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ScienceDirect

Neuromuscular Disorders 29 (2019) 776–785

www.elsevier.com/locate/nmd

Report of a novel ATP7A mutation causing distal motor neuropathy

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Received 17 April 2019; received in revised form 9 August 2019; accepted 20 August 2019

Abstract

We describe a novel ATP7A gene mutation associated with distal motor neuropathy, mild connective tissue abnormalities and autonomic disturbances. Next-generation sequencing analysis of a lower-motor neuron diseases gene panel was performed in two sibs presenting with distal motor neuropathy plus an autonomic dysfunction, which main manifestations were retrograde ejaculation, diarrhea and hyperhydrosis. Probands underwent dysmorphological, neurological, electrophysiological as well as biochemical evaluations and somatic and autonomic innervation studies on skin biopsies. A novel missense mutation (p.A991D) was identified in the X-linked ATP7A gene, segregating in both brothers and inherited from their healthy mother. Biochemical studies on patients' blood samples showed reduced serum copper and ceruloplasmin levels. Clinical and neurophysiological evaluation documented dysautonomic signs. Quantitative evaluation of skin innervation disclosed a small fiber neuropathy with prevalent autonomic involvement. Mutations in the ATP7A gene, encoding for a copper-transporting ATPase, have been associated with the severe infantile neurodegenerative Menkes disease and in its milder variant, the Occipital Horn Syndrome. Only two ATP7A mutations were previously reported as causing, a pure axonal distal motor neuropathy (dHMN-SMAX3). The phenotype we report represents a further example of this rare genotype-phenotype correlation and highlights the possible occurrence in SMAX3 of autonomic disturbances, as described for Menkes disease and Occipital Horn Syndrome.

Keywords: ATP7A mutation; Peripheral neuropathy; Autonomic neuropathy.

1. Introduction

ATP7A is a transporting P-type ATPase representing a key component of the copper homeostatic machinery and expressed in a wide range of tissues [1]. In all cells, the protein is located within the trans-Golgi network (TGN) to supply copper to cuproenzymes. To regulate metal homeostasis, ATP7A constitutively cycles between the TGN and plasma membrane (PM). ATP7A trafficking to the PM is elevated in response to increased copper load (in order to eliminate the excess of metal from the cell) and is reversed

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https://doi.org/10.1016/j.nmd.2019.08.008
0960-8966/

when copper concentrations are lowered [2]. Mutations in ATP7A have been classically associated to two X-linked conditions, Menkes Disease (MD - MIM #309400) and its allelic variant, the occipital horn syndrome (OHS-MIM #304150). MD is a multisystem disorder characterized by progressive neurodegeneration and marked connective tissue abnormalities. The clinical heterogeneity is high and the severity of neurological symptoms varies greatly from the classical severe form causing death in early childhood, to long-surviving patients [3]. OHS is the mildest form of MD, in which the neurodevelopmental component is less evident and connective tissue manifestations predominates, with the pathognomonic feature of symmetrical exostosis protruding downward from the occipital bones [4]. Symptoms of dysautonomia occur in OHS and are related to the reduced activity of dopamine-β-hydroxylase (DβH), a copper dependent enzyme [5]. Both in MD and OHS specific biochemical abnormalities, as low serum copper and ceruloplasmin and abnormal plasma and CSF neurochemicals, point out a global derangement of copper metabolism. ATP7A mutations underline MD and OHS and are mainly loss-of-function, causing different degrees of reduction in protein activity and copper trafficking, which translate in a clinical spectrum including MD-OHS intermediate phenotypes [6].

In 2010 a third phenotype was described to be associated with two novel missense ATP7A mutations, consisting of a pure axonal distal motor neuropathy (X-linked dHMN-SMAX3), adult in onset and with slowly progressive weakness and atrophy of distal muscles. In the described families, carrying the ATP7A mutations p.A944I and p.P1386S affecting transmembrane (TM) domains in the carboxyl half of the protein, the copper-related biochemical parameters were normal and autonomic function was preserved [7].

2. Methods

2.1. Family ascertainment

Two sibs, aged 47 (PT1) and 43 (PT2) years, were evaluated in our Neurogenetic Clinic, presenting with a distal axonal motor neuropathy and autonomic dysfunction, which main manifestations were retrograde ejaculation, diarrhea and hyperhidrosis . The participants in this study gave informed consent according to the Ethical Committee University Hospital of Ferrara given to the Neuromics project (May 30, 2013, approval number 05/2013, P. 58-2013).

2.2. Neurological examination

The patients underwent neurological clinical examination. A Small Fiber Neuropathy Symptoms Inventory Questionnaire (SFN-SIQ) [8] was assessed in order to provide data on clinical symptoms of small nerve fiber and autonomic impairment.

2.3. Neurophysiological examination

Nerve conduction studies were performed by a standard technique exploring the compound motor action potentials (CMAP) of median, tibial and peroneal right nerves, relative nerve conduction velocities (NCV) and sensory nerve action potentials (SNAP) of sural right nerve. The autonomic nervous system (ANS) was studied by performing standard Ewing tests [9] and cutaneous sudomotor and vasomotor reflexes. Beat to beat non invasive Blood Pressure (BP) was recorded by using a Portapress poligraphic device. Heart rate variability in response to Valsalva maneuver, deep breathing (DB) were evaluated to test parasympathetic innervation, whereas BP variations during 60° tilt test, Valsalva and handgrip manoeuvres to test sympathetic innervation. Skin autonomic cholinergic and adrenergic functions were studied by sympathetic skin responses (SSR) and cutaneous flowmetry (CF) reflexes, respectively. The SSR were recorded at the mid-palm of both hands and feet following random electrical stimulation of the right median nerve, acoustic stimuli and inspiratory gasp. Adrenergic sympathetic vasoconstrictor system was studied by Laser Doppler flowmetry (LDF) (dual channel Periflux, 785 nm wavelength laser, 0.5 mm fiber separation combined optical and temperature probe, Perimed, Sweden) in relation to DB, at 32 °C fixed skin temperature [10]. One probe was positioned at the distal tip pulp of the index finger of the non-dominant hand and one in the hallux. The percentage fall in CF in response to DB was calculated between the minimal CF and the basal one. Somatic C and A-delta nerve fibres functions were assessed by studying thermal Quantitative Sensory Testing (QST) and local skin axonal reflexes (AR). Thermal QST (MEDOC TSA II) was performed by applying a 30 × 30 mm probe in the dorso-lateral aspect of the feet, in the antero-medial region of the leg and in the hand (thenar eminence), with limits method [11]. Local axonal reflexes mediated by C-fibres were evoked by noxious heat and postural stimuli. Vasodilation response was recorded by two probes positioned in the dorsal aspect of the foot during local skin raising temperature at 44 °C. The increment of CF after one and five minutes in comparison to basal condition was calculated as percentage. Veno-arterioral response (VAR) in the lower limb was evoked after passive positioning the foot hanging 50 cm down the heart level and measuring percentage flow changing after 3 min.

2.4. Genetic analysis

Genomic DNA from the two brothers and parents was obtained after informed consent. Next generation sequencing (NGS) was performed in all subjects using an in-house-designed lower motor neuron diseases gene panel. The panel includes 21 ALS, 12 SMA, 16 HMN and 14 CMT-associated genes (Table 1). The coding regions, as well as the 5′ and 3′UTRs of the 65 neuromuscular selected genes, were enriched, starting from 10 ng genomic DNA, using the Ion Ampliseq Enrichment Kits, according to manufacturer

Table 1
Genes in the NGS panel.

ALS	# OMIM	SMA	# OMIM	HMN	# OMIM	CMT	# OMIM
SOD1	147450	IGHMBP2	600502	HSPB8	608014	KIF1B	605995
ALS2	606352	DYNC1H1	600112	HSPB1	602195	MFN2	608507
SETX	608465	VAPB	605704	HSPB3	604624	DNM2	602378
SPG11	610844	BICD2	609797	KIF1A	601255	LMNA	150330
FUS	137070	ATP7A	300011	TRPV4	605427	MED25	610197
ANG	105850	AR	313700	KIF1C	603060	NEFL	162280
TARDBP	605078	UBA1	314370	FBXO38	608533	MPZ	159440
FIG4	609390	ASAH1	613468	GARS	600287	GDAP1	606598
OPTN	602432	PLEKHG5	611101	BSCL2	606158	AARS	601065
ATXN2	601517	SCO2	604272	REEP1	609139	LRSAM1	610933
VCP	601023	DNAJB2	604139	SLC5A7	608761	KLHL9	611201
UBQLN2	300264	MUSK	601296	DCTN1	601143	CCDC78	614666
SIGMAR1	601978			MYH14	608568	MYH7	160760
CHMP2B	609512			TFG	602498	SLC18A3	600336
PFN1	176610			FBLN5	604580		
DAO	124050			HINT1	601314		
PRPH	170710						
NEFH	162230						
TAF15	601574						
LUM	600616						
DCTN2	607376						

instruction. Sequencing was performed bidirectionally as single-end sequencing at a mean coverage of >3000X, on a Ion-Torrent Personal Genome Machine. Data analysis, including comparison with public databases as Human Gene Mutation Database (www.hgmd.cf.ac.uk), Inherited Neuropathy Variant Browser (http://hihg.med.miami.edu/code/http/cmt/public_html/index.html#/ PMID 2947324) and Exome Variant Server (evs.gs.washington-edu/), was performed using an in house pipeline. Potentially pathogenic variants were tested in silico using Human Mutation Taster (www.mutationtaster.org) and Polyphen2 (genetics.bwh.harvard.edu/pph2/) algorithms. The detected variant in exon 15 of ATP7A gene (OMIM 300011; NCBI reference sequence: NM_000052.6) was retested by sanger sequencing.

2.5. Hair electron microscopy analysis

Hair samples approximately 0.8 cm long were mounted on a luminium holders by bi-adhesive tape and conductive silver glue at both ends. Control hairs were taken from an age matched consenting volunteer. The sample holders were gold coated by sputtering and examined in a Zeiss EVO MA 10 scanning electron microscope operated at 20 kV. The chemical composition of the hairs was analyzed by EDS microanalysis at 2000x. As a parameter, the internal ratio between copper (Cu) and sulfur (S, basic component of hair proteins) was chosen. The Cu/S ratio was determined by Energy Dispersive (EDS) X-ray microanalysis (Oxford INCA Energy 200) performed at 8.5 mm WD.

2.6. Skin biopsy

Three mm punch biopsies were taken from the thigh (15 cm above the patella) and distal leg (10 cm above the lateral malleolus) hairy skin sites in both probands. According to previously published procedures [12,13] skin samples were immediately fixed in cold Zamboni's fixative and stored at 4 °C overnight. Fifty μm thick sections were obtained using a freezing sliding microtome (2000R, Leica, Deerfield, IL, USA) and the corresponding free-floating sections were incubated overnight with a panel of primary antibodies including the mouse or rabbit pan-neuronal marker PGP 9.5 (1:800; Biogenesis, Poole, UK), mouse ColIV (1:800, Chemicon, Temecula, CA, USA) and autonomic markers like rabbit DβH (1:150, Chemicon, Temecula, CA, USA) to identify the noradrenergic fibers and rabbit VIP (1:1000, Incstar, Stillwater, MN, USA), usually co-localized in the sudomotor cholinergic fibers [14]. Sections were then washed and secondary antibodies, labeled with cyanine dye fluorophores 2 and 3.18 (1:400; Jackson ImmunoResearch, West Grove, PA, USA), were added for an overnight incubation. Washed sections were mounted onto coverslips in agar, dehydrated through alcohols, cleared with methylsalicylate and embedded in slides with DPX (VWR International PBI, Milan, Italy). Sections were initially viewed under a Zeiss fluorescent microscope (model Axioskop 40; Jena, Germany). For a 3D study of the autonomic innervation pattern digital images were also acquired using a laser-scanning confocal microscope (Nikon confocal microscopy – Eclipse Ti A1) [14,15].

Epidermal nerve fiber density (ENFs) was calculated by considering a single epidermal fiber marked by PGP 9.5 crossings of the dermal–epidermal junction [16]. Autonomic innervation density was quantified using the previously described automated method showing a high interobserver and intraobserver reliability [12]. Briefly, this method is based on a technique known as the 'unsharp mask filter' which creates a composite image by subtracting the

background color in the out of focus image from the base image expressing the autonomic innervation staining (Image Pro Plus, Media Cybernetics). Adrenergic innervation was analysed considering DβH staining in the muscle erector pilorum (MEP) and arterioles. By contrast, cholinergic innervation was analyzed by VIP staining around the sweat gland (SG) [15,17,18]. Target autonomic structures were identified by using a ColIV staining. Autonomic innervation score in each skin site usually represented the mean of three different target structures.

3. Results

3.1. Clinical history

In the two sibs, aged 47 (PT1) and 43 (PT2) years, first symptoms arose around 27 years of age and presentation was with cramps, progressive difficulties in feet dorsiflexion, lower limbs distal muscle weakness and distal muscle atrophy. EMG and muscular biopsy showed neurogenic patterns. CK values were constantly slightly elevated (300–400 U/L, normal reference values: <190). Urological assessment documented in both sibs retrograde ejaculation with anejaculation due to dysfunction of the internal urethral sphincter at the bladder neck. The younger sib suffered since young age from a chronic diarrhea refractory to anti-diarrheal drugs. Gastro and colon endoscopy were negative. Laboratory study of copper metabolism showed a reduction of serum copper and ceruloplasmin level in both brothers in two different dosages, (copper PT1 47 and 63 μg/dl; PT2 69 and 63 μg/dl (normal range 70–140); ceruloplasmin PT1 14,7 and 19.1 mg/dl; PT2 20 and 19,8 mg/dl (normal range 20–60). The two brothers didn't receive any medications that could explain biochemical results. Plasma catecholamine testing revealed values in the normal range (Epinephrin PT1 0,32 nmol/L – PT2 0,10 nmol/L (normal range 0,10–0,46); Norepinephine PT1 0,54 nmol/l; PT2 0,57 nmol/L (normal range 0,10–2,49). Family history was unremarkable and clinical assessment of proband's parents was negative. They are non-consanguineous.

3.2. Dismorphological evaluation

Main auxological parameters of PT1 (47-year-old) were: weight 78 kg (90–97th centile), height 187 cm (75–90th centile), OFC 61,5 cm (>97th centile), arm span length 190 cm, total hand length 21 cm (97th centile) with palm 11 cm (75th centile). Main auxological parameters of the PT2 (43-year-old) were: weight 69 kg (50–75th centile), height 177 cm (25–50th centile), OFC 62 cm (>97th centile), arm span length 180 cm, total hand length 19,5 cm (75th centile) with palm 11 cm (75th centile).

Clinical signs shared by both patients, but more pronounced in PT1 were: regular head shape with a slight occipital protuberance, slightly sloping forehead, mild widows pick with coarse hairs, prominent eyebrows, anteverted nares, malar hypoplasia, mild micrognatia, long and thin face, long and stocky neck (Fig. 1(A) and (B)) . An anterior prominence of the hyoid bone and a slight asymmetry of clavicles were observed as well as limited elbow extension, muscle atrophy of the distal parts of arms and feets, bilateral pes cavus and a profuse sweating were noted in both brothers (Fig. 1(C)). Furthermore, in the younger brother, a mild extensible and redundant skin, in particular in the abdominal area, and a large hyperpigmented dysomogeneous area in the right side of the back were noted. Patients hairs examined at 64× and 2000× showed no torsion of the hair shaft which could resemble "pili torti" (data not shown). The Cu/S ratio appeared abnormally low in the hairs of PT1 when compared with the control, while patient PT2 had normal values (Fig. 1(D)).

3.3. Neurological evaluation

PT1 showed decreased mass of tibialis anterior (TA), gastrocnemius (G) and soleus (S) muscles with foot drop, severe weakness in foot dorsiflexion (MRC 0/5) and mild weakness in plantar flexion (MRC 3/5). He had Achillei areflexia; the representation of hands muscles was normal as well as the sensitivity. Severe plantar hyperhidrosis and mild palmar hyperhidrosis were present. Gait was hackney without aid. Cognitive function and intellect were normal. PT2 showed gait difficulties, calves hypotrophy, and leg weakness. The pattern of muscle involvement was similar to his sib with prevalent weakness in foot dorsiflexion (MRC 1/5), less in plantar flexion (MRC 3/5), hackney gait and Achillei areflexia. He also showed interossei muscles mild hypotrophy without hand weakness. Mild plantar hyperhidrosis was present. Cognitive function and intellect were normal. Both patients reported at least 3 symptoms related to dysfunction of small nerve autonomic fibers at SFN-SIQ. Sweating disorders (hyperhidrosis), diarrhea, and impotence due to retrograde ejaculation, in the absence of any urological diseases as confirmed by specialistic clinical evaluations, were reported, without pain or sensory symptoms.

3.4. Neurophysiological examination

Results of nerve conduction studies confirmed the diagnosis of distal axonal motor neuropathy as shown in Table 2. Functional cutaneous small fiber studies showed small fiber neuropathy involving A delta and C fibers, including cutaneous sympathetic cholinergic and adrenergic autonomic fibers.

For more details: CMAPs were significantly reduced or absent in tibial and peroneal nerve, less in median nerve. One of the two sibs has also reduction in the SNAP of the sural nerve (PT 1). Cardiovascular reflexes were normal in both sib, no orthostatic hypotension was detected, while SSR showed a multiple pattern responses in relation to hyperhidrosis.

Thermal QST showed increase of cold and warm threshold in feet and in warm threshold in the hands of PT1, resembling small fiber neuropathy involving C and A delta fibers. PT2 showed alteration of warm threshold in feet and legs following a disto-proximal gradient of severity, without involvement of the upper limbs. None of them reported positive symptoms such as allodynia, hyperalgesia or aftersensation.

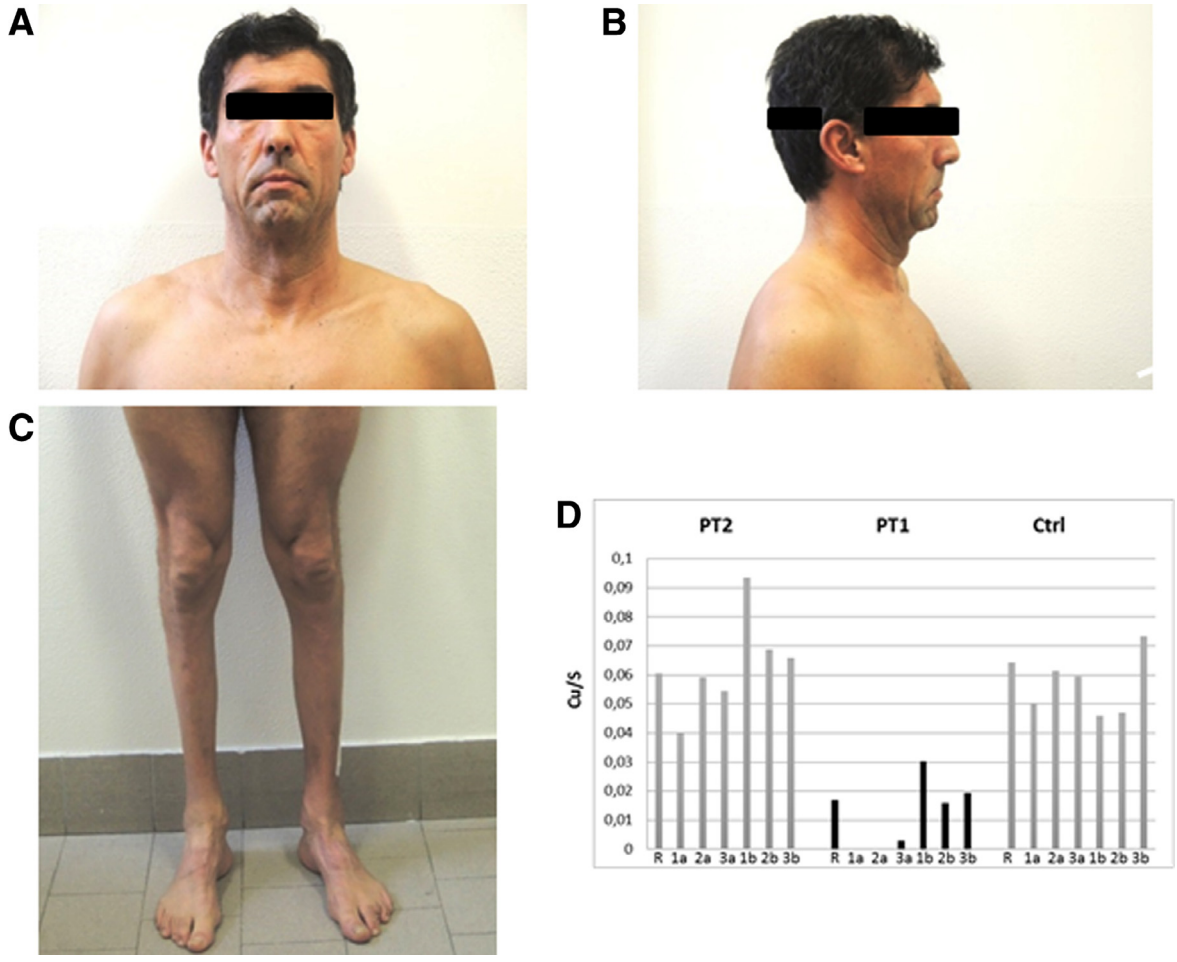

Fig. 1. Clinical findings.
(A–B–C) Clinical findings in PT1: regular head shape with slightly sloping forehead, mild widows pick with coarse hairs, prominent eyebrows, anteverted nares, malar hypoplasia, mild micrognatia, long and thin face, long and stocky neck, anterior prominence of the hyoid bone and a slight asymmetry of clavicles. Distal muscle wasting of lower limbs: decrease mass of the vastus lateralis, vastus medialis, tibialis anterior, gastrocnemius and soleus. Pes cavus deformities in both feets; (D) Hair chemical analysis: the chemical composition of the hairs was analyzed by EDS microanalysis at 2000× in three different areas along the shaft (1–2–3), repeated on two distinct hairs for each patient and the control (a–b), plus on one randomly chosen area for each sample (R).

Table 2
Electrophysiological data.

Patient	cMAP amplitude (mV)			NCV (m/s)	SNAP amplitude (μV)
	Median nerve (nv ≥ 6 mV)	Tibial nerve (nv ≥6 mV)	Peroneal nerve (nv ≥ 4 mV)	Peroneal nerve (nv ≥ 43 m/s)	Sural nerve (nv ≥ 8 μV)
PT1	7,3	1,1	0,1	45.9	5,4
PT2	15	0,8	0,2	34,1	10,9

UL: upper limbs; LL: lower limbs; + distal weakness MRC>4; ++ distal weakness MRC<4; +++ proximal weakness; DTR deep tendon reflexes; NCV: nerve conduction velocity; SNAP: sensory nerve action potentials; cMAP: compound motor action potentials; nv: normal values.

LDF study showed severe alterations with absence of sympathetic-mediated vasoconstriction response after DB and reduced local axon reflex C fiber-mediated vasodilatation during warming at 44 °C. VAR was normal in both of the subjects.

3.5. Genetic analysis

NGS analysis identified in both affected brothers a hemizygous missense mutation (c.2972C>A; p.A991D) in exon 15 of the X-linked ATP7A gene. The unaffected mother was heterozygous for the mutation (Fig. 2(A)). The c.2972C>A variant was absent in the Exome Variant Server and in silico evaluation (Mutation taster, Polyphen2) supported pathogenicity. The identified missense change lies in the same ATP7A a-helix/TM domain of the reported c.2981C>T (p.T994I) mutation [7] in a family with SMAX3, being located only 3 residues upstream, and involves a highly conserved alanine residue across species (Fig. 2(B) and (C)).

3.6. Skin innervation studies

Small fiber neuropathy was disclosed in both patients. Skin innervation dysfunction mainly involved the autonomic

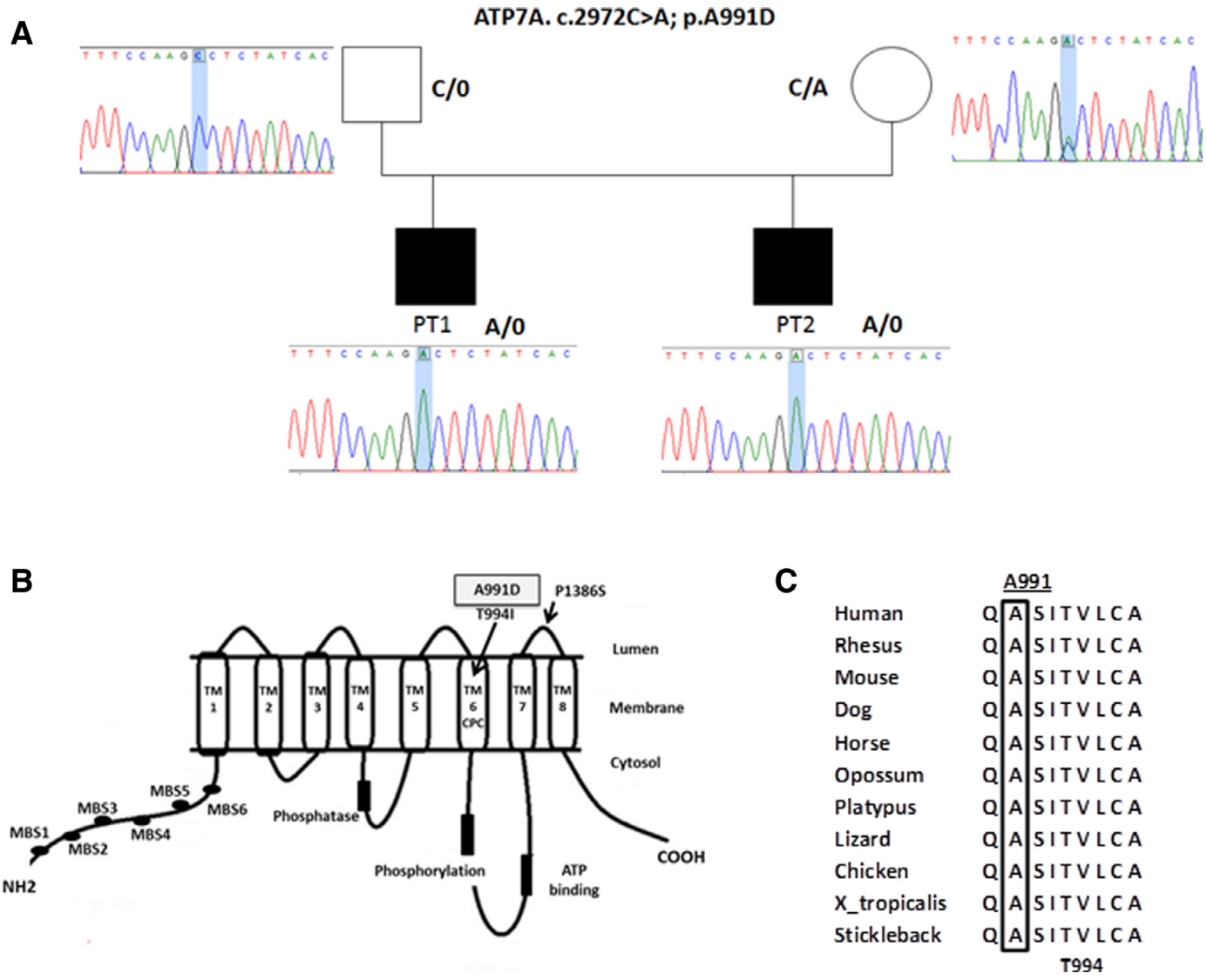

Fig. 2. ATP7A mutation details.
(A) Family tree and sequence chromatograms of the p.A991D mutation identified in the ATP7A gene. The mutation is present in hemizygosity in probands PT1 and PT2 and in heterozygosity in proband's mother. (B) Location of the p. A991D mutation in the TM6 domain of the ATP7A copper-ATPase and depiction of the two previously described SMAX3 mutations (p.T994I and p.P1386S); (C) alignment analysis of the p.A991D mutation (boxed) for ATP7A orthologs in different species.

fibers with a prevalent adrenergic damage. DβH staining was absent in the muscle erector pilorum (MEP) but VIP, although decreased and deranged, was found around sweat gland (Table 3 and Figs. 3 and 4). Somatic innervation also showed a significant epidermal nerve fiber density decrease (ENFs) in the leg and thigh (Table 3).

4. Discussion

Classical phenotypes associated to ATP7A mutations include Menkes Disease (MD), (MIM: 309400), Occipital Horn Syndrome (OHS) (MIM: 304150) and X-linked recessive distal spinal muscular atrophy (SMAX3) (MIM: 300489). MD and OHS are known as being part of the same clinical spectrum including MD-OHS intermediate

Table 3
Skin somatic and autonomic innervation scores.

Patients	*Leg*			*Thigh*		
	ENFs[a]	*SG*[b]	*MEP*	*ENFs*[c]	*SG*[d]	*MEP*
	Mm	FD%	FD%	mm	FD%	FD%
PT1	**3,2**	**4,5**	**Absent**	**13,8**	**6,3**	**Absent**
PT2	**6,7**	**6,8**	**Absent**	**10,9**	**6,5**	**Absent**

ENFs = epidermal nerve fiber density; SG = sweat gland; MAP = muscle erector pilorum.
[a] Sex and age-matched mean normal value 10.3 EFNs/mm (cut-off 9.7 EFNs/mm) [16].
[b] Normal values 10 ± 3% (cut-off 7.5%) [12].
[c] Mean normal value for our laboratory 27.1 EFNs/mm (cut-off of 15 EFNs/mm).
[d] Normal values 11 ± 2% (cut-off 9.5%) [12].

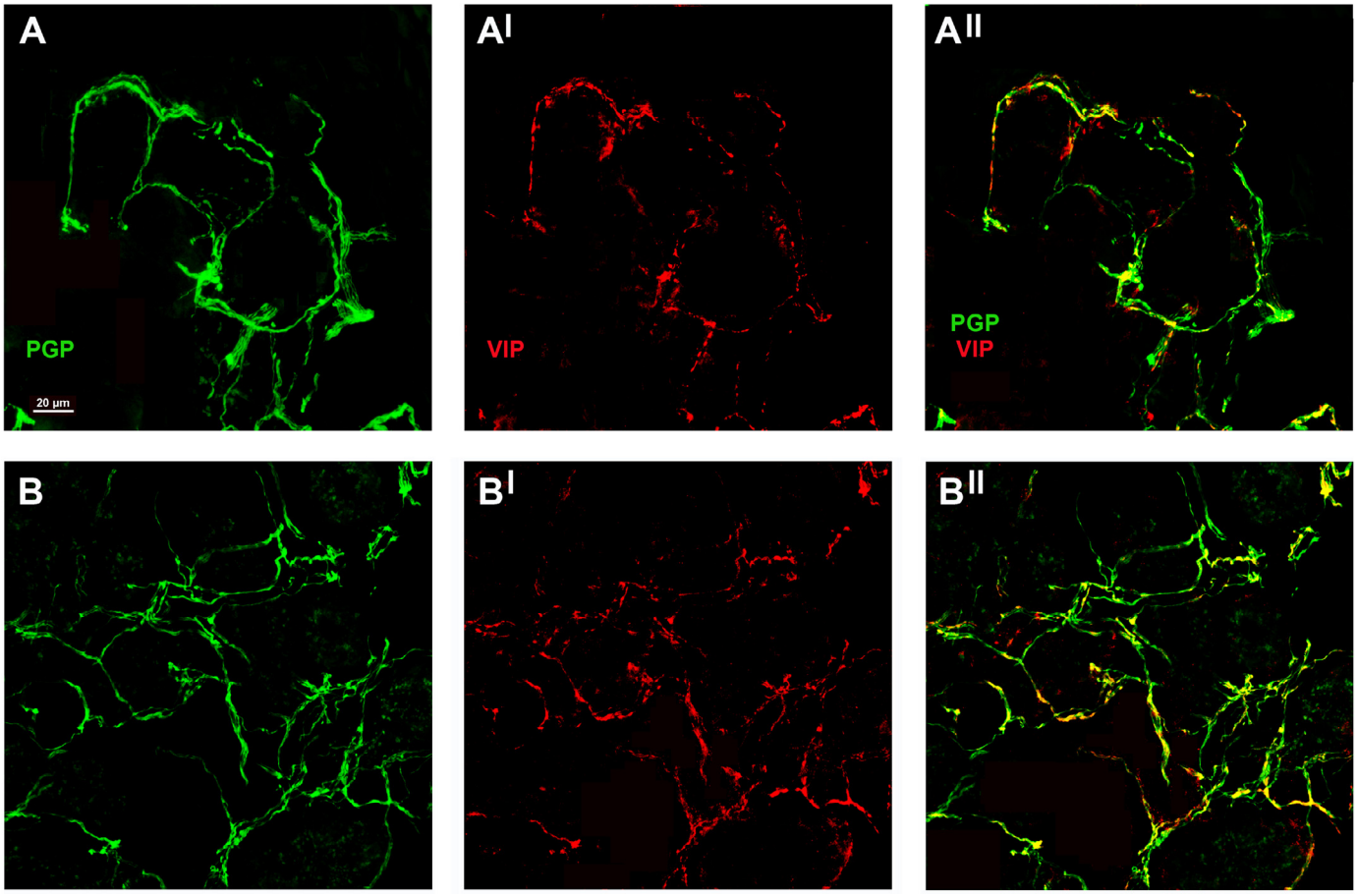

Fig. 3. Confocal study of autonomic cholinergic innervation of a sweat gland in the proband with ATP7A mutation and a healthy control.
Leg cholinergic autonomic innervation disclosed by confocal microscope (×40) in the 43 years-old proband with ATP7A mutation (A, AI and AII) and an age-matched control subject (B, BI and BII). Nerve fibers are marked in green using a pan-neuronal marker, PGP 9.5 or in red with a specific cholinergic marker, VIP. (A) In the proband sweat gland shows a reduction in cholinergic fiber density with several fibers presenting morphological abnormalities such as nerve fiber fragmentations (AI). However the cholinergic pattern of innervation was recognizable with nerve fibers encircling the sweat tubules (AII). (B) The control subject shows an abundant sweat gland innervation with nerve fibers mainly cholinergic VIP positive (BI) encircling the sweat tubules (BII).

phenotypes, but neither condition features overt motor neuropathy occurring in SMAX3.

Copper deficiency is the main pathogenic mechanism for MD and OHS and the early institution of copper-replacement therapy affects prognosis for MD patients [19]. Animal models have shown that in the peripheral and central nervous systems, copper is essential for the normal functions of cuproenzymes such as ceruloplasmin, hephaestin, copper-zinc superoxide dismutase (SOD1), cytochrome c oxidase (CCO), dopamine-β-hydroxylase (DβH), peptidyl α-amidating monooxygenase (PAM) and tyrosinase [20,21] and that axonal ATP7A expression during synaptogenesis is essential for normal neuronal development. Further underlining the relevance of copper metabolism in Charcot-Marie-Tooth disease, very recently compound heterozygous variants in the mitochondrial copper-binding protein SCO2, were identified in two unrelated patients with axonal polyneuropathy [22].

Dysautonomic aspects described in OHS are thought to be related to the reduced activity of DβH and to the consequent reduction of dopamine conversion in norepinephrine in norepinephrinergic neurons. Accordingly, OHS dysautonomic disturbances mirror those of congenital DβH deficiency with severe orthostatic hypotension and noradrenergic failure.

The SMAX3 phenotype consists of a pure motor neuropathy (X-linked dHMN) [7] and the copper-related biochemical parameters were reported as normal in described patients. This evidence, together with the absence of central nervous system, renal tubular, skeletal and connective tissues involvement, and the lack of autonomic signs, prompted to consider SMAX3 as a clear cut separated ATP7A phenotype with distinctly different mechanism(s) of disease than for MD and OHS [23].

We describe a novel ATP7A mutations causing SMAX3. Our patients show a distal motor neuropathy, which is associated with mild signs and symptoms described in OHS [4]. In particular, the presence of a peculiar facial morphology andredundant skin, point out a connective tissue involvement.

Reduced serum copper and ceruloplasmin levels attest copper metabolism impairment. As described in OHS patients, both sibs in our family present dysautonomic signs.

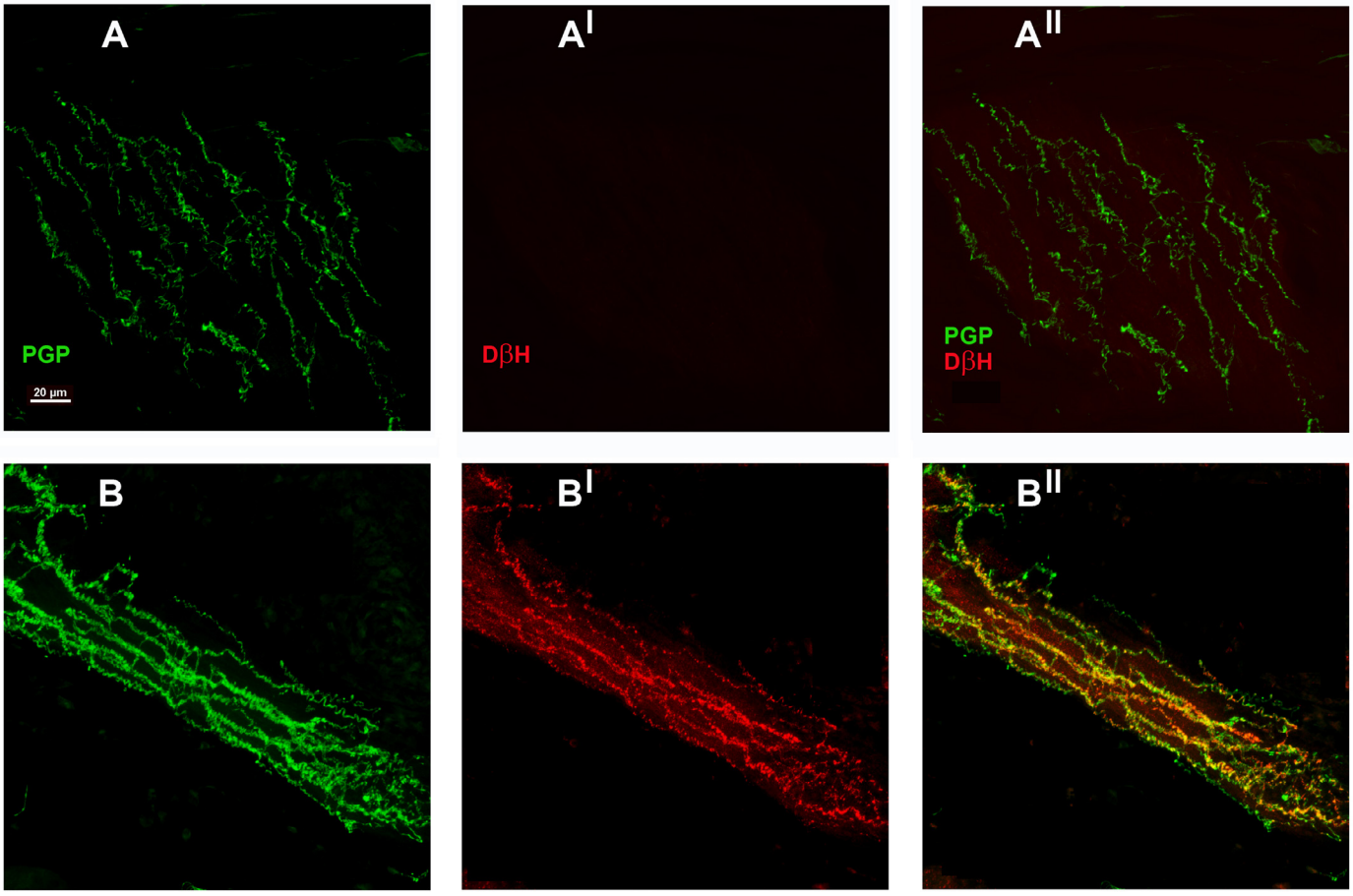

Fig. 4. Confocal study of autonomic adrenergic innervation of a muscle errector pilorum in the proband with ATP7A mutation and a healthy control.
Leg adrenergic autonomic innervation disclosed by confocal microscope (×20) in the 43 years-old proband with ATP7A mutation (A, AI and AII) and an age-matched control subject (B, BI and BII). Nerve fibers are marked in green using a pan-neuronal marker, PGP 9.5 or in red with a specific adrenergic marker, DβH. (A) In the proband muscle errector pilorum shows a slight reduction in PGP positive nerve fiber density but interestingly the antibody against DβH does not show any substantial staining (AI) suggesting that autonomic dysfunctions are mainly due to absent DβH activity. In any case the pattern of innervation was recognizable with PGP positive nerve fibers running in a longitudinal wavy pattern (AII). (B) The control subject shows a muscle errector pilorum richly innervated by nerve fibers mainly DβH positive (BI) presenting a classical pattern of innervation with nerve fibers running in a longitudinal wavy shape (BII).

The ATP7A p.A991D mutation, we identified in our patients, affecting a highly conserved residues in a transmembrane domain (TM6) of the protein, is located only three residues upstream from the previously reported missense ATP7A variant (p.A994I) causing a pure motor neuropathy [7]. Missense mutations in the TM6 domain were also associated to MD either in its classical or long survival form [24]. Our observation confirms the lack of genotype–phenotype correlation in ATP7A related clinical spectrum, with similar mutations underlying a wide range of disease severity [25].

A striking intra-familial variability in the phenotype associated with ATP7A mutations was reported, with dramatic examples of mild OHS coexisting with a classic severe MD phenotype in the same family. This variability was related to an increased expression of the mutant ATP7A protein in the less affected individual possibly due to a difference in the activities of ATP7A transcription factors [26]. Very recently the ATP7A interactome was delineated revealing that ATP7A belongs to a network of proteins located in different cell compartments [27].

As described in OHS patients, but not reported in SMAX3, both sibs in our family present dysautonomic signs referable to an adrenergic involvement related to the DβH deficiency. Our patients actually show a severe alteration of vasomotor control in the skin, documented by Laser Doppler Flowmetry, and suffer from retrograde ejaculation. Furthermore, the skin biopsy studies disclosed a prevalent damage of skin adrenergic fibers with absent staining for DβH. In spite of a severe skin adrenergic dysfunction, both patients did not present orthostatic hypotension during cardiovascular reflexes suggesting a mild involvement of the overall autonomic adrenergic outflow, and consistent with a normal level of catecholamines in the plasma. A possible explanation of this discrepancy could rely on a residual DβH functional activity in different peripheral sympathetic fibers such as cardiac, muscle and splanchnic branches, which may functionally compensate the marked involvement of skin adrenergic fibers

and sustain blood pressure and other adrenergic functions. A more focused study exploring the functional activity in other peripheral sympathetic branches, i.e. cardiac (with uptake of [123-I]-MIBG) and muscle (using microneurography), is needed to confirm this hypothesis.

Both sibs in our family also show hyperhidrosis and skin biopsy demonstrated that autonomic dysfunctions may be wider also involving the skin cholinergic subdivision which could be involved in the sweating impairment.

In addition to dysautonomic hypothesis, the bowel dysfunction, with chronic diarrhea, could be related to a local damage of enteric mucosae caused by copper accumulation or to the high dopamine level caused by the lack of substrate catalase due to DβH deficiency, in response to various stimuli able to activate noradrenergic system [28].

The clinical and neurophysiological aspects of PNS pathology in our patients mirror those described in SMAX-3, and consist of an axonal neuropathy involving prevalently but not exclusively the motor fibres. Our patients suffer from distal weakness and muscle hypotrophy, foot drop, absent Achillei reflexes. CMAPs are of small amplitude. However a mild sensitive involvement is documented by the small SNAP and by the pathologic thermal thresholds reflecting small fibers involvement.

The somatic small fiber neuropathy was also confirmed by the skin biopsy disclosing a proximal and distal decrease of the epidermal innervation. The involvement of large and small sensory fibers could be explained by the abnormal neural synaptogenesis induced by the abnormal function of ATP7A [20,29].

5. Conclusion

The described family refines the phenotype related to ATP7A mutations. Our data demonstrated that ATP7A mutations induced a generalized damage of autonomic and sensory neurons together with motor axons dysfunctions possibly due to the effect of axonal ATP7A on normal synaptogenesis.

CRediT authorship contribution statement

Francesca Gualandi: Conceptualization, Data curation, Writing - original draft. **Elisabetta Sette:** Investigation, Writing - original draft. **Fernanda Fortunato:** Investigation, Writing - original draft. **Stefania Bigoni:** Investigation, Data curation. **Domenico De Grandis:** Conceptualization, Writing - review & editing. **Chiara Scotton:** Investigation, Methodology. **Rita Selvatici:** Investigation, Data curation. **Marcella Neri:** Investigation, Methodology, Writing - review & editing. **Alex Incensi:** Investigation, Methodology, Writing - original draft. **Rocco Liguori:** Supervision, Writing - review & editing. **Markus Storbeck:** Investigation, Data curation, Writing - original draft. **Mert Karakaya:** Investigation, Data curation. **Valentina Simioni:** Investigation, Writing - review & editing. **Stefano Squarzoni:** Investigation, Writing - original draft. **Vincent Timmerman:** Supervision, Writing - review & editing. **Brunhilde Wirth:** Supervision, Writing - review & editing. **Vincenzo Donadio:** Supervision, Writing - review & editing. **Valeria Tugnoli:** Supervision, Writing - review & editing. **Alessandra Ferlini:** Supervision, Funding acquisition, Writing - review & editing.

Acknowledgments

The Neuromics EU Project to AF, BW and VT (grant agreement no 2012-305121, www.neuromics.eu) is acknowledged.

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