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Screening for *SH3TC2* gene mutations in a series of demyelinating recessive Charcot-Marie-Tooth disease (CMT4)

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Abstract

Charcot-Marie-Tooth disease type 4C (CMT4C) is an autosomal recessive (AR) demyelinating neuropathy associated to *SH3TC2* mutations, characterized by early onset, spine deformities, and cranial nerve involvement.We screened 43 CMT4 patients (36 index cases) with AR inheritance, demyelinating nerve conductions, and negative testing for *PMP22* duplication, *GJB1* and *MPZ* mutations, for *SH3TC2* mutations. Twelve patients (11 index cases) had CMT4C as they carried homozygous or compound heterozygous mutations in *SH3TC2*. We found six mutations: three nonsense (p.R1109*, p.R954*, p.Q892*), one splice site (c.805+2T>C), one synonymous variant (p.K93K) predicting altered splicing, and one frameshift (p.F491Lfs*32) mutation. The splice site and the frameshift mutations are novel. Mean onset age was 7 years (range: 1–14). Neuropathy was moderate-to-severe. Scoliosis was present in 11 patients (severe 7 in 4), and cranial nerve deficits in 9 (hearing loss in 7). Scoliosis and cranial nerve involvement are frequent features of this CMT4 subtype, and their presence should address the clinician to look for *SH3TC2* gene mutations. In our series of undiagnosed CMT4 patients, *SH3TC2* mutation frequency is 30%, confirming that CMT4C may be the most common AR-CMT type.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Keywords

Charcot-Marie-Tooth disease; CMT type 4C; hereditary motor sensory neuropathy; recessive demyelinating CMT; *SH3TC2* gene

Introduction

Charcot-Marie-Tooth neuropathy type 4C (CMT4C) is a demyelinating neuropathy, frequently characterized by spine deformities and cranial nerve involvement, associated with recessive mutations in the *SH3TC2* (SH3 domain tetracotripeptide repeats 2) gene (Senderek et al., 2003). CMT4C might be the most frequent autosomal recessive (AR) form of CMT overall (Laššuthová et al., 2011; DiVincenzo et al., 2014). Neuropathy usually develops in the first decade of life or adolescence, but occasionally causes motor delay during infancy. Within the context of AR-CMT, it has a mild-to-moderate severity and a slowly progressive course. Rarely, patients become wheelchair restricted because of proximal lower limb involvement (Laššuthová et al., 2011).

Affected subjects usually develop early and severe scoliosis or kyphoscoliosis in the first decade of life or, less commonly, early in the second decade (Senderek et al., 2003). Indeed, spine deformities appear to be the presenting sign in several patients (Kessali et al., 1997; Gabreëls-Festen et al., 1999; Azzedine et al., 2006). In a large series of patients reported by Azzedine and coauthors, almost all patients (27 out of 28) presented scoliosis (Azzedine et al., 2006). The degree of scoliosis severity is broad, ranging from moderate to very disabling, may cause respiratory difficulties (Gosselin et al., 2008) and required surgery in 7%–39% (Kessali et al., 1997; Gabreëls-Festen et al., 1999).

Cranial nerve involvement is also often reported, with variable occurrence of diplopia, facial weakness, hearing loss, dysphagia, tongue atrophy with fasciculation, and vocal cord paresis (Senderek et al., 2003; Azzedine et al., 2006; Colomer et al., 2006; Yger et al., 2012). Reviewing previous series, Azzedine and coauthors found that hearing loss was reported in 7 out of 46 patients and complete deafness in other 7 (Azzedine et al., 2008).

Electrophysiological studies show nerve conduction velocity (NCV) in the demyelinating range and variable evidence of axonal loss (Gosselin et al., 2008; Houlden et al., 2009; Yger et al., 2012).

Nerve biopsy shows rather characteristic myelin abnormalities, with loss of large myelinated fibers, very thin myelin sheaths and even completely demyelinated axons, rare focal myelin outfoldings, extensive Schwann cell proliferation with small onion bulbs as well as excess basal lamina membrane to form basal lamina onion bulbs. The presence of T-lymphocytes cell infiltrates has been reported (Gabreëls-Festen et al., 1999; Houlden et al., 2009).

Several, mainly nonsense, *SH3TC2* mutations have been described thus far (see Human Gene Mutation Database, HGMD, http://www.hgmd.cf.ac.uk/). The function of SH3TC2, localized both in Schwann and neuronal cells, is still poorly understood and is likely related to intracellular vesicular trafficking (Pareyson et al., 2014).

We report clinical, electrophysiological, and pathological features, and molecular data in our CMT4C cases identified from a cohort of recessive demyelinating CMT patients (CMT4).

Patients and Methods

Inclusion criteria

Forty-three patients (36 index cases and 7 affected relatives) were selected among CMT subjects seen during the last 10 years (2006–2015) with information archived in the inherited peripheral neuropathy database of the IRCCS Foundation "C. Besta" Neurological Institute. Patients were selected for *SH3TC2* gene screening according to the following inclusion criteria: (1) pedigree compatible with AR inheritance, (2) demyelinating pattern on NCS (motor conduction velocity [MCV] less than 38 m/s in upper limb motor nerves), (3) previous exclusion of the *PMP22* duplication, *GJB1* and *MPZ* mutations.

Genetic tests

Written informed consent was obtained from all patients or the relative guardian in pediatric cases. *SH3TC2* gene was analyzed by Sanger sequencing and next-generation sequencing (NGS) technology. In the first set of 23 index cases, we used an amplicon-based customized NGS panel (Illumina Inc., San Diego, CA USA TruSeq Custom Amplicon, TSCA) covering 54 CMT causative genes. In the second set of 13 index cases, we used a probe-based customized panel (Illumina Nextera Rapid Capture Custom kit) which included a larger number of CMT causative genes (n=94). Sequencing was performed using the NGS MiSeq sequencer (Illumina). The entire *SH3TC2* targeted region (17 coding exons ± 20 bp of flanking introns) was sequenced by NGS with a depth of coverage >20×. Reads were mapped against the hg19 standard reference genome to detect single nucleotide polymorphisms (SNPs), single nucleotide variants (SNVs), short deletions and insertions. Data analysis was performed using: (1) MiSeq Reporter software (Illumina), for quality control, alignment and variant calling, (2) VariantStudio software (Illumina), for variants annotation, (3) CLC Genomics Workbench software (CLCbio, Qiagen, Venlo, Netherlands), for quality control and coverage analysis. All sequence variants were verified using Sanger methods. Sequence variants were annotated and filtered with public human genetic variation/mutation databases (SNP database 137, NHLBI Exome Sequencing Project 6500, 1000 Genomes project, Human Gene Mutation Database). *In silico* prediction of synonymous or intronic variants effect on splice site was performed using NNSplice predictor (Reese et al., 1997; http://www.fruitfly.org/seq_tools/splice.html) and Alternative Splice Site Predictor (Wang and Marín, 2006; http://wangcomputing.com/assp/).

Results

Genetic and clinical data of the patients carrying *SH3TC2* mutations are summarized in Table 1 and electrophysiological findings in Table 2.

Genetic findings

SH3TC2 mutations were found in 11 out of 36 index cases and in the affected sibling of Pt4. NGS analysis of all the 11 index cases using a customized disease-specific multi-gene panel

showed a number of heterozygous nonsynonymous variants with minor allele frequency (MAF) <1% in other genes known to be associated with hereditary neuropathies (CMT1–4, CMT2, HMN, and HSAN) (Table S1, Supporting Information). However, none of these variants were pathogenic or likely pathogenic according to the variant classification criteria of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards et al., 2015). All subjects but the two sisters Pt4 and Pt5 were unrelated. Overall, we found six pathogenic mutations, two of which are novel (p.F491Lfs*32 and c.805+2 T>C). Three mutations were found in homozygosity in five unrelated patients: the recurrent mutations p.R954* (Pt7) and p.R1109* in Pt1 and Pt6, and the novel mutation c.805+2 T>C in Pt2 and Pt10. The remaining seven patientswere compound heterozygous for different mutations. Pt3 had the two most common mutations (p.R954* and p.R1109*) in compound heterozygosity. The rare p.Q892* mutation was found in two unrelated index cases from Albania, each one carrying a second different mutation (p.R954* or p.R1109*). The p.F491Lfs*32 mutation, found in association with p.R954* in two unrelated patients (one from Albania), has never been described so far. The c.805+2 T>C mutation is also a novel intronic sequence alteration, deleting the constitutive donor site of intron 7. Interestingly, the silent c.279G>A mutation (p.K93K) in exon 3 is also predicted to alter splicing, eliminating the donor site of this exon (Laššuthová et al., 2011).

Clinical findings

Mean age at examination was 39 years (range: 16–68); three patients were aged less than 18 years. Mean onset age was 7 years (range: 1–14) and mean disease duration at examination was 33 years (range: 15–60). Four index cases had a pedigree compatible with AR inheritance, whereas seven were sporadic cases. All were sporadic cases but Pt4 and her sister Pt5, and Pt10, whose affected brother never came to visit.

Foot deformities and walking difficulties related to neuropathy starting in lower limbs were the presenting symptoms in almost all patients (11 out of 12). Neuropathy onset occurred in the first decade in nine patients, and two of them had motor development delay. In one subject (Pt5), scoliosis was the heralding disease manifestation at the age of 6 years, while the neuropathy became evident later during adolescence. Four patients developed lower limb proximal weakness (Pt1, Pt6, Pt11, Pt12) and one (Pt11) had also involvement of proximal upper limbs. Three subjects (Pt6, Pt7, Pt11) showed marked difficulty in standing without support, positive Romberg sign, and sensory gait ataxia.

Scoliosis was present in 11 patients, manifesting within the first two decades of life. Four patients had severe scoliosis, requiring surgery in two cases (Pt6, Pt7) after prolonged bracing during childhood. Currently, one young patient (Pt3) still wears braces. Marked foot deformities required surgery in two cases (Pt3, Pt11).

Cranial nerve involvement was present in nine patients. Hearing loss was the most frequent manifestation, occurring in seven patients, while four had diplopia and strabismus related to oculomotor nerves' dysfunction, and one had also tongue atrophy with fibrillation (Pt1). Two patients (Pt8, Pt9) had subclinical optic pathway involvement as documented by mild prolongation of P100 wave latency at Visual Evoked Potentials (VEPs).

Mean CMT examination score (CMTES) and CMT neuropathy score (CMTNS) were in the interval of moderate severity: 15 out of 28 (range: 6–25) and 19 out of 36 (range: 8–28), respectively. Disease severity according to the CMTES/CMTNS was variable: mild in one, moderate in seven, and severe in four patients (Pt1, Pt6, Pt11 need a cane and Pt12 a double support to stand and walk).

Electrophysiological data

Electrophysiological data are summarized in Table 2. We observed a variable degree of temporal dispersion, but no evidence of conduction block. Ulnar and median motor nerves showed the same degree of impairment, as did peroneal and tibial nerves. Lower limbs were more affected than upper limbs. Overall, sensory nerves were more affected than motor nerves, as sensory action potentials (SAPs) were often undetectable.

Neuropathological data

Sural nerve biopsy from two patients (Pt1, Pt6) showed marked loss of myelinated fibers. The residual fibers were thinly myelinated and surrounded by Schwann cell proliferations resulting in small onion bulbs. Occasional axonal regeneration clusters, myelin outfoldings, and focal thickenings were found. Most teased fibers (Pt6) showed evidence of paranodal or intrasegmental de-remyelination; 5%–10% of the internodes had focal thickenings. In Pt6, we also observed endoneural edema, lymphocyte infiltrates, and few complement (Cd3) deposits in vessel wall.

Discussion

We report the clinical, electrophysiological, pathological, and genetic findings of 12 CMT4C patients. Mean onset age was early (7 years) and all subjects developed symptoms before the age of 15 years, findings largely in keeping with data from other series (Table 3) (Kessali et al., 1997; Senderek et al., 2003; Gosselin et al., 2008; Houlden et al., 2009; Laššuthová et al., 2011; Yger et al., 2012), although later onset during adulthood has been described (Gooding et al., 2005; Yger et al., 2012).

Gait difficulty related to neuropathy was the presenting symptom in almost all subjects, as only one developed scoliosis before neuropathy. These data are partially in contrast with previous reports describing early and severe scoliosis as presenting sign in most individuals (Kessali et al., 1997; Gabreëls-Festen et al., 1999; Azzedine et al., 2006). However, spine deformities appeared comparably early and were very common (92% of subjects), similarly to previous reports (Table 3) (Kessali et al., 1997; Gabreëls-Festen et al., 1999; Azzedine et al., 2006; Gosselin et al., 2008; Houlden et al., 2009; Yger et al., 2012). Four patients had severe spine deformities, requiring surgery in two cases. Usually scoliosis is severe when CMT neuropathy is highly disabling, whereas in CMT4C, early and marked spine deformities suggest dissociation between neuropathy and scoliosis severity. The pathophysiology of scoliosis in this context is poorly understood as it has been attributed either to premature motor deficit in the paravertebral muscles or to a role of SH3TC2 in spine development (Azzedine et al., 2006).

Cranial nerve involvement was evident in nine patients and occurred independently from disease duration and neuropathy severity (Tables 1 and 3). Hearing loss (seven cases) and oculomotor dysfunction (diplopia in four patients) were the most frequent symptoms. Cranial nerve involvement may occur in CMT patients, but its characteristic presence in CMT4C, useful for diagnosis, suggests a distinctive association and possibly a specific pathogenic role of SH3TC2 in these nerve tracts. Interestingly, Pt11 had ocular flutter associated to eyelid flickering, both not reported so far. Although we cannot be sure that this phenomenon is related to CMT4C and to peripheral nerve damage, as it is usually due to brainstem degenerative lesions which however were not found in the present case, nystagmus was previously reported in two patients (Senderek et al., 2003). The subclinical optic pathways abnormalities at VEPs detected in two patients are also unprecedented in CMT4C.

Although some authors hypothesized an early proximal involvement (Gooding et al., 2005; Yger et al., 2012), we found proximal lower limb weakness only in one-third of the patients, and it was correlated with disease duration. Sensory ataxia was evident in three patients and contributed to disability. Four subjects lost independent deambulation (Pt1, Pt6, Pt11, Pt12) needing single support to stand and walking, mainly because of sensory ataxia rather than to lower limb proximal weakness. Overall, neuropathy was moderate-to-severe according to the CMTES and CMTNS. These data suggest that CMT4C is less severe than other AR-CMT (Azzedine et al., 2006; Pareyson et al., 2014).

All our patients showed clear-cut electrophysiological evidence of sensorimotor demyelinating neuropathy with variable degrees of axonal loss. Slowing of motor and sensory nerve conduction was homogeneous in different nerves and in all limbs. However, based on amplitudes of motor and sensory responses, lower limb nerves were definitively more compromised than upper limbs and sensory nerves appeared to be more affected than motor nerves, as also reported by other authors (Senderek et al., 2003; Yger et al., 2012). Unlike Azzedine and colleagues (Azzedine et al., 2006), we found no correlation between nerve conduction slowing and disease duration.

The most frequent mutations were p.R954* (8 out of 24 mutated alleles) and p.R1109* (6 out of 24 mutated alleles). These data are largely in agreement with the literature (Laššuthová et al., 2011). The p.R954* was the most prevalent *SH3TC2* mutation in the Czech (63% of pathogenic alleles) (Laššuthová et al., 2011), Dutch (5 out of 6 families) (Gabreëls-Festen et al., 1999; Azzedine et al., 2006), and French (10 out of 16 patients) populations (Yger et al., 2012). Laššuthova and coauthors suggested a genotype–phenotype correlation for patients with homozygous or heterozygous p.R954* mutation as their patients manifested an early onset and mild-to-moderate neuropathy with foot deformities and scoliosis (Laššuthová et al., 2011). However, we and others observed a great variability in disease severity for R954* mutation carriers (Kessali et al., 1997; Gabreëls-Festen et al., 1999; Senderek et al., 2003; Azzedine et al., 2006; Varley et al., 2015). The p.R1109* sequence variation is reported as the most frequent mutation in the Gypsy population. Claramunt and coauthors found this mutation in 20 out of 21 *SH3TC2* alleles in CMT4C patients, with a likely founder effect (Claramunt et al., 2007). However, we found it in some Italian and Albanian unrelated patients without any known Gypsy ancestry. Haplotype

analysis could help clarifying whether all the p.R1109* alleles stem from the same founder allele, regardless of the ethnic origin of the patients. We also observed the previously described p.Q892* mutation (Yger et al., 2012), found in the two sisters (Pt4 and Pt5) and in an unrelated patient (Pt8). All are from the same region of Albania, suggesting a possible founder effect. The p.F491Lfs*32 is a novel mutation and was found in association with the p.R954* mutation in two unrelated sporadic cases, both with very early onset (1 year) and motor development delay, although disease severity at the same age of examination (17 years) was quite different (Pt9 and Pt11, CMTNS: 14 and 28, respectively). The c.805+2 T>C sequence change is also novel and was found in homozigosity in two unrelated patients from Calabria, a region of Southern Italy. The mutation changes the consensus donor splice site GT to GC. Consistently, *in silico* analyses predict the abolishment of the site (NNSplice Site Predictor score: wild type: 0.30, mutated: 0; Alternative Splice Site Predictor: wild type: 8,47, mutated: 0). Unfortunately, no patient's cells were available to verify the effect of this mutation on the mRNA. In conclusion, in our well-selected cohort of undiagnosed CMT4 patients, *SH3TC2* mutations accounted for about 30% of the index cases (11 out of 36), a percentage similar to that reported by Azzedine and coauthors in a series from Northern Europe and Northern Africa (10 out of 38 families, 26%) (Azzedine et al., 2006). Therefore, CMT4C appears to be the most frequent form not only of the demyelinating CMT4 but also of all AR-CMT, as suggested also by gene mutation distribution in other series (Murphy et al., 2012; DiVincenzo et al., 2014; Fridman et al., 2015; Zimoń et al., 2015), although in some regions of Southern Europe (i.e., Spain and Southern Italy) CMT4A associated with *GDAP1* mutations appears the most common AR-CMT subtype (Sivera et al., 2013; Manganelli et al., 2014), probably because of founder effects in these populations. Our Italian CMT4C patients came from all parts of the country, suggesting that overall in Italy CMT4C may be more prevalent that CMT4A. Finally, our data further emphasize that scoliosis associated with cranial nerve involvement in demyelinating CMT are important diagnostic findings to prompt molecular screening for *SH3TC2* mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Clinical and genetic findings of CMT4C patients.

Pt, gender	Age		Inherit. pattern	Motor delay	Spine deformities*	Cranial nerves involved	Foot/spine surgery	CMTES/ CMTNS	*SH3TC2* mutation	
	Current	Onset							cDNA†	Protein
Pt1, F	53	10	S	No	+++, #	8th; 12th	Prescribed for scoliosis	25/28≠	c.3325C>T	p.R1109*
									c.3325C>T	p.R1109*
Pt2, F	34	6	S	No	++	No	No	13/17≠	c.805+2T>C	AS
									c.805+2T>C	
Pt3, M	16	1	S	No	+++, #	No	Foot	6/8	c.2860C>T	p.R954*
									c.3325C>T	p.R1109*
Pt4, F	43	14	AR§	No	No	8th	No	14/18≠	c.2674C>T	p.Q892*
									c.2860C>T	p.R954*
Pt5, F	47	6	AR§	No	++	Oculomotor; 7th; 8th	No	12/17≠	c.2674C>T	p.Q892*
									c.2860C>T	p.R954*
Pt6, M	55	3	AR¶	No	+++	Oculomotor; trigeminal neuralgia; 8th	Spine (18 years)	21/27	c.3325C>T	p.R1109*
									c.3325C>T	p.R1109*
Pt7, M	40	13	AR¶	No	+++	No	Spine (26 years)	12/18	c.2860C>T	p.R954*
									c.2860C>T	p.R954*
Pt8, F	37	14	S	No	++	Abnormal VEPs; oculomotor	No	10/11	c.2674C>T	p.Q892*
									c.3325C>T	p.R1109*
Pt9, M	17	1	S	Yes	++, #	Abnormal VEPs; 8th	No	9/14	c.1470_1473delCTTC	p.F491Lfs*32
									c.2860C>T	p.R954*
Pt10, F	43	3	AR§	No	+	8th	No	13/17	c.805+2T>C	AS
									c.805+2T>C	
Pt11, M	17	1	S	Yes	+	Oculomotor; nystagmus; 7th	Foot	21/28	c.1470_1473delCTTC	p.F491Lfs*32
									c.2860C>T	p.R954*
Pt12, F	68	8	S	No	++	8th	No	19/na	c.279G>A	p.K93K (AS)
									c.2860C>T	p.R954*

AR, autosomal recessive; AS, predicted abnormal splicing; F, female; M, male; na, not available; S, sporadic; VEPs, Visual Evoked Potentials.

[*] Spine deformities: +, mild; ++, moderate; +++, severe; #, kyphoscoliosis.

[†] cDNA numbering relative to the coding sequence.

[‡] Version 1 of CMTNS (left ulnar SAP).

[§] One or more affected siblings.

[¶] Consanguineous parents.

Table 2

Electrophysiological findings of CMT4C patients.

	Left upper limb NCS										Left lower limb NCS					
	Median nerve				Ulnar nerve				Radial nerve		Peroneal nerve		Tibial nerve		Sural nerve	
	Motor		Sensory		Motor		Sensory		Sensory		Motor		Motor		Sensory	
	CV	CMAP	CV	SAP	CV	CMAP	CV	SAP	CV	SAP	CV	CMAP	CV	CMAP	CV	SAP
Pt1, F	25	5.2	27	4.6	24	4.3	25	5.3	np	np	–	Abs	14	0.1	–	Abs
Pt2, F	np	np	–	Abs	27	6.3	–	Abs	np	np	np	np	23	0.3	–	Abs
Pt3,M	38	10.3	38	3.8	30	9.2	38	6.6	42	6.5	18	0.9	24	1.4	–	Abs
Pt4, F	np	np	np	np	38	7.2	45	13.3	np	np	–	Abs	24	1.4	–	Abs
Pt5, F	np	np	np	np	31	6.6	–	Abs	np	np	27	1.2	22	0.8	–	Abs
Pt6, M	26	5.4	–	Abs	31	5.8	–	Abs	–	Abs	21	4.4	20	1.1	–	Abs
Pt7, M	20	6.9	–	Abs	21	3.5	–	Abs	–	Abs	11	0.5	13	1.0	–	Abs
Pt8, F	34	2.6	np	np	43	6.0	44	7	63	12	28	0.7	np	np	–	Abs
Pt9, M	17	2.1	–	Abs	21	2.0	–	Abs	31	1.1	9	0.1	19	0.3	–	Abs
Pt10,F	30	6.6	–	Abs	31	3.5	np	np	38	8.2	23	1.0	26	0.8	–	Abs
Pt11, M	17	2.5	–	Abs	18	3.8	–	Abs	–	Abs	18	3.6	9	0.2	–	Abs
Mean	26	5.2	33	1.1	29	5.3	38	3.2	44	4.0	19	1.2	19	0.7	–	–

Abs, absent; CV, conduction velocity (m/s); CMAP, compound muscle action potential amplitude (mV); F, female; M, male; Np, not performed; SAP, sensory action potential amplitude (μV).

Table 3

Comparison between our series and literature reports.

Series	Number of pts.	Mean age at onset (years)	Scoliosis % (present/total subjects)	Cranial nerve involvement (most frequently affected nerve and relative %)
Kessali et al., 1997	12	5.2	83 (10/12)	Present
Gabreëls-Festen et al., 1999	11	Unknown	82 (9/11)	Unknown
Senderek et al., 2003	18	Unknown*	61 (11/18)	Present (8th, 17%)
Gooding et al., 2005	12	Unknown†	25 (3/12)	Present
Azzedine et al., 2006	28	Unknown	96 (27/28)	Present (8th, 12%)
Gosselin et al., 2008	17	6.6	94 (16/17)	Present (8th, 47%)
Houlden et al., 2009	6	5.3	100 (6/6)	Present (8th, 33%)
Laššuthová et al., 2011	17	<10	59	Present (8th, 18%)
Yger et al., 2012	14	7	86	Present (8th, 57%)
Our series	12	7	92 (11/12)	Present (8th, 58%)

*Mean age at first examination is 8 years (when reported); in seven subjects the first examination was during infancy.

†mean age at first examination is 17.5; in two subjects the first examination occurred during infancy.