females. These values were similar to those of the patients as a whole, suggesting that the functional properties of Cx32 are not directly correlated with the degree of demyelination in CMTX patients.

This study extends the number of differences between males and females affected with CMTX and provides preliminary evidence that the type of *CX32* mutation influences the phenotype. However, a systematic functional study of *CX32* mutations should be performed to confirm these effects and determine their mechanism.

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