Supplementary material

The CAPOS mutation in *ATP1A3* alters Na/K-ATPase function and results in auditory neuropathy which has implications for management

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Histories

Classification of the degree of hearing impairment is described according to Mazzoli M et al, 2003, where 20-40 dB HL is mild; 41-70 dB Hl is moderate; 71-95 dB HL is severe; and >95 dB Hl is profound hearing impairment as calculated on the Pure Tone Average ($PTA_{0.5,1,2,4\ kHz}$**)**

Family 1 (AG34) (cases 1, 2, 3, and 4) is a two generation Swedish family with affected proband and her three children from two different marriages (Fig. 2). The father of case 2 had profound hearing impairment after meningitis at age 2 years. Cases 3 and 4 had a different father, who had normal hearing. They all present cardinal features of CAPOS. In addition they have movement abnormalities, namely athetosis. Their hearing impairment is of AN type.

Molecular genetics investigations included sequencing of *WFS1; TIMM8A; ZCD2; PRPS1*; and for the mitochondrial mutations, m.8993T>G/C (associated with NARPP), m.3243A>G; m.1555A>G, and all were normal. Heterozygosity for the exon 18 c.2452G>A mutation in *ATP1A3* was identified in all four. The mutation was not found in the mother's mother, and her father was not available for examination. He was said to be healthy.

Audiograms from Case 1 at age 7y and 26y are shown in Fig. 3, showing the progressive nature of the hearing loss. See Table 1 and 2 and Fig. S2 for summary of clinical findings. $PTA_{0.5,1,2,4\ kHz}$ was moderate-severe at age 7 years (64 dBHL and 74 dBHL for the right and the left ear), and progressed to severe (81 dBHL and 74 dBHLfor the right and the left ear) at age 26 years.

Case 2: $PTA_{0.5,1,2,4\ kHz}$ was moderate at age 5 years (55 dBHL and 68 dBHL for the right and left ear respectively) and age 15 years (51 dBHL and 68 dBHL for the right and left ear respectively).

Case 3: $PTA_{0.5,1,2,4\ kHz}$ was moderate/mild at age 5 years (40 dBHL and 34 dB HL for right and left ear), fluctuating to mild at age 12 years (20 dBHL and 23 dBHL for right and left ear).

Case 4: $PTA_{0.5,1,2,4\ kHz}$ was mild at age 11,5 years (38 dBHL and 29 dBHL for right respectively left ear).

Family 2 (AG349) (cases 5 and 6) is a two generation Swedish family with affected proband and her oldest daughter (Fig. 2). The proband had four episodes of febrile disease during all of which the severity of disease progressed, both with regard to neurological, ophthalmological and auditory presentation. She remained intellectually bright. A large number of molecular genetic investigations have been performed, including *OPA1* (sequencing and MLPA); *WFS1; PRPS1*, *ZCD2*, m.3243A>G; m.1555A>G and all were normal, until heterozygosity for the exon 18 c.2452G>A

mutation in *ATP1A3* was identified in both patients. The audiological phenotype was compatible with AN. Audiograms are shown from Cases 5 and 6 in Fig. 3 showing how low frequencies are affected. The parents of the proband were clinically healthy and only the biological mother was genetically tested., and did not have the mutation. See Table 1 and 2 for summary of clinical findings. In case 5 $PTA_{0.5,1,2,4\ kHz}$ was severe at age 3,5 years (75 dBHL and 79 dBHL), progressing to profound at age 37 years (90 dB HL and 94 dB). Case 6: $PTA_{0.5,1,2,4\ kHz}$ was mild at age 4 years (34 dBHL and 30 dBHl for right and left ear respectively) progressing to severe at age 15 years (73 dBHL and 78 dBHL for right and left ear).

Family 3 (AG310) (cases 7 and 8) is a two-generation Danish family with affected proband and her mother (Fig. 2). The proband was hospitalized briefly at age 8 months with fever, hypotonia and reduced consciousness, but had a severe febrile episode at age 5 years, diagnosed as encephalitis, and was hospitalized for 1 month, with very slow partial recovery several months after discharge from hospital. During her hospital stay she had seizures, ataxia, areflexia, and severely reduced level of consciousness, but no agent was identified underlying the "encephalitis". Genetic testing excluded *MELAS1* and *2*, *MERRF, and NARPP*. Sequencing of the *SLC26A4* and *WFS1* genes was normal. Heterozygosity for the exon 18 c.2452G>A mutation in *ATP1A3* was demonstrated. Audiogram from Case 7 at age 25 years is shown in Fig. 3, demonstrating moderate hearing impairment most pronounced in the low frequencies. ($PTA_{0.5,1,2,4\ kHz}$ was 51 and 55 dB HL for right and left ear, respectively). Audiometry at age 33 years showed evidence of progression of the hearing impairment with $PTA_{0.5,1,2,4\ kHz}$ 60 and 69 dB HL for right and left ear, respectively. Speech recognition testing at most comfortable loudness level using the Danish monosyllabic speech material Dantale 1 (Elberling C et al, 1989) revealed a near total loss of speech discrimination. (Speech Recognition Score (SRS) was 19% and 26% for right and left ear respectively). ABR testing showed absent auditory brainstem response at high intensity stimulation, but cochlear microphonics were present at 75 dB nHL. Robust transient evoked otoacoustic emissions (TEOAEs) were present bilaterally as were distortion product emissions (DPOAEs) recorded with a stimulus level of 65/55 dB SPL at 1, 2, 3, 4 and 6 kHz (DPOAE 20, ver. 1.03.1, Eclipse, Interacoustics). Thereby the criterion for AN was met. MRI at age 33 years demonstrated bilateral cochlear nerve hypoplasia and otherwise normal structures of the temporal bone. No significant benefit of hearing aid use in terms of speech understanding was reported. Accordingly, SRS in quiet with hearing aids measured at 65dB SPL was 24%.

Her otherwise healthy sister was born with anal atresia, which was surgically corrected early in infancy. Her mother (case 8) is less severely affected, but with similar combination of all features of CAPOS. Her mother's parents, who were first cousins were reported unaffected, but were both deceased and therefore not available for testing. The audiological phenotype was compatible with AN. See Table 1 and 2 for summary of clinical findings. Case 8: $PTA_{0.5,1,2,4\ kHz}$ was severe at age 45 years (75 dBHL and 79 dBHL for right and left ear), progressing to profound at age 57 years (96 dBHL, and 108 dBHL for right and left ear) .

Family 4 (AG214) (cases 9, 10 and 11) is a two-generation Danish family unrelated to family 3 or family 5. The proband, her older sister and her mother were affected, whereas her younger sister was unaffected as well as her maternal grandparents (Fig. 2). The father of cases 9 and 10 was congenitally deaf, reported to be due to maternal rubella with an otherwise negative family history regarding hearing impairment. The proband is wheelchair bound. Her disease was triggered by a serious febrile illness at age 20 months, and "encephalitis" was the diagnosis given following two months hospitalization, but no infectious agents were identified. She had seizures, and athetoid hand movements. Optic atrophy and abnormal VEP were demonstrated.

Case 9 has minimal benefit from CI. No formal testing was possible. $PTA_{0.5,1,2,4\ kHz}$ was moderate at age 17 years (56 dBHL and 57 dBHL for right and left ear respectively).

Her elder sister (case 10) became unwell at age 3, with similar encephalitis-like course and has subsequently had a second attack at age 13 years, and has severe dystonia and ataxia. She has been treated with a Baclofen pump for extensive periods with questionable effect, and is wheelchair bound. She, her sister and their mother all have the features of CAPOS, but of variable degree of severity. Optic atrophy and abnormal VEP were demonstrated. See Table 1. Both the proband and her affected sister are wheelchair bound. $PTA_{0.5,1,2,4\ kHz}$ was moderate at age 21 years (45 dBHL and 58 dBHL for right and left ear respectively). No formal testing of speech perception or benefit form HA could be performed due to severe dystonia.

The mother (case 11) of the proband (case 9) and her affected sister (case 10), had hearing impairment from age 4 years and optic atrophy from age 30 years and had thyroid cancer at age 28 years. She received a cochlear implant at age 45 years before CAPOS was diagnosed and the mutation confirmed. Case 11 experiences minimal benefit from her CI (detailed testing not performed). The maternal grandparents of the proband were reportedly healthy, and the grandmother did not have the *ATP1A3* mutation. The maternal grandfather of the proband is

deceased. Because of the severe dystonia in the sister of the proband (case 10) the entire *ATP1A3* gene was sequenced and showed only the single c.2452G>A mutation in exon 18. Discordant segregation of a known heterozygous *WFS1* disease-causing mutation (exon 8: c.2649delC; p.F884Sfs*68) in the proband, her mother and an unaffected sister, that was not present in the older affected sister, resulted in investigation of the family members further back in the family. Some of the healthy siblings of the proband's deceased maternal grandfather had the same genotype. Reconsideration of the diagnosis after the initial identification of the two Swedish families led to testing of the *ATP1A3* gene. The mutation causing CAPOS syndrome was identified and co-segregated in perfect accordance with disease status. The mother had optic atrophy and poor color vision. See Table 1 for further ophthalmological details and Table 2 for audiological details. In case 11 $PTA_{0.5,1,2,4\ kHz}$ was severe at age 33 years (95dB HL and 99dB HL for right and left ear, respectively.

Family 5 (AG350) (case 12) is a Danish family with one affected son, aged 21 years at investigation. He is unrelated to the other two Danish families. His early development was normal, and at age 11 months he had a high temperature, was hypotonic, and was admitted to hospital. He recovered after two months. At the age of 16 months he became severely ill. He was clinically septic with high fever, lactic acidosis and reduced consciousness. He had seizures, dystonia and painful movements accompanied by vomiting. The episode was considered sepsis without detectable underlying agents. The dystonia was impossible to treat; he stopped thriving and lost weight. After more than six months in hospital he received a Baclofen pump with good effect on the dystonia and spasms, and he began to thrive again. He was treated with the Baclofen pump from age 3 to 7 years (1997-2001). At age 12 years DYT12 (RPD) was considered as a possible diagnosis, and he has since been treated as having dystonic cerebral palsy. He is wheelchair dependent but intellectually well-functioning despite a lack of verbal language and severe visual impairment (light perception), and is considered deaf-blind (See Fig. S3). He is able to communicate very well by means of tactile sign language. He lives in a community for deaf-blind individuals. Optic atrophy was demonstrated at age 8 years (in 2002) and again at age 21 (2015). VEPs were severely abnormal with almost extinguished responses. It was not possible to perform OCT and formal testing of benefit of HA due to spasticity (Table 1).

The audiological phenotype was compatible with AN. $PTA_{0.5,1,2,4\ kHz}$ was moderate at age 18 years (53 dBHL and 56 dbHL for right and left ear). CI was considered but it was decided not to proceed, since he uses tactile sign language well.

Despite many investigations, no underlying metabolic disorder was identified. Mitochondrial biochemical analysis based on a muscle biopsy (oximetry) at age 4 years was normal, as were CSF metabolites and neurotransmitters; sequencing the *GJB2* gene was normal, and *TIMM8A* sequence analysis was normal. Heterozygosity for the exon 18 c.2452G>A mutation in *ATP1A3* was demonstrated at age 21 years. His parents and two siblings are healthy. The parents do not have the *ATP1A3* mutation. See Table 1 and 2 for summary of clinical findings.

Family 6 (AG351) (case 13) is a French family with one affected male age 32 years. He has healthy parents and two healthy sisters. He became unwell at 14 months of age with a second febrile episode at age 20 years. He presented all of the features of CAPOS. He had ataxia from age 14 months and at age 20 years had severe vestibular dysfunction. He had thorough ophthalmological examination that disclosed normal ERG, abnormal VEP and OCT which showed thinned retinal nerve fiber layer (see Fig. S7). Mitochondrial dysfunction was suspected and muscle biopsy and other tests were performed with normal results (*OPA1, OPA3, WFS1, MFN2, PMP22* sequencing were normal no LHON mtDNA primary mutation was present and he did not have the NARPP-associated mutation m.8993T>G/C. Heterozygosity for the exon 18 c.2452G>A mutation in *ATP1A3* was demonstrated subsequently. His parents did not have the mutation. The audiological phenotype was compatible with AN with very poor speech discrimination. See Table 1 and 2 for summary of clinical findings.

$PTA_{0.5,1,2,4\ kHz}$ was severe at age 26 years (88 dBHL and 93 dBHL for right and left ear respectively)

Family 7 (case 14) was a British male patient, diagnosed at age 34 years with CAPOS. He is the only affected person in his family. He was born after normal pregnancy, and had normal early development including spoken language. At age 22 months he had measles encephalitis, with floppiness and semi-consciousness for 10 days. He regained ability to crawl and walk, but remained with horizontal pendular nystagmus and poor vision. The second episode at age 9 years along with tonsillitis was followed by dramatic loss of hearing ability, demonstration of abnormal brainstem responses but preserved OAEs; subsequently there was deterioration of vision and hearing in his twenties, and development of cerebellar ataxia, dysarthria, absent peripheral reflexes, but no pes cavus. The hearing loss has been gradually progressive and is now moderate to severe, 'flat' on the right and upsloping (low frequencies predominantly affected, severe to mild) on the left. Genetic testing confirmed *de novo ATP1A3* c.2452G>A; p.Glu818Lys in the patient. See Table 1 and 2 and

Fig. S4 for further clinical details. $PTA_{0.5,1,2,4\ kHz}$ at 29 years was moderate (54 dBHL on the right and 49 dBHL on the left) and at 32 years was severe/moderate (70 dBHL on the right and 54 dBHL on the left)

Family 8 (case 15) was a 12 year old British girl, born to healthy parents, after a normal pregnancy and delivery. Early development was normal. She was the only affected person in the family. At 18 months she had a high fever, was dystonic and had reduced consciousness. This resolved completely in hospital, except for poor balance. A second episode occurred at age 3.5 years during a febrile illness; there was reduced consciousness, nystagmus and weakness, which was diagnosed as "cerebellitis". Her condition improved, but the ataxia remained and hearing impairment was diagnosed at age 5 years, initially affecting low frequencies. It progressed and there was dramatic loss of hearing ability at age 9 years in association with another febrile illness (Audiogram from age 10½ years in Fig. 2); there was poor speech recognition, abnormal ABRs and preserved OAEs. She underwent cochlear implant at 12 years. At age 9 years pale optic discs and delayed VEPs were noted and there was thinning of nerve fiber layers on OCT. EMG and NCV studies were normal. After extensive metabolic and biochemical investigations (including amino acids, VLCFA, biotinidase, caeruloplasmin, copper, manganese, iron, white cell ubiquinone, adenosine deaminase, ammonia, lactate, blood spot free and acyl carnitine profile, plasma purines and uric acid, riboflavin, FAD, FMN, and vitamin B2) as well as genetic investigations (including *OPA1* and *WFS1*, and a 55 gene sensorineural hearing loss panel), genetic testing confirmed the *ATP1A3* c.2452G>A; p.Glu818Lys CAPOS mutation which was *de novo* in the patient. See Table 1 and 2, Fig. 3 and Fig. S5 for summary of clinical findings. $PTA_{0.5,1,2,4\ kHz}$ was moderate (53 dBHL on the right and 48 dBHL on the left).

Speech recognition was assessed pre and post implantation in this patient using BKB sentences automated test. (Bench J et al, 1979). This varies the presentation level of the speech and records the point at which the patient is able to identify >70% of key words correctly. Results are below; pre-implant the patient needed speech at 64.4 dBA in a quiet background and could not complete the test in background noise of 55dBA. In contrast, 1 year post unilateral implant, she only required voice at 40 dBA in quiet background and could achieve a score >70% with a voice at 62 dBA when background noise was 55 dBA. She has since undergone a second side implant but post CI results are not yet available.

	Speech presentation level at which score >70% key words correct (dBA)	Noise level (dBA)	Signal to noise ratio (dB)
Pre implant; bilateral hearing aids; presented in quiet	64.4	n/a	n/a
Pre implant; bilateral hearing aids; presented in noise	Unable to complete the test at 70.0	55	+15
6 months post CI; unilateral implant alone; presented in quiet	53.8	n/a	n/a
6 months post CI; unilateral implant alone; presented in noise	64.3	55	+9.3
1 year post CI; unilateral implant alone; presented in quiet	40.0	n/a	n/a
1 year post CI; unilateral implant alone; presented in noise	62.0	55	+7.0

Family 9 (case 16) is a 17 year old British girl of South Asian origin. She had four episodes of fever followed by neurological deterioration between 18 months and 10 years of age. During one episode she had documented elevation of mycoplasma titres, one episode with HPV6 titres and one with streptococcal throat infection. By age 17 years optic atrophy, nystagmus, ataxia and titubation of the head were noted. Hearing impairment was diagnosed at age 5 years as auditory neuropathy with abnormal brain stem responses and cochlear microphonics present, OAEs present and poor speech discrimination. There was progressive loss of hearing. She had her first CI at age 10 years and the second one at age 16 years. The proband has multiple café au lait patches, but no other features of neurofibromatosis and no pathogenic mutation has been found in *NF1*. Over the years she underwent extensive neurometabolic testing including open muscle biopsy (with normal

respiratory chain enzymes), CSF neurotransmitters, lactate and glucose as well as genetic investigations which included *FMR1*, *SCA1*, *SCA2*, *SCA3*, *SCA6*, *SCA7 PDH*, *SLC52A2*, *SLC52A3* and *GJB2*.

Multiple MRI scans of the brain and internal auditory meati were performed over the years for the neurological features and were normal. EMG and NCV studies were also normal.

Eventually the CAPOS mutation was identified and found to be *de novo* in the proband. See Table 1 and 2 and Fig. S6 for summary of clinical findings. $PTA_{0.5,1,2,4\ kHz}$ was mild at age 8 years (34 dBHL on the right and 34 dBHL on the left) and mild/moderate at age 9 years (38 dBHL on the right and 59 dBHL on the left).

Speech recognition was assessed using AB word lists (Boothroyd, 1968). Pre-cochlear implant, the patient was able to achieve 59% of phonemes correctly using live voice without lip reading with lower scores in background noise; 3 years post implant she was able to identify 97% correctly without lip reading.

AB word lists			
		% phonemes correct with lip reading	% phonemes correct without lip reading
Pre CI	Bilateral aided – live voice	87	59
	Bilateral aided – live voice +25dB SNR		43
	Bilateral aided – live voice +20dB SNR		55
	Bilateral aided – live voice +15dB SNR		42
NB. Testing was attempted with recorded voice pre-CI but patient was unable to carry out the test and reported it all as 'noise'			
3 years post unilateral CI	Live voice in quiet		97

Family 10 (case 17) This male patient has been published previously (Rosewich *et al*., 2014) but audiological features were not described. He was diagnosed at age 12 years with CAPOS and the associated *ATP1A3* mutation. Detailed neurological examination showed ataxia, dysarthria, and mild dystonia, starting at age 4-6 years with several febrile episodes. He has mild-moderate hearing impairment with characteristics of auditory neuropathy (near absence of ABR, abnormal CAP, presence of some cochlear microphonic potentials and large OAE responses). See Fig. 4. Speech

perception tests showed threshold increases for numbers (4 syllables) RE: 30 dB; LE: 24dB Normal value for 50% correct identification of numbers is 0 dB). Monosyllable words 65 dB: RE: 50%; LE: 60%. Monosyllable words at 80 dB: RE: 95%; LE: 100%. Speech test in noise was reduced (Oldenburger Sentence test (Brand and Wagener, 2017)), speech and noise from loudspeaker in front): at a noise level of 65dB, 50% correct word identification was achieved at a signal level of 65.3 dB (normal level would be 58 dB). The patient is now 14 years old and attends a mainstream middle school with a small class (15 pupils). He did not benefit from hearing aids. He had used an FM communication system only for foreign language classes (English, French), which was abandoned because of little additional benefit when sitting close to the teacher. The problems are most obvious for speech perception and in particular speech in noise (0.3 dB signal to noise ratio required for 50% correct). This matches the parents' reports that a high attention level and visual contact were important for efficient communication. Moreover, his parents stated that sound localization was difficult which remains to be tested in the clinic. $PTA_{0.5,1,2,4\ kHz}$ was mild at age 12 years (27dB HL and 29dB HL at right and left ear, respectively) and not progressing at age 14 years.

Extensive genetic testing (sequencing of *PLA2G6, POLG, TWNK* and other genes) was performed until the CAPOS mutation was found. His parents were healthy and did not have the mutation.

See Fig. 4, Table 1 and 2 for summary of clinical findings.

Family 11 (case 18) The proband was born in Germany after an uncomplicated pregnancy to a healthy Italian mother and Spanish father. In contrast to other patients, proband 18 has followed a slightly different clinical course, since he was diagnosed with auditory neuropathy and no obvious neurological deficits at age 6 years, and is currently 8 years of age.

His newborn hearing screening examination was repeated several times until it yielded a passing result. He had physiotherapy from age 1 to 2 years and learned to walk at the age of 15 months. Since 2 years of age, he suffered from episodes of significant tiredness with reduced physical activity and communication, during which he complained of headache. He would be confined to bed and was unable to go to school. He was diagnosed with 'episodic migraine'. He had no episodes of fever leading to deterioration despite having typical childhood illnesses. At age 6 years, he was diagnosed with bilateral moderate low-frequency hearing impairment with auditory neuropathy that has not shown signs of progression (8½ years). $PTA_{0.5,1,2,4\ kHz}$ was at age 6,5 years mild (24 dBHL and 18 dBHL for right and left ear, respectively), and similar results were obtained

at age 7 years. He falls/trips often undertaking sporting activities (such as soccer) and was slow to learn how to ride a bicycle; he is still less stable, on a bicycle compared to his younger brother.

He has used hearing aids since age 6½ years. He attends a mainstream school but with reduced learning goals and extra assistance in German and arithmetic. He has been diagnosed with dyscalculia. His intellectual abilities are within normal limits. Notably, his "Test for Reception of Grammar" in German (TROG-D) (Kampfhaus RW, 2005) scored his speech understanding in the 18th percentile at 8½ years with hearing aids. Trilingual German, Italian and Spanish language education proved very difficult for him and was stopped. On examination he has mild anisocoria and a small number of café au lait macules that are not connected to any other syndrome. Clinical examination shows no nystagmus, visual dysfunction, areflexia, dystonia or autistic features. He does not wear glasses and does not have signs of peripheral neuropathy. Free field speech audiometry (the Göttinger speech intelligibility test II) showed poor speech discrimination. Left and right ears were subjected to this test at noise levels of 65 and 80 dB. The patient was able to discriminate 30% and 70% of words on the right ear, as well as 50% and 70% of words on the left ear at these respective thresholds.

Routine molecular genetic diagnostic work-up at $6^1/_2$ years of age included sequencing of *GJB2*, *GJB6*, *SLC26A4*, and *MT-RNR1* and yielded normal results. Two years later, whole exome screening revealed the c.2452G>A mutation in *ATP1A3* in a heterozygous state. The unaffected parents and younger brother were negative for this mutation.

Method

Whole Exome Sequencing (Family 11, case 18)

Whole exome sequencing was performed using Nextera Rapid Capture exome enrichment according to the manufacturer's protocol and sequenced with a NextSeq 500 (Illumina). Paired-end reads (2 x 75 bp) were subjected to bioinformatics processing, alignment, variant calling, and filtering which were performed using a locally established in-house exome analysis pipeline using human reference genome hg19/GRCh37. Frequency-based variant filtering according to a 0.01 minor allele frequency was used. Variant prioritization utilized multiple prediction algorithms (PolyPhen-2, SIFT, MutationTaster, fathmm, LRT and GERP).

ATP1A3 was analyzed using an *in silico* gene panel. The c.2452G>A variant was Sanger sequence validated according to standard protocols with primers available upon request.

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