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BRIEF REPORT

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Identification of a novel homozygous *synthesis of cytochrome c oxidase 2* variant in siblings with early-onset axonal Charcot-Marie-Tooth disease

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Abstract

The *synthesis of cytochrome c oxidase 2* (*SCO_2*) gene encodes for a mitochondrial located metallochaperone essential for the synthesis of the cytochrome *c* oxidase (COX) subunit 2. Recessive mutations in *SCO_2* have been reported in several cases with fatal infantile cardioencephalomyopathy with COX deficiency and in only four cases with axonal neuropathy. Here, we identified a homozygous pathogenic variant (c.361G > C; p.[Gly121Arg]) in *SCO_2* in two brothers with isolated axonal motor neuropathy. To address pathogenicity of the amino acid substitution, biochemical studies were performed and revealed increased level of the mutant SCO_2-protein and dysregulation of COX subunits in leukocytes and moreover unraveled decrease of proteins involved in the manifestation of neuropathies. Hence, our combined data strengthen the concept of *SCO_2* being causative for a very rare form of axonal neuropathy, expand its molecular genetic spectrum and provide first biochemical insights into the underlying pathophysiology.

KEYWORDS
axonal neuropathy, Charcot-Marie-Tooth disease, *synthesis of cytochrome c oxidase 2 (SCO2)*, white blood cell proteomics

The *synthesis of cytochrome c oxidase 2* (*SCO_2*) gene is located on chromosome 22q13.33 and encodes for a gene product, the cytochrome c oxidase (COX) assembly protein (SCO_2), which is needed for the assembly of COX (also known as complex IV of the mitochondrial respiratory chain) (Papadopoulou et al., 1999). COX is the unique terminal oxidase of the mitochondrial respiratory chain and is located in the inner mitochondrial membrane of all eukaryotes and has two main functions: (i) reduction of molecular oxygen to water by means of electron transfer from the cytochrome *c* to oxygen and (ii) pumping protons across the inner mitochondrial membrane into the cytoplasm (Rak et al., 2016). COX is composed of 13 subunits that need multiple assembly factors. Mutations in some of those (*SCO1*, *SCO_2*, *COX10*, and *SURF1*) are associated with a broad spectrum of clinical phenotypes.

SCO_2 contains a highly conserved copper-binding motif (CXXXC), which is important for the insertion of copper into subunit 2 of COX (Banci et al., 2007). Copper dysregulation is a key pathological feature in neurodegenerative disorders (Alzheimer's, Parkinson's, prion diseases as well as Menke's and Wilson's disease). Along this line, mutations in *ATP7A* and *ATP7B* causative for Menke's and Wilson's disease, respectively (Telianidis et al., 2013) are good examples. Kennerson and colleagues first described *ATP7A* mutations in two families restricted to progressive distal hereditary motor neuropathy (dHMN) without overt signs of systemic copper deficiency (Kennerson et al., 2010).

In 1999, the association between mutations in *SCO_2* and fatal cardioencephalomyopathy (MIM# 604377) with severe COX deficiency was first described by Papadopoulou and colleagues in three unrelated infants (Papadopoulou et al., 1999). The children described in this report were severely affected (increased lactate and lactate/pyruvate ratio, hypertrophic cardiomyopathy, brain abnormalities, neuropathic and myopathic signs in electrophysiology, dysmorphic features, and hepatomegaly). Due to the cardiomyopathy, they died at the age of 53 days, 11 weeks, and 6 months, respectively. Afterward, further reports, which now comprise more than 50 patients with the phenotype of fatal cardioencephalomyopathy due to homozygous or compound heterozygous mutations in SCO_2 (Freisinger et al., 2004; Gurgel-Giannetti et al., 2013; Jaksch, 2001) followed. In addition, *SCO_2* mutations were linked to a phenotype resembling spinal muscular atrophy type I (Jaksch et al., 2001; Tarnopolsky et al., 2004).

In 2018, Rebelo and colleagues first described two unrelated patients with axonal polyneuropathy, also known as Charcot-Marie-Tooth (CMT) neuropathy type 4 (Shy, 2018), due to a compound heterozygous variant in *SCO_2* (p.E140K/p.P169T and p.D135G/p.R171Q) (Rebelo et al., 2018). CMT in general is the most common inherited neuromuscular disorder, affecting 1 in 2500 individuals (Skre, 1974), with until today >100 genes described as being causative by means of autosomal dominant, autosomal recessive, or X-linked inheritance (Benarroch et al., 2020). Patients typically present with distal accentuated muscle wasting, distal onset of weakness, and sensory loss, often with foot deformities (Murphy et al., 2011).

Here, we describe two brothers with an isolated motor peripheral neuropathy with a novel homozygous mutation in *SCO_2* (c.361G>C; p.[Gly121Arg]). Thus far, these siblings are the fifth and sixth patients with a *SCO_2* variant leading to an axonal motor neuropathy, and the second family described with a homozygous variant in *SCO_2*. This combined molecular genetics and clinical observation strengthen the concept that *SCO_2* is a gene being causative for the manifestation of a neuropathic phenotype. Biochemical studies were performed to address the pathogenicity of the p.(Gly121Arg) missense variant and revealed an impact on protein stability as well as on general protein composition with a profound dysregulation of mitochondrial respiratory chain-related proteins as well as of such crucial for neuronal function.

We recruited both patients from the Department of Pediatric Neurology, Centre for Neuromuscular Disorders, University Hospital Essen, Germany. All data concerning patients II.1 and II.4 was extracted from their medical routine files. Informed consent including permission to publish data and photographs was obtained for both patients and the ethics committee of University Medicine Essen (19-9011-BO) had granted ethical approval. All procedures involving human subjects were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Concerning genetic analyses, the most frequent hereditary neuropathy, CMT1A, due to a duplication of *PMP22*, was excluded by MLPA (MRC Holland, SALSA MLPA Probemix P033). Next-generation sequencing (gene panel analysis including 1.678 genes with relevance in clinical diagnostics, custom-made target enrichment, Agilent Sure Select) was carried out on an Illumina NextSeq 500 system (Illumina, San Diego, CA) as 150 bp paired-end runs using v2.0 SBS chemistry. Sequencing reads were aligned to the human reference genome (GRCh37/hg19) using BWA (v0.7. 13-r1126) with standard parameters. Statistics on coverage and sequencing depth on the clinical targeted regions (i.e., RefSeq coding exons and ±5 intronic region) were calculated with a custom script. SNV and INDEL calling on the nuclear genes was conducted using SAMtools (v1.3.1) with subsequent coverage and quality-dependent filter steps. Variant annotation was performed with snpEff (v4.2) and Alamut-Batch (v1.4.4). Only variants (SNVs/small INDELs) in the coding and flanking intronic regions (± 50 bp) were evaluated.

For segregation studies, the relevant part of exon 2 of the *SCO_2* gene was amplified using the Immolase™ DNA polymerase Kit (Firma Bioline) and the following primers: 2F: CCTGTTAAGCCTGAACGTGG and 2R: AGGGTTGAGCAGGTAGATGG. PCR conditions: standard protocol with 40 cycles of amplification and 40 ng of genomic DNA for each sample. Sanger sequencing was performed using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) on a 3100 genetic analyzer (Applied Biosystems).

Three-millimeter skin punch biopsies were taken distally, 10 cm above the lateral malleolus of the leg in the patient and his father. Skin samples were fixed with Zamboni fixative (2% paraformaldehyde, 15% picrin acid, phosphate-buffered saline [PBS]) for at least 24 h, washed in PBS, transferred to 10% sucrose, and stored at −80°C freezer until further use. From each biopsy 50-µm thick frozen sections were stained using a free-floating protocol. Primary antibody anti-protein gene product (PGP 9.5, 1:1000, Zytomed) was incubated overnight at room temperature in a PBS solution, containing 0.1% Triton X-100, 0.1% Tween 20, and 0.1% bovine serum albumin. The goat anti-rabbit Alexa Fluor 488 secondary antibody (1:1000, Thermo

Fisher Scientific) was incubated for 3 h at room temperature. Sections were mounted with DAPI Fluorshield (Abcam).

The samples were examined using a Leica DM 2000 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at magnification x400 with a Leica DFC450C camera. Morphological analysis was performed with Leica Application-Suite Version 4.7.1 and Fiji ImageJ (version 1.51n). The investigator (AS) was blinded to the specimen category during the morphologic evaluation of biopsies.

The snap-frozen white blood cell fractions were lysed by adding 200 µl of 50 mM Tris-HCl (pH 7.8) buffer, 5% SDS, and cOmplete ULTRA protease inhibitor (Roche) using the Bioruptor® (Diagenode) for 10 min (30 s on, 30 s off, 10 cycles) at 4°C followed by centrifugation at 4°C and 20,000 g for 15 min. Protein concentration of the supernatant was determined by BCA assay according to the manufacturer's protocol. Disulfide bonds were reduced by addition of 10 mM TCEP at 37°C for 30 min, and free sulfhydryl bonds were alkylated with 15 mM IAA at room temperature (RT) in the dark for 30 min. Hundred microgram protein of each sample was used for proteolysis using the S-Trap protocol (Protifi) and using a protein to trypsin ratio of 20:1. The incubation time for trypsin was changed to 2 h at 37°C. Proteolysis was stopped using FA to acidify the sample (pH < 3.0).

All proteolytic digests were checked for complete digestion after desalting by using monolithic column separation (PepSwift monolithic PS-DVB PL-CAP200-PM, Dionex) on an inert Ultimate 3000 HPLC (Dionex) by direct injection of 1 µg sample. A binary gradient (solvent A: 0.1% TFA, solvent B: 0.08% TFA, 84% ACN) ranging from 5% to 12% B in 5 min and then from 12% to 50% B in 15 min at a flow rate of 2.2 µl/min and at 60°C, was applied. UV traces were acquired at 214 nm (Burkhart et al., 2012).

All samples for DIA-LC-MS/MS analysis were analyzed using an Ultimate 3000 nano RSLC system coupled to an Orbitrap Fusion Lumos mass (all Thermo Scientific) operating in data-independent mode (DIA). One microgram of peptides for each sample was preconcentrated on a 100 µm × 2 cm C18 trapping column for 10 min using 0.1% TFA (v/v) with a flow rate of 20 µl/min, followed by separation on a 75 µm × 50 cm C18 main column (both Pepmap, Thermo Scientific) with a 120 min LC gradient ranging from 3% to 35% B (84% ACN in 0.1% FA) at a flow rate of 250 nl/min. Each sample contained an appropriate amount of iRT standard (Biognosys) peptides. MS survey scans were acquired from 300 to 1100 m/z at a resolution of 60,000 FWHM followed by MS/MS using 24 DIA windows, each covering a range of 25 m/z (with 1 m/z overlap) at a resolution of 30,000. The polysiloxane ion at 445.12 m/z was used as lock mass (Olsen et al., 2005) CID spectra were acquired with normalized collision energy of 32% and an activation time of 10 ms. For the analysis of the samples acquired with nano-LC-MS/MS in DIA mode, the data were introduced to the Spectronaut software (Biognosys) and analyzed with a library-based search. As library an in-house created spectral library was used. Search and extraction settings were kept as standard (BGS Factory settings). As proteome background, the human proteome data were selected from UniProt (www.uniprot.org) containing 20,374 entries. For reliable label-free quantification, only proteins identified with ≥2 unique peptides were considered for further analysis. Next, the average normalized abundances (obtained by Spectronaut) were calculated for each protein and were used to determine the ratios between patient muscle samples with their respective controls. Last, for each protein log2 transformation of the generated ratios and Student's *t*-test *p*-values were calculated using MS Excel.

Parents are healthy, distantly related, of south Asian descent (Afghanistan). Neurologic, neuromuscular, or metabolic disorders in family history were denied. They have two nonaffected/healthy children (son 9 years and daughter 11 years) and two affected sons described in the following paragraphs and listed in Table 1, as well as in the pedigree as II.1 and II.4, respectively.

Concerning the Index (II.1): Pregnancy was uneventful, birth and postnatal period were uncomplicated. Early childhood psychomotor development was normal, as remembered by parents. Beginning at the age of 6 years, parents noticed frequent falls. He developed a joint contracture of the right ankle, requiring a tendotomy. Since the gait abnormalities persisted, a second operation of the right foot was performed (no further information was available, both surgeries were performed in Afghanistan). In the course, a reduced range of motion of the left ankle in combination with weak dorsiflexion of both feet and beginning atrophy of both lower legs were noted. At the age of 9 years, he developed atrophy of the hand muscles resulting in weakness of finger abduction and extension. At the age of 11 years and 7 months, the patient showed mild resting tremor. He was not able to stand or walk without help (muscle strength of legs: 1–2 of 5 medical research council [MRC]), due to a physical assault on the flight from Afghanistan (no further information). Severe atrophy of both lower legs, moderate atrophy of hands and feet, high-arched feet, and contractures of ankles and first metacarpophalangeal joints were observed.

At 12 years of age, parents reported worsening of the hand function (inability of thumb abduction and adduction) but improvement of muscle strength in both legs (3–4 of 5 MRC). Diagnostic work-up showed a borderline creatine kinase (CK) of 183 U/l (normal range <180 U/l), severe abnormality of peripheral nerve conduction consistent with severe axonal neuropathy (for details see Table 1). Electrocardiography (ECG), echocardiography, and pulmonary function test were normal. Brain magnet resonance imaging (MRI) revealed diffuse white matter alterations (Figure 1b). At 12 years and 8 months, he developed persisting severe back pain and profound disturbance of fine motor abilities as major issues. After a period of intensive physiotherapy, the patient was able to walk short distances without help but was wheelchair depended for longer distances. At 13 years and 9 months, his muscular strength further worsened: he had distal pronounced muscle weakness (no finger extension and no foot dorsiflexion, both 0 of 5 MRC), was only able to walk a few steps without help and showed a significant steppage gait. Both lower leg muscles were nearly completely atrophied (Figure 1c, 1 and 2), he had distinct high-arched feet with claw toes, was not able to extend and abduct his fingers (Figure 1c, 3 and 4) and a clear resting tremor of both hands was evident. In the CMT neuropathy score-version 2 (CMTNSv2), he reached 13 of 28 points, indicating moderate neuropathic symptoms. At 14 years, additional diagnostic workup was done, revealing normal results for copper in serum and urine

TABLE 1 Detailed comparison of patients described in literature (Barcia et al., 2019; Rebelo et al., 2018) and the two patients described in this study

	Rebelo and colleagues, 2018		Barcia and colleagues, 2019		This study	
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5: Index (II.1)	Patient 6: Affected brother (II.4)
Sex	**Female**	**Male**	**Male**	**Female**	**Male**	**Male**
Age at last appointment	9 years	26 years	8 years	6 years	14 years	6 years
Country of origin	Unknown	Unknown	West African origin		Afghanistan	
Inheritance	Recessive (compound heterozygous)	Recessive (compound heterozygous)	Recessive (homozygous)		Recessive (homozygous)	
Type of mutation	Missense	Missense	Missense		Missense	
cDNA	c.418G>A and c.505C>A	c.404A>G and c.512G>A	c.763C>T		c.361G>C	
Protein change	p.Glu140Lys and p.Pro169Thr	p.Arg171Gln and p.Asp135Gly	p.Arg255Trp		p.(Gly121Arg)	
First symptom	Unable to lift her head up from prone position at 6 months	Inability to keep up with peers, heel cord tightness and foot drop at 12 years	Motor and speech delay	Cerebellar syndrome (ataxic gait, mild tremor), distal muscular atrophy at 28 months	Frequent falls and reduced range of motion of the right ankle at 6 years	Motor delay: walking unassisted with 24 months
Developmental delay	Yes	Unknown	Yes	Unknown	No	Yes
Walking ability status	Started toe walking at 15 months, needed ankle-foot orthotics since 3 years of age, full-time wheelchair dependant since 5 years of age	Ambulatory with full-time use of ankle-foot orthotics	Started walking with frequent falls at 19 months, at 2 years, he could walk a few steps with gait ataxia	Started walking unaided at 16 months; ataxic gait at 28 months	"Normal" age of walking; at 14 years: able to walk 10 m unaided, wheelchair dependant for longer distances	Started walking at 24 months; at 6 years ambulatory, maximum walking distance according to age
Speech development	Unknown	Unknown	No speech at 8 years	Could say a few words	Normal	Mild delay
Intellectual disability	Unknown	Unknown	Special school	Normal school	No test performed	Yes, IQ 50
Muscle relief	Significant distal upper and lower extremity muscle wasting	Striking lower leg atrophy	Severe distal myoatrophy of all four limbs	Distal muscular atrophy	Profound atrophy of lower legs, moderate atrophy of hands and feet	Normal
Muscle strength	Distal > proximal, can bring hands to mouth, needs help eating and writing	Progressive distal lower extremity weakness with inability to move feet	Unknown	Unknown	Distal >proximal, muscle weakness, inability to extend fingers or dorsiflex feet	Normal
Reflexes	Absent	Normal at the biceps, triceps, and knees, absent at the ankles	Lack of ankle reflexes, normal patellar reflexes	Weak, yet present deep tendon reflexes	Normal at the biceps, triceps, and knees, absent at the ankles	Normal
Tremor	Unknown	Unknown	Yes	Mild	Yes	Yes

TABLE 1 (Continued)

	Rebelo and colleagues, 2018		Barcia and colleagues, 2019		This study	
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5: Index (II.1)	Patient 6: Affected brother (II.4)
Sex	Female	Male	Male	Female	Male	Male
Sensory exam	Mildly diminished vibratory sense at the toes	Decreased vibratory sensation in toes and ankles	Unknown	Unknown	Normal	Normal
CMTNSv2	17/28	7/28	Unknown	Unknown	13/28	3/28
Nerve conduction velocity	Length dependent, motor predominant axonal polyneuropathy	Length dependent axonal sensorimotor polyneuropathy	Severe axonal neuropathy	Unknown	At the age of 11 years 7 months severe axonal neuropathy, lower extremities predominant	At 3 years and 10 months motor axonal neuropathy
Brain MRI	Normal at 24 months	Unknown	Bilateral cerebellar vermis atrophy, T2 hyper-intensities of the posterior white matter at 6 years	Cerebellar atrophy with posterior hyperintensities of the white matter at 6 years	Diffuse white matter alterations at 13 years	Not performed
Serum lactate	Midly elevated, lactate to pyruvate ratio normal	Normal	Elevated	Unknown	Normal	Not performed
Serum copper	Normal	Normal	Unknown	Unknown	Normal	Not performed
COX deficiency	Muscle biopsy: COX normal at 24 months	Not performed	Skeletal muscle homogenate: isolated COX deficiency	Circulating lymphocytes: COX activity defective	Not performed	Not performed
Echocardiography/ ECG	Normal/normal	Normal/premature ventricular contractions	Normal/unknown	Normal/unknown	Normal/normal	Normal/normal

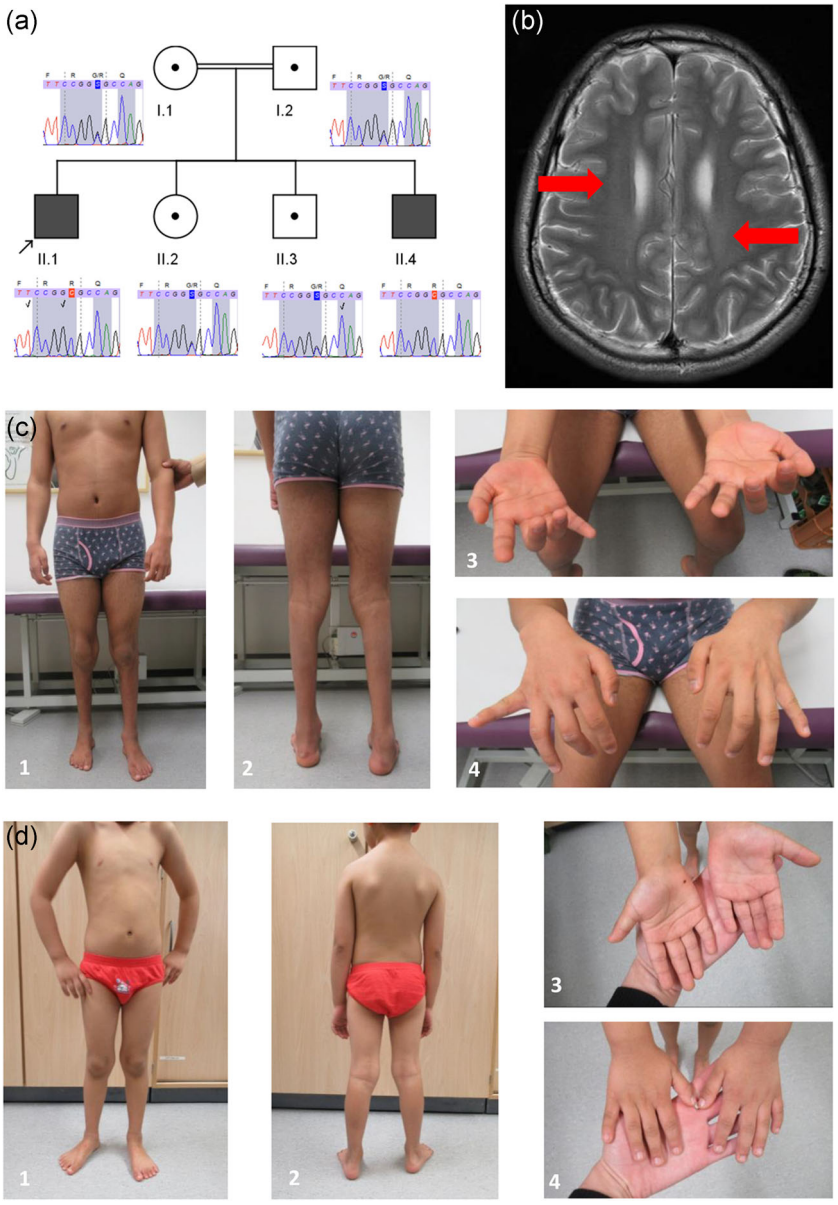

FIGURE 1 (a) Pedigree of the family with a homozygous variant in SCO_2 (c.361G > C; p.[Gly121Arg]) in the Index (II.1) and his affected brother (II.4) and a heterozygous state in both parents (I.1 and I.2) as well as in both healthy siblings (II.2 and II.3). (b) Brain MRI of the index patient (II.1) at the age of 12 years shows diffuse white matter alterations (red arrows). (c) Index (II.1) aged 13 years and 8 months: (1 and 2) Profound atrophy of both lower legs (3 and 4) Inability to extend and abduct fingers. (c) Affected younger brother (II.4) aged 5 years and 6 months: (1 and 2) Unremarkable muscle relief and (3 and 4) regular hand function

(including 24-h collection urine), as well as normal lactate (pre- and postprandial) and normal intraepidermal nerve fiber density (Table 1).

Concerning the affected brother (II.4): Pregnancy was uneventful, birth and postnatal period were uncomplicated. Early childhood motor development was abnormal. He showed a gross motor delay (walking unassisted with 2 years) and reduced fine motor skills (not being able to eat with a fork at 4 years). Speech development was also mildly delayed, considering that he is raised trilingual (Dari, English, and German). Cognitive development seemed to be normal as reported by parents. Beginning at the age of 3 years parents observed shaking of both legs during walking and physical exercise. He had to hold on the railing when climbing stairs to prevent tripping and was not able to jump or run. At the age of 3 years and 10 months, he was able to climb stairs, showed tremor of both hands, slim calves but no joint contractures, no foot deformities, no atrophy of hands or feet. Peripheral nerve conduction velocities were abnormal (for details see Table 1) consistent with motor neuropathy (no further measurements possible due to noncompliance). At the age of 4 years and 4 months, parents reported normal walking distances but poor balance, causing frequent falls (5–6 per day). Climbing stairs was still problematic, he was not able to jump nor to eat unaided due to the persisting tremor of both hands.

At his last clinical examination, at 5 years and 6 months, he was able to run short distances but got exhausted quickly. Balance improved under physiotherapy and occupational therapy, but frequent falls and problems climbing stairs persisted, as well as problems eating unaided. He presented moderate intentional and resting tremor of both hands, bipedal bouncing was only hinted, standing on one leg was only possible for a short moment and very unstable, as well as balancing on a line. Mild contractures were present in both ankle joints (10°/0°/50°). Additionally, a hinted scapula winging was noted (Figure 1d). Muscle strength, muscle tone, and muscle relief were normal (Figure 1d). CMTNSv2 showed mild neuropathic

symptoms (3 of 28 points). At the same age, the intelligence quotient (IQ) was measured using the Wechsler Preschool and Primary Scale of Intelligence—Third Edition (WPPSI-III) and revealed a heterogeneous performance profile far below the average (verbal part IQ 51, action part IQ 61, processing speed IQ 52, overall test 50) (normal value 85–115). For further details, see Table 1.

To obtain a molecular genetic diagnosis, first, a single gene dosage analysis of *PMP22* was performed showing a normal result. Second, a multi-gene panel was conducted revealing normal results for *BSCL2*, *DYNC1H1*, *GDAP1*, *GJB1*, *GNB4*, *HSPB1*, *IGHMBP2*, *MFN2*, *MPZ*, *TTR*, but a homozygous variant in *SCO_2* (c.361G > C; p.[Gly121Arg]) was found, which was classified as class 3 variant (variant of uncertain significance, VUS) according to the American College of Medical Genetics and Genomics (ACMG) classification criteria. The case-related assessment was "possibly pathogenic." The variant was not listed in gnomAD, ClinVar, HGMD, or LOVD. Third, segregation analysis of the *SCO_2* variant was performed in the parents and siblings uncovering the same homozygous variant in his affected brother (II.4) and a heterozygous status in his parents (I.1 and I.2) and the healthy siblings (II.2 and II.3), respectively (Figure 1a).

To evaluate the pathogenicity of the identified missense variant, immunoblot studies were carried out on protein extracts of white blood cells isolated from EDTA-blood. These studies revealed a profound increase of SCO_2 in patient-derived cells compared to those derived from the father (Figure S2e). Additionally, an increase of TIM23 (component of a complex that mediates the translocation of proteins across the mitochondrial inner membrane) was identified accompanied by a decrease of MICU1 (key regulator of mitochondrial calcium uniporter) and MFN2 (mitochondrial outer membrane GTPase that mediates mitochondrial clustering and fusion) (Figure S2e). Coomassie-blue staining was performed to visualized protein loading (Figure S2e).

Proteomics enables the identification of a variety of protein dysregulations in a single experiment and thus holds the potential to identify pathophysiological processes in an unbiased manner (Roos et al., 2018). Here, we applied proteomic profiling on whole protein extracts derived from white blood cells of the two index cases in addition to the healthy siblings and parents (serving as controls). Based on a data-independent approach, we quantified 2358 proteins with a p-ANOVA below 0.05. A total of 61 proteins (2.6% of the statistically significant quantified proteins) showed a dysregulation: 46 proteins are decreased whereas 15 are increased. Proteomic data have been uploaded to ProteomeXchange (identifier: PXD026283). A GO-term based in silico analysis aimed to pinpoint pathophysiological processes upon expression of aberrant SCO_2 (Figure S4). Increased proteins are indicative for altered protein binding and processing whereas decreased proteins hint toward (among others) mitochondrial activity and extracellular matrix composition. Of note, six COX subunits are significantly decreased whereby subunit 2 (Figure S2d) shows the most profound downregulation (81% decrease compared to controls) (Table S1).

Based on reported increased pain perception and thus to address the question if sensory nerves are also affected in terms of a "small-fiber-neuropathy," PGP9.5 staining was performed on skin biopsies derived from index patient II.1 and his father I.2. Intraepidermal nerve fibers crossing the dermal-epidermal junction were counted according to published counting recommendations (Lauria, Bakkers, et al., 2010). At minimum, six sections were analyzed from each biopsy. Intraepidermal nerve fiber density (IENFD) was assessed as the density of the total length of the epidermis (nerve fibers/mm). The IENFD in the patients was 14.4 IENF/mm. Compared to recommended reference values for 20- to 29-year-old subjects (median: 10.9, 5% quantile 6.1) (Lauria, Bakkers, et al., 2010) the IENFD of the patient (14.4 IENF/mm) (Figure S3a) and the father (8.6 IENF/mm) (Figure S3b) was not reduced. For the 14-year-old boy the normal values might be little higher but the IENFD is still in the normal range (Görlach et al., 2020).

The SCO_2 protein is an essential component of COX (complex IV), the unique terminal oxidase of the mitochondrial respiratory chain. Its main role is provision of monovalent copper (Cu^+) and assembly of COX. In the vast majority of described cases, pathogenic *SCO_2* variants are associated with fatal infantile cardioencephalomyopathy with COX deficiency (MIM# 604377). This common phenotype presents with multiorgan involvement including cardiomyopathy, progressive encephalopathy, and muscular weakness. The vast majority of patients die during infancy (Verdijk et al., 2008). Other manifestations of *SCO_2* mutations include Leigh syndrome, hypertrophic cardiomyopathy, lactic acidosis, stridor with ventilator insufficiency, and a phenotype resembling spinal muscular atrophy (Gurgel-Giannetti et al., 2013).

Up to date, there are only four further cases with a motor-predominant neuropathic phenotype caused by recessive variants of *SCO_2* described (Barcia et al., 2019; Rebelo et al., 2018). Both of our patients extend the phenotypic spectrum of *SCO_2* by adding the fifth and sixth patient with a motor-predominant neuropathic phenotype due to a novel homozygous variant in *SCO_2*. Clinical data of all reported patients are summarized in Table 1 presenting a detailed comparison of patients described in literature (Barcia et al., 2019; Rebelo et al., 2018) and the two patients described in this study.

Remarkably, onset of symptoms shows a wide range (6 months to 12 years), as well as the severity of neuropathy grading using the CMNTSv2 (3/28 to 17/28). Of note, 3 out of 6 patients showed a distal muscle weakness, 5 out of 6 presented with muscle atrophy in combination with (partially) absent reflexes. One might assume, that the sixth patient II.4 (at 5 ½ years the youngest patient) will still develop the same symptoms at a later disease stage. Increased pain perception is only reported in one of our cases and was not associated with small fiber pathology. Interestingly, our index patient is the only patient with proven intellectual disability (IQ 50) reported so far, his brother has not yet been tested. In two patients reported by Barcia et al speech development was severely delayed implicating some intellectual impairment (Barcia et al., 2019). MRI revealed white matter alternations in 4/6 patients, 2/6 presented with cerebellar atrophy. So we postulate that the intellectual impairment may be a part of the phenotypic spectrum of *SCO_2*-patients and thus recommend intellectual testing these patients. Almost in all patients (4/5) a primarily axonal neuropathy was proven by altered nerve conduction velocities (see Table 1). In our patient II.1, this was also evident by absent deep tendon reflexes and distal atrophy in

lower extremities (Figure 1). None of the patients developed hypertrophic cardiomyopathy, strengthening the concept of a different phenotype compared to patients presenting with early onset cardioencephalomyopathy.

The homozygous variant in *SCO$_2$* (c.361G>C; p.[Gly121Arg]) found in our index patient was initially classified as class 3 variant (VUS) according to the ACMG classification. The case-related assessment was "possibly pathogenic," it was not listed in gnomAD. To prove our theory of the variant being pathogenic, we performed a segregation analysis of the *SCO$_2$* variant found in the family, showing (i) a homozygous constellation in his affected brother (II.4), in conformity with (ii) both parents (I.1 and I.2) and (iii) both healthy brothers and sisters (II.2 and II.3) being heterozygous carriers. In combination with the fitting CMT phenotype and the concordance with the two patients described by Rebelo et al. (2018), this clearly supports the concept of this newly discovered homozygous *SCO$_2$* variant being pathogenic and causing a CMT phenotype.

However, to strengthen this concept, additional biochemical studies were performed on protein extracts of white blood cells: immunoblot studies revealed increased abundance of SCO$_2$ in cells derived of one of the index patients analyzed compared to the level in cells of the heterozygous father. This finding might either hint toward altered (increased) protein stability based on the amino acid substitution or arise from a compensatory mechanism in terms of increased expression of the mutant gene. In this context, it is important to note that in other pure *SCO$_2$*-related neuropathy cases quantification of the SCO$_2$ protein was not performed, and more systematic studies are needed to investigate whether increase of mutant SCO$_2$ protein has a toxic effect. However, decrease of cytochrome c oxidase subunit II (MT-CO$_2$/COX$_2$) as identified by our proteomic profiling hints toward a loss of function of the mutant protein. In addition, by immunoblotting we identified dysregulation of further mitochondrial proteins including TIM23, MFN2, and MICU1 suggesting a greater impact of the p.(Gly121Arg)-*SCO$_2$* variant on mitochondrial protein composition and most likely function. However, one might assume that dysregulation of this protein might reflect a secondary effect of mitochondrial vulnerability. Important to note that Mitofusin-2 (MFN2) is one of two ubiquitously expressed homologous proteins in eukaryote cells, playing a critical role in mitochondrial fusion. Mutations in MFN2 (most commonly autosomal dominant) cause Charcot-Marie-Tooth disease type 2A (CMT2A), the commonest axonal form of CMT (Pipis et al., 2020). Recessive mutations in the *MICU1* gene cause a very rare neuromuscular disease characterized by myopathy with extrapyramidal signs, due to primary alterations in mitochondrial calcium signaling (Bitarafan et al., 2021; Kohlschmidt et al., 2021). However, further functional studies on patient-derived cells such as fibroblasts or neuronal cells would be needed to study the precise effect of the p.(Gly121Arg)-*SCO$_2$* variant on mitochondrial fusion and calcium signaling. Nevertheless, results of our unbiased proteomic profiling moreover support the assumption of perturbed mitochondrial function upon p.(Gly121Arg)-SCO$_2$ expression by showing a decrease of six COX subunits whereby subunit 2 shown the most profound downregulation in addition to TXNRD2 controlling reactive oxygen species levels and the regulation of mitochondrial redox homeostasis. Of note, SCO$_2$ acts a copper metallochaperone which is essential for the synthesis and maturation of COX subunit 2 (MT-CO2/COX2) (Leary et al., 2009), thus in turn not only suggesting an impact on mitochondrial function but also underlying the pathogenicity of the p.(Gly121Arg) amino acid substitution. Interestingly, also other proteins identified as being decreased by proteomics are known to be involved in neurological processes: ABHD16A mediates the hydrolysis of phosphatidylserine to generate lysophosphatidylserine (LPS), a signaling lipids that regulating neurological processes (www.uniprot.org/uniprot/O95870). PRUNE1 plays a role in the regulation of neurogenesis (Zollo et al., 2017) and RAB11FIP1 acts as a Rab11 effector protein involved in the endosomal recycling process (Jin & Goldenring, 2006). Perturbed endosomal recycling via Rab11 was already linked to the pathophysiology of a recessive form of peripheral neuropathy (Stendel et al., 2010). TXNRD2 is involved in the control of reactive oxygen species levels and the regulation of mitochondrial redox homeostasis. Mutations of *TXNRD2* are causative for familial glucocorticoid deficiency-5 which is characterized by resistance to adrenocorticotropic hormone (ACTH) and isolated glucocorticoid deficiency which may lead to long-term neurological damage (Prasad et al., 2014). MICU1 was identified as being dysregulated by our immunoblot studies. Recessive *MICU1* mutations were linked to myopathy with extrapyramidal signs and learning disability in pediatric cases (Kohlschmidt et al., 2021; Logan et al., 2014).

Up to date, there is no causative therapy for *SCO$_2$*-associated phenotypes. In 2004 Freisinger et al. reported the case of a 13-month-old girl with hypertrophic cardiomyopathy due to a *SCO$_2$* mutation. By subcutaneously supplementing copper histidine over a time period of 60 days, a temporary reversion of hypertrophic cardiomyopathy could be achieved. The therapy was very well tolerated, and no copper toxicity was noted. The patient died aged 45 months due to a severe pneumonia (Freisinger et al., 2004).

In patients with *ATP7A*-related dHMN, copper replacement was considered by Kaler in 2013—despite normal serum copper levels—based on the known relationship between acquired copper deficiency and peripheral neuropathy. He assessed this treatment consideration as especially relevant for pediatric patients in dHMN families who possess a mutant allele but have not yet developed neurological symptoms (Kaler, 2013). In contrast, Natera-de Benito and colleagues reported a patient with dHMN due to an *ATP7A* mutation who experienced a severe clinical and neurophysiologic worsening when he was treated with copper replacement therapy, with a subsequent fast recovery after the copper histidinate was withdrawn (Natera-de Benito et al., 2021).

We discussed the possible option of an individual healing attempt of both boys including a risk-benefit assessment with the family. Normal results for copper in serum and urine (including 24-h collection urine) as well as lack of cardiomyopathy in combination with observations of Natera-de Benito et al. (2021) did not encourage us in treating the boys with copper histidine. In addition, the family was very reluctant about an individual healing attempt.

To conclude, our data expand the phenotypic spectrum of *SCO$_2$*-associated mutations by adding the fifth and sixth patient with the rare phenotype of axonal polyneuropathy, describing intellectual disability as part of the disease for the first time. Our proteomic data

helped us to confirm the pathogenicity of the mutation. Further investigations of copper metabolism and the development of polyneuropathy are necessary to evaluate the option of a treatment with copper histidine for these patients.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The variant data concerning the novel *SCO_2* variant described here have been uploaded to Leiden Open Variation Database (https://www.lovd.nl/).

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