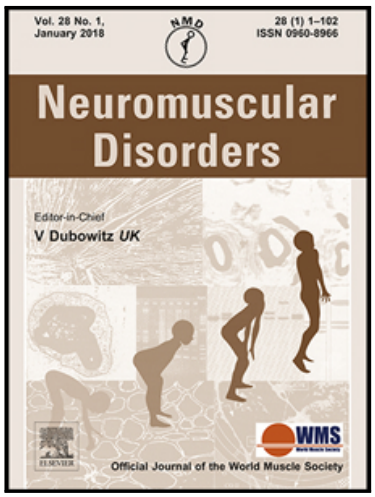

Hereditary polyneuropathy with optic atrophy due to PDXK variant leading to impaired Vitamin B6 metabolism

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Highlights:

- Pathogenic variants in *PDXK* cause hereditary axonal polyneuropathy and optic atrophy.
- Symptoms manifest in (pre)adolescent age and slowly progresses to blindness and ambulation loss.
- Pyridoxal 5'-phosphate supplementation might prevent disease progression.

Hereditary polyneuropathy with optic atrophy due to *PDXK* variant leading to impaired Vitamin B6 metabolism

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ABSTRACT

PDXK encodes for a pyridoxal kinase, which converts inactive B_6 vitamers to the active cofactor pyridoxal 5'-phosphate (PLP). Recently, biallelic pathogenic variants in *PDXK* were shown to cause axonal Charcot-Marie-Tooth disease with optic atrophy that responds to PLP supplementation. We present two affected siblings carrying a novel biallelic missense *PDXK* variant with a similar phenotype with earlier onset. After detection of a novel *PDXK* variant using Whole Exome Sequencing, we confirmed pathogenicity through *in silico* protein structure analysis, determination of pyridoxal kinase activity using liquid chromatography-tandem mass spectrometry, and measurement of plasma PLP concentrations using high performance liquid chromatography. Our *in silico* analysis shows a potential effect on PDXK dimer stability, as well as a putative effect on posttranslational ubiquitination that is predicted to lead to increased protein degradation. We demonstrate that the variant leads to almost complete loss of PDXK enzymatic activity and low PLP levels. Our patients' early diagnosis and prompt PLP replacement restored the PLP plasma levels, enabling long-term monitoring of clinical outcomes. We recommend that patients presenting with similar phenotype should be screened for *PDXK* mutations, as this is a rare opportunity for treatment.

Keywords

hereditary neuropathy, optic atrophy, pyridoxal kinase, *PDXK*, pyridoxal phosphate, vitamin B6

Abbreviations:

PLP: pyridoxal 5'-phosphate, **PN:** pyridoxine, **PL:** pyridoxal, **PM:** pyridoxamine, **PDXK:** pyridoxal kinase, **PNPO:** pyridoxamine 5'-phosphate oxidase, **PDXP:** pyridoxal phosphatase

1. Introduction

Pyridoxal 5'-phosphate (PLP) is an essential co-factor involved in vital metabolic pathways, including neurotransmitter production and amino acid biosynthesis (1, 2). Like all other mammals, humans cannot synthesize PLP *de novo* and therefore require the dietary uptake of the inactive B_6 vitamers pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM) which are subsequently converted into catalytically active PLP (3). Bioregulation of PLP involves three enzymes namely, pyridoxal kinase (PDXK), pyridoxamine 5'-phosphate oxidase (PNPO) and pyridoxal phosphatase (PDXP). PDXK catalyzes the ATP-dependent phosphorylation of the B_6 vitamers PN, PL and PM to their phosphorylated counterparts PNP (pyridoxine 5'-phosphate), PLP and PMP (pyridoxamine 5'-phosphate) (4). PNPO catalyzes the conversion from PNP and PMP into PLP, which is the metabolically active form of vitamin B6 (3, 5). PDXP dephosphorylates the cytosolic excess of catalytically active PLP.(6) Mutations in *PNPO* are known to cause neonatal epileptic encephalopathy responsive to PLP substitution (OMIM 610090) (7).

Only recently, Chelban *et al*. published the first report of biallelic variants in *PDXK*, causing PLP-responsive hereditary motor and sensory neuropathy type 6C with optic atrophy (HMSN6C, OMIM 618511) (8). They reported five affected individuals from two unrelated families presenting with axonal peripheral polyneuropathy and optic atrophy progressing to ambulation loss and blindness. Importantly, PLP supplementation was shown to improve the biochemical and motor phenotype of the affected individuals. The reported mutations (p.Arg220Gln and p.Ala228Thr) were shown to affect PDXK ATP-binding through conformational rearrangements, leading to reduced PDXK enzymatic activity and low plasma PLP concentrations.

Here, we report two affected individuals from a consanguineous family presenting with a childhood-onset sensorimotor axonal neuropathy and first signs of optic atrophy with a novel

biallelic *PDXK* mutation. In addition, we characterize the functional effect of our missense variant on the PDXK enzyme activity, compare with the effect of previously published variants and therefore provide a full picture of the biochemical consequences of the reported mutations on enzyme level.

2. Material and methods

2.1. Whole Exome Sequencing

Informed consent for next-generation sequencing (NGS) and photos depicting relevant clinical features were obtained from the parents and the affected individuals following the regulations of the ethics committee of the University of Cologne. For index patient (II.2), we first performed an NGS-based gene panel comprising 479 genes associated with neuromuscular diseases. After negative results, we expanded our analysis with Whole Exome Sequencing (WES). We used the Agilent SureSelect All Exon v7 target enrichment method with sequencing on an Illumina NovaSeq^{TM} 6000 platform. For data analysis, we used the new version of our Varbank analysis tool (Varbank 2.0) (https://varbank.ccg.uni-koeln.de/varbank2/). WES data processing was performed as previously described (9). The analysis based on runs of homozygosity (ROH) resulted in a ROH score of 299, which strongly supports the consanguinity between parents.

2.2. Protein modelling

Protein structures of human PDXK (2F7K and 3KEU) were acquired from RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Cartoon representations were generated using PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

2.3. Pyridoxal kinase activity measurement

The pyridoxal kinase activity present in dried blood spots (DBS) was determined by using the protocol described previously by Chelban *et al* (8). In summary, 3 mm discs punched from dried blood spots were incubated for 10 min at 37 °C with shaking at 300 rpm in a reaction buffer containing 20 mmol/L potassium phosphate (pH 6.1), 10 μmol/L pyridoxal and 300 μmol/L MgATP (all purchased from Sigma-Aldrich, Gillingham, UK), prior to addition of 120 μl of a reaction stop mix identical to that used for the determination of pyridoxamine 5'-phosphate oxidase activity from DBS.

DBS were collected from the affected siblings, the heterozygous carrier parents and the unaffected sibling, two previously published *PDXK* pathogenic variants and six healthy controls with *PDXK* variants previously excluded. Pyridoxal kinase activity of dried blood spots was determined by using liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to determine the formation of pyridoxal 5'-phosphate after incubation of these with the enzyme substrate pyridoxal and expressed as pmol PLP (3 mm DBS)$^{-1}$ h^{-1} from DBS.

Data collection and statistical analysis were performed using Waters MassLynx and OriginPro 2017 software packages.

2.4. HPLC measurement of B_6 vitamers

Pyridoxal phosphate was measured by HPLC with fluorescence detection using a Chromsystems kit as described previously (10).

3. Results

3.1. Clinical description

Both affected siblings (Patient II.1, currently 17 years old; Patient II.2, currently 14 years old) presented with progressive lower and upper distal limb weakness beginning at the age of 9 and 10, respectively. The initial lower limb weakness progressed with upper limb weakness. At their current age, both patients show gait difficulty but remain able to walk independently.

In both patients, neurological examination showed atrophy and weakness of the thenar, hypothenar, and intrinsic hand muscles (Fig 1B to F). Patient II.1 had joint contractures in both fifth fingers (DIPs). Both patients showed *pes cavus* and hammertoe deformity (Fig 1D). The MRC muscle score was 55 (of 60) in both affected siblings. Reflexes were moderately decreased in the upper limbs and significantly decreased in the lower limbs. Sensory examination showed reduction to light touch, pinprick, temperature, joint position and vibration in lower extremities up to knees as well as in the upper limbs up to elbows. Visual acuity was reduced in both patients (6/10 in II.1 and 7/10 in II.2), yet colour and peripheral vision remained intact. Moreover, fundoscopic examination revealed bilateral optic disk pallor, and optical coherence tomography confirmed optic atrophy in both patients, showing peripapillary thinning of the combined retinal nerve fibre layer (NFL), ganglion cell layer (GCL) and inner plexiform layer (IPL). In Patient II.2, the NFL, GCL and IPL thickness was significantly below average in all six paripapillary sectors in both eyes, with an average thickness of 83 µm and 84 µm in the right and left eye, respectively (Fig 1G). The remaining cranial nerve examination was normal.

Electromyography was consistent with chronic length-dependent denervation in both patients (Supplementary Table 1). Nerve conduction studies in Patient II.2 showed absent compound muscle action potential (CMAP) of the right peroneal and tibial nerves and reduced CMAP of the median and ulnar nerves. Sensory nerve action potentials (SNAPs) were absent in the right sural nerve. Motor nerve conduction velocity was normal (47 m/s), whereas sensory nerve conduction velocity was reduced in the right median (29 m/s) and ulnar (26 m/s) nerves. These results are consistent with chronic axonal sensorimotor neuropathy with predominance in the lower limbs (Supplementary Table 1).

3.2. Genetic testing

First, we filtered for homozygous variants spanning the ROH tracts and found 36 rare homozygous variants (Supplementary Table 2). After exclusion of variants that are phenotypically and functionally irrelevant or not co-segregated, we identified a homozygous missense variant in *PDXK* (NM_003681.4), c.225T>A (p.Asn75Lys), as the most promising candidate. The latter was located in a homozygous stretch of 5.2 Mb in the 21q22.3 region. The variant was not reported in the in-house database including 12,000 exomes, in gnomAD (https://gnomad.broadinstitute.org/), in the Greater Middle East Variome database (http://igm.ucsd.edu/gme/), or in the Iranome database (http://www.iranome.ir/) and predicted to have a score of 0.6 (0=benign; 1=pathogenic) according to 31 different *in silico* prediction algorithms (Supplementary Data). Segregation analysis by Sanger sequencing confirmed the homozygote variant in both siblings, and heterozygosity in the unaffected sibling and the parents (Supplementary Data).

3.3. Potential effect of PDXK p.Asn75Lys mutation on protein structure and function

Asn75 is located in the loop between helix α2 and strand β4 (11). Our *in silico* modelling analyses indicated that the p.Asn75Lys mutation compromises hydrophobic interactions, which could in turn have a destabilizing effect on WT-Met74 and thus on the dimeric conformation of PDXK (Fig 2A). Secondly, the p.Asn75Lys substitution has a putative impact on WT-Lys76 (Fig 2A-B), which is a posttranslational ubiquitin attachment site. The latter could have a major effect on protein degradation and turnover and thus, function.

3.4. Confirmation of pathogenicity via reduced enzymatic activity and low PLP

To confirm the pathogenicity of the *PDXK* p.Asn75Lys mutation we measured the PDXK enzymatic activity and the end product, PLP, in patients' blood samples. We then measured the PDXK activities from samples with previously published pathogenic PDXK variants for

comparison. Each sample was analysed in duplicate. We show that the novel p.Asn75Lys mutation leads to almost complete loss of PDXK enzymatic activity in the two homozygote siblings whereas there is higher activity in the heterozygote carrier parents and sibling, although still below the published normal range of 2.6 – 14.7 pmol/hr. There is an overlap between the enzymatic activity of heterozygote carriers reported here (p.Asn75Lys) and the enzymatic activities in one previously reported pathogenic variant (p.Ala228Thr). The novel mutation reported here is predicted to have a more severe impact and this could explain the low enzymatic activity seen in heterozygote carriers (Fig 2C).

Finally, we measured plasma PLP concentrations in the affected individuals. Low plasma PLP levels were detected in both affected siblings (Table 1).

3.5. Supplementation with PLP

After confirmation of low vitamin PLP levels in both patients (10.8 and 9.6 nmol/l, respectively), oral PLP supplementation (50 mg/day) was started. Four weeks after starting the PLP supplements plasma levels had increased to above-average levels of 350.3 nmol/l (II.1) and 144.2 nmol/l (II.2), respectively.

4. Discussion

Here we report two siblings at adolescent age (14 and 17 years) with autosomal recessive axonal sensorimotor polyneuropathy and optic atrophy caused by a biallelic *PDXK* variant. The optic atrophy and motor symptoms began concurrently at 10 years of age. Unlike the mild decrease in the visual acuity, colour vision was normal at the time of the assessment. The patients' young age at the time of diagnosis enabled an early start of PLP supplementation treatment, which not only allows patient's long-term monitoring but will also reveal the potential efficiency of prompt PLP treatment in preventing the progression of the disease.

Pyridoxal kinase activities measured from DBS samples were almost absent in our patients with homozygous p.Asn75Lys mutation and significantly reduced in the heterozygous carriers. Interestingly, the enzymatic reduction in the samples from the previously reported patients with homozygous p.Ala228Thr mutation were less remarkable than in our patients (Fig 2C). As Asn75 is located in the loop between the helix α2 and strand β4, which is a posttranslational ubiquitin attachment site, changes at this position may explain the more severe impact seen on the enzymatic activity when compared to mutations located at Ala228 position. Furthermore, we were unable to perform NCS in the heterozygous p.Asn75Lys carriers; therefore, a subclinical phenotype with minor NCS changes in these individuals cannot be excluded. Further work, investigating heterozygous *PDXK* carriers with PDXK enzymatic activity below the normal range, is necessary in order to elucidate the full *PDXK*-related disease processes.

Chelban *et al.* reported a motor improvement with ambulation regain and alleviation of neuropathic pain in their two elderly probands after 24 months of PLP treatment, despite starting the therapy more than 60 years after disease onset. However, no improvement was observed in sensation or vision, most likely due to the advance disease stage. In the peripheral nervous system (PNS), the upregulation of numerous growth-promoting genes (like *SPRR1A* (12), *GAP-43* and *CAP-23* (13), and *Sox-11* (14)) after nerve damage allows axonal regeneration and functional recovery, which is why a gain of motor function can be observed after vitamin B_6 supplementation. Nevertheless, a recovery in visual symptoms is not expected, since axon regeneration is extremely limited in the central nervous system (CNS), mainly due to the regeneration-inhibitory CNS environment (15) generated by the expression of growth-inhibiting molecules by oligodendrocytes and astrocytes (16), as well as the lack of upregulation of growth-promoting genes after nerve damage (17). As the probands reported by Chelban (8) were both over 75 years old at the start of treatment and had lost their visual capacity significantly, the possibility to regain the vision was comprehensively low.

Therefore, in our cases, it will be especially interesting to see whether an early start of vitamin B_6 supplementation could prevent further optic nerve damage and keep patients from losing their visual capacity, and whether a long-term treatment will allow improvements in sensation as well as in motor function.

5. Conclusion

This third, unrelated family confirms that biallelic mutations in *PDXK* lead to axonal peripheral polyneuropathy with optic atrophy via reduced PDXK enzymatic activity and low PLP. As this is a rare opportunity for treatment intervention, it is essential that variants in *PDXK* should be investigated particularly in patients with autosomal recessive, early-onset polyneuropathy, enabling PLP supplementation in time to prevent loss of ambulation and vision.

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Author Contributions

NK and MK for acquisition and analysis of data and drafting a significant portion of the manuscript and figures. RM, VC, HH, YK and PM for acquisition of data, enzymatic assays and drafting the clinical table. RB, PNT and EGK, recruitment and clinical evaluation of the family, following up and data collection on PLP measurement. NMF for data acquisition and protein analyses. HT for acquisition and analysis of the data, providing the sequencing platform. MK and BW for conception and design of the study and leading the study. All authors have read and approved the manuscript.

Potential Conflicts of Interest

The authors declare no conflict of interest.

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FIGURE LEGENDS:

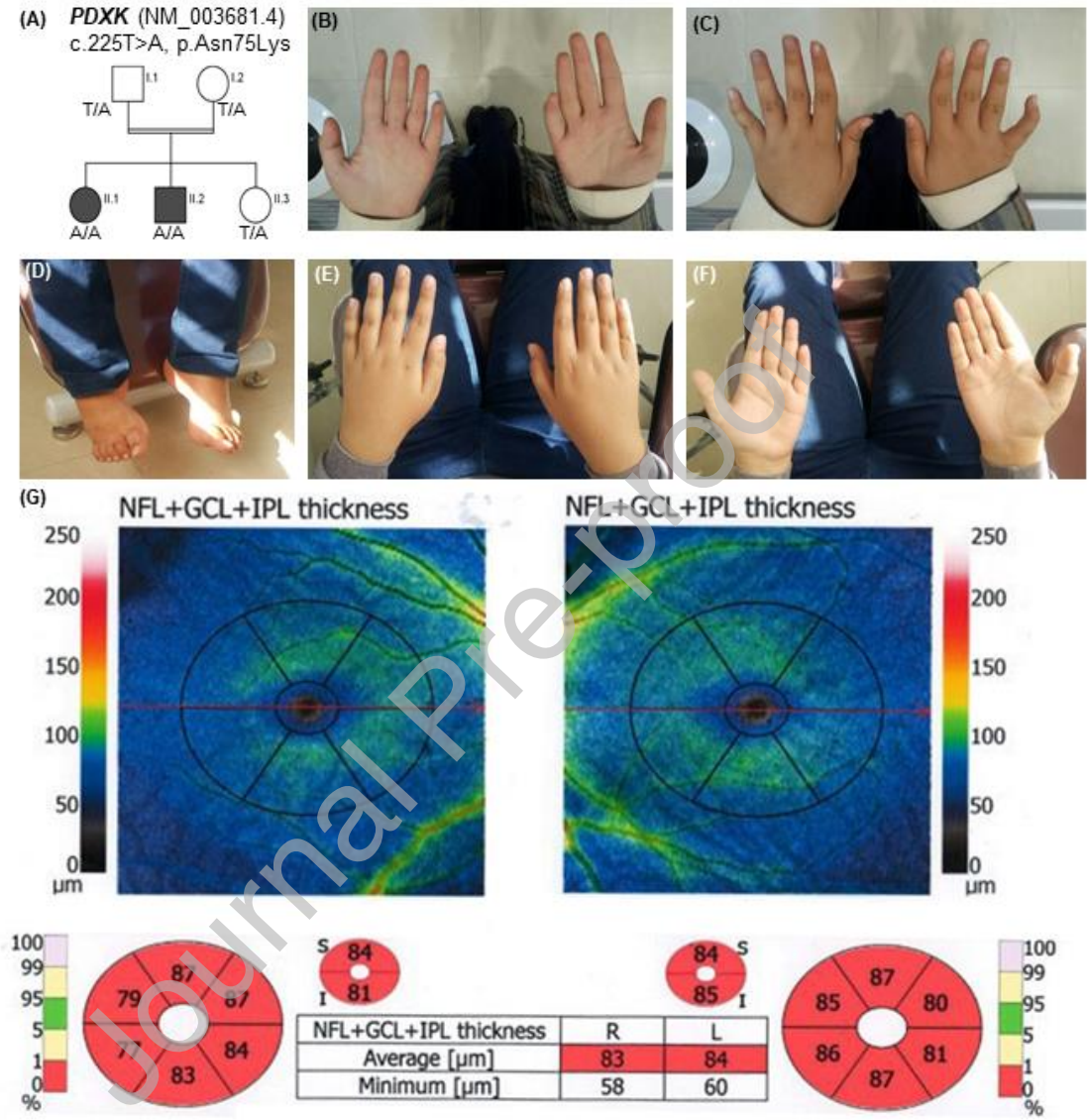
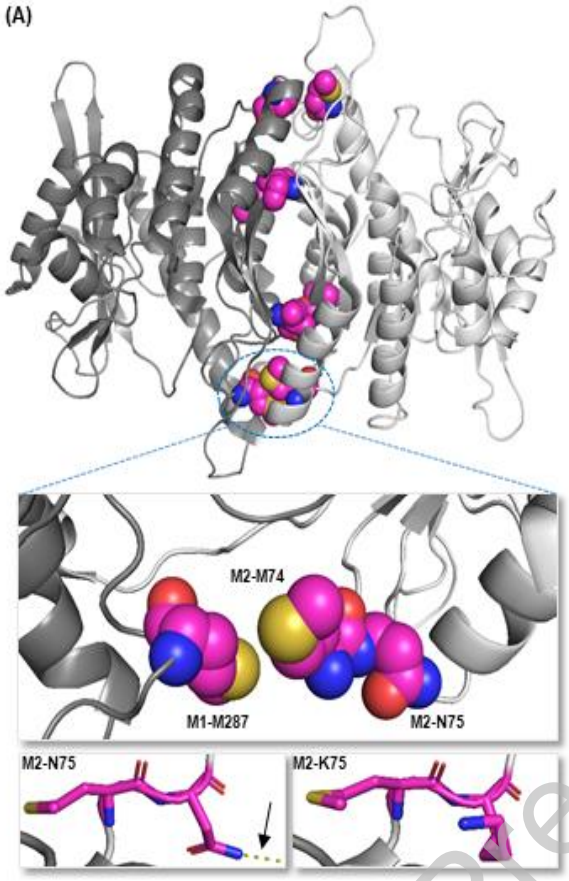


Figure 1: Clinical findings of observed patients (A) Pedigree of the family. II.1 and II.2 are the affected individuals. **(B-C)** Atrophy of the hand muscles and finger contractures of individual II.1. **(D)** Pes cavus and hammertoe deformity of individual II.2. **(E-F)** Atrophy of the hand muscles of individual II.2. **(G)** Optical coherence tomography (OCT) results of individual II.2. Abnormal thinning of the retinal nerve fibre layer (NFL), ganglion cell layer (GCL) and inner plexiform layer (IPL) taken together.

(A)



(B)

	70			74	75	76				80
H. sapiens	R	L	N	N	M	N	K	Y	D	Y	V
H. sapiens (mut)	R	L	N	N	M	**K**	K	Y	D	Y	V
P. troglodytes	R	L	N	N	M	N	K	Y	D	Y	V
M. mulatta	***K***	L	N	N	***V***	N	K	Y	D	Y	V
F. catus	***K***	L	N	N	***V***	N	K	Y	D	Y	V
M. musculus	***K***	***V***	N	**D**	***V***	N	K	Y	D	Y	V
G. gallus	***K***	L	N	**K**	***V***	N	**Q**	Y	D	Y	V
D. rerio	***K***	L	N	N	***V***	N	***H***	Y	D	Y	V

(C)

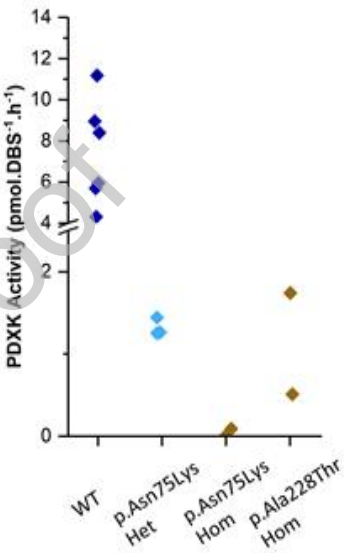


Figure 2: Potential functional effect on protein and enzyme levels (A) Cartoon representation of the crystal structure of *Homo sapiens* PDXK (X-Ray diffraction, 2.8 Å resolution, PDB ID 2F7K). In the upper structure, PDXK monomers are depicted in dark and light grey. The hydrophobic residues at the dimer interface (Ile15, Ile35, Met74, and Met287) are depicted as spheres in both monomers. The middle panel shows a magnification (12 Å Sphere) into the Met74-Asn75 area. M1 and M2 indicate monomer 1 and 2, respectively. The lower images depict the WT-Asn75 and mutant Lys75 residues depicted as magenta sticks. Notice the absence of a hydrophobic bond (dotted yellow line) in the mutant residue. **(B)** Conservation of Asn75 in *PDXK* across species. Bold&underlined: amino acid changes causing a charge alteration. Bold&*italic*: amino acid changes within similar groups. (**C**) Erythrocyte PDXK activity in dried blood spots from cases homozygous for the novel p.Asn75Lys and previously published, known pathogenic p.Ala228Thr versus controls. Patients homozygous for p.Asn75Lys have lower activity than all controls. The two different values from p.Ala228Thr mutation come from different siblings, in which the more severely affected sibling has the lower pyridoxal kinase activity (0,5 vs. 1,7 pmol.DBS^{-1}.h^{-1}). Activity measured as PLP formed after incubation of a 3mm dried blood spot punch with pyridoxal. Each sample was analysed in duplicate and the mean is shown.

TABLE 1: Clinical features of affected individuals with *PDXK* variants compared with the reported affected individuals in Chelban *et al.* 2019

	Present family		Chelban (2019)
Phenotype/Case	**II.1**	**II.2**	**5 individuals / 2 families ***
Demographics			
Origin	Iran	Iran	Cyprus, Italy/Scotland
Gender	F	M	1 M, 4 F
Age at examination,yrs	17	14	29 to 79 years
Age at onset, yrs	9	10	2 to 9 years
Progression			
Symptoms at onset	Lower limb weakness	Upper and lower limb weakness	Lower limb weakness and wasting
Upper limb weakness	At 10 yrs	At 10 yrs	12 to 20 yrs
Optic atrophy	At 10 yrs	At 10 yrs	29 to 50 yrs
Neurological examination			
Fundoscopy	Pale optic discs	Pale optic discs	Pale optic discs (4/5)
Other cranial nerves	Normal	Normal	Normal
Skeletal deformities	Pes cavus, hammer toes, clawing of hands	Pes cavus, hammer toes, clawing of hands	Pes cavus (5/5), hammer toes (5/5), clawing of hands (3/5)
Power	Moderate weakness of dorsiflexion/plantar flexion, long finger extensors and intrinsic muscles of the hands	Moderate weakness of dorsiflexion/plantar flexion, long finger extensors and intrinsic muscles of the hands	Moderate to severe weakness of dorsiflexion/plantar flexion, long finger extensors; mild to moderate-severe weakness of intrinsic muscles of the hands
MRC power score	55	55	44; 44; 54; 56
Reflexes	Upper limbs: reduced Lower limbs: reduced	Upper limbs: reduced Lower limbs: reduced	Areflexia; mute plantar responses (4/5)
Sensation	Reduced sensation, upper and lower distal: light touch, pinprick, temperature, joint position, vibration	Reduced sensation, upper and lower distal: light touch, pinprick, temperature, joint position, vibration	Reduced pain and vibration sense in distal limbs (4/5)
Romberg sign	Negative	Negative	Present (4/5)
Coordination	Normal	Insecure in handling things	Normal (4/5)
Visual acuities	6/10	7/10	Reduced (2/5); NA(2/5)
Colour vision	Normal	Normal	Impaired (4/5)
Peripheral vision	Normal	Normal	Normal (4/5)
Cognitive function	Normal	Normal	Normal (4/5)
Seizures	Absent	Absent	Absent (4/5)
Investigation results			
MRI head	Normal	Normal	Normal (2/5); NA (2/5)
Optic nerve and chiasm CT	Optic atrophy	Optic atrophy	Normal (2/5); NA(2/5)
VEPs	NA	NA	Attenuated with anomalous waveform on flash VEPs (2/5); NA (2/5)
Somatosensory evoked potentials	Poorly formed due to severe polyneuropathy	NA	Poorly formed due to severe polyneuropathy (1/5); NA (3/5)*
Nerve conduction study	NA	Severe sensorimotor axonal neuropathy	Severe sensorimotor axonal neuropathy (4/5)
Electromyography	NA	Chronic denervation in a length-dependent pattern; no myopathic changes	Chronic denervation in a length-dependent pattern; no myopathic changes (4/5)
Renal function	Normal	Normal	Normal (4/5)
Biochemical profile			
Liver function	Normal	Normal	Normal (4/5)
GI tract	NA	NA	Normal (4/5)
Plasma amino acids **	NA	Normal	Normal (1/5); NA (3/5)
Vitamin B_1, normal range 3-17 ng/ml	9.7	11.2	Normal (3/5); NA (1/5)
Vitamin B_6, normal range 20-125 nmol/l	10.8	9.6	7.8 and 9
Vitamin B_{12}, normal range 200-835 pg/ml	192	167	Elevated (1/5); normal (3/5)

* one of the reported patient died at 71 year-of-age from an unrelated medical condition and could therefore not be included in the examination. ** Plasma amino acids tested methionine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, lysine, histidine, and arginine. CT = computed tomography; F = female; GI = gastrointestinal; M = male; MRC = Medical Research Council; MRI = magnetic resonance imaging; NA = not available; VEP = visual evoked potential; yrs = years