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Difficulties in recognition of pyruvate dehydrogenase complex deficiency on the basis of clinical and biochemical features. The role of next-generation sequencing

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ABSTRACT

Pyruvate dehydrogenase complex (PDHc) defect is a well-known cause of mitochondrial disorders (MD) with at least six responsible genes (*PDHA1*, *PDHB*, *DLAT*, *DLD*, *PDHX*, *PDP1*). The aim of this work was to assess the diagnostic value of biochemical methods in recognition of PDHc defect in Polish patients with suspicion of MD. In the first step, Western blot of the E1α subunit was performed on 86 archive muscle bioptates with suspicion of MD. In the second step, Sanger *PDHA1* sequencing was performed in 21 cases with low E1α expression. In the third step, 7 patients with negative results of *PDHA1* sequencing were subjected to whole-exome sequencing (WES). This protocol revealed 4 patients with *PDHA1* and one with *DLD* mutations. Four additional probands were diagnosed outside the protocol (WES or Sanger sequencing).

The molecular characterization of PDHc defect was conducted in a total of 9 probands: 5 according to and 4 off the protocol. Additionally, two affected relatives were recognized by a family study. Altogether we identified seven different *PDHA1* changes, including two novel variants [c.464T > C (p.Met155Thr) and c.856_859dupACTT (p.Arg288Leufs*10)] and one *DLD* variant.

The lactate response to glucose load in the *PDHA1* subset was compared to a subset of non PDHc-related MD. Opposite responses were observed, with an increase of 23% and decrease of 27%, respectively.

The results show that determining lactate response to glucose load and muscle E1α expression may contribute to distinguishing PDHc-related and other MD, however, WES is becoming the method of choice for MD diagnostics.

1. Introduction

Pyruvate dehydrogenase complex (PDHc) deficiency is a frequent cause of mitochondrial disorders. Progressive neurological symptoms usually start in infancy, but may be evident at birth or in later childhood, and adult onset is very rare. They may include developmental delay, brain malformations, microcephaly, poor muscle tone, seizures, intermittent ataxia, West syndrome, and Leigh-like syndrome.

There are six forms of PDHc deficiency, depending on the genetic background and damaged subunit of the enzyme complex. A few cases are known to result from mutations in genes encoding subunits: E1β (*PDHB*), E2 (*DLAT*), E3 (*DLD*), and E3BP (*PDHX*) or PDH phosphatase (*PDP1*). The most common causes are mutations in X-linked *PDHA1*, encoding the E1α subunit [1]. *PDHA1* maps to the Xp22.1 region and consists of eleven exons. The majority of mutations in this gene occur *de novo*. Hemizygous males are generally symptomatic, whereas heterozygous females present variable expression of the mutant and normal genes in different tissues as a result of the X-inactivation pattern [2].

This is the first genetic study of PDHc deficiency in Poland and we report novel pathogenic variants and recurrent causal mutations in the genes *PDHA1* and *DLD*. Our aim was also to check the utility of Western

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Table 1
Clinical, biochemical and molecular data of 11 patients with pathogenic variants in PDHc related genes.

Data and symptom(s)	Patient KI (p)	Patient KW (b)	Patient SzO (p)	Patient BF (p)	Patient KBS (m)	Patient PM (p)	Patient GP (p)	Patient KG (p)	Patient PM (p)	Patient ZJ (p)	Patient PZ (p)
Patient no.	1	2	3	4	5	6	7	8	9	10	11
Family no.	1		2	3		4	5	6	7	8	9
Sex	M	M	F	M	F	M	F	F	F	F	F
Age of onset	2 y	ND	Neonatal	Neonatal	2 y	15 m	3 m	7 m	Birth	4 m	1.5 y
Age at diagnosis	8.5	ND	2	4 y	ND	8 y	2.5 y	4.5 y	25 y	2 y	9 y
Neurological findings											
Psychomotor retardation/developmental delay	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Developmental delay (>3 y)	No	No		Yes	No	ND	Yes	Yes	Yes	Yes	Yes
Dysarthria	No	No	Nd	Yes	Yes	Yes	ND	ND	ND	ND	Yes
Microcephaly	No	ND	No	ND	No	No	Yes	Yes	Yes	Yes	No
Seizures	No	ND	No	No	No	No	Yes	Yes	No	Yes	No
Ataxia	No	ND	Yes	Yes	Yes	Yes	ND	No	ND	No	No
Hypotonia/hypertonia	No	ND	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Peripheral neuropathy	Yes	ND	ND	ND	Yes	Yes	ND	ND	ND	ND	No
Brain malformations											
Brain atrophy	ND	ND	No	Yes	No	ND	Yes	ND	Yes	Yes	Yes
Corpus callosum hypoplasia	ND	ND	No	ND	No	ND	Yes	Yes	ND	Yes	Yes
Cerebellum atrophy	ND	ND	No	No	No	No	ND	No	ND	VH	VH
Demyelinisation	ND	ND	No	Yes	No	ND	ND	ND	ND	ND	Yes
Basal ganglia abnormalities	ND	ND	Yes	ND	No	ND	ND	ND	ND	ND	Yes
Brain stem involvement	ND	ND	Yes	ND	No	ND	ND	ND	ND	ND	ND
Ocular findings											
Nystagmus	No	No	Yes	No	No	No	ND	No	Yes	No	No
Ptosis	No	No	No	No	No	No	ND	No	Yes	No	Yes
Oculomotor apraxia	No	No	Yes	No	No	Yes	ND	No	Yes	No	No
Biochemical findings											
Blood lactate (fasting) [mg/dL]	42.66	NA	26	20.8	47.4	62	36.2–65.6	39	42	54	104
Blood pyruvate (fasting) [mg/dL]	2.2	NA	2	1.7	2.3	3.7	NA	2.8	NA	NA	NA
Blood lactate (after carbohydrate) [mg/dL]	39	NA	25	29.6	NA	92	NA	NA	51	53	NA
Blood pyruvate (after carbohydrate) [mg/dL]	2.6	NA	1.5	2.1	NA	4.1	NA	NA	NA	4.5	NA
Blood lactate/pyruvate ratio	18	NA	13	12	20.7	17	NA	14	7.5	12	NA
CSF lactate [mg/dL]	40	NA	NA	NA	NA	67	75	47	87	NA	58.8
Alanine [μmol/dL]	NA	NA	559	180	NA	238	952	NA	222–1338	806	407
GC/MS urine	LA, PA, 2-KGA	NA	NA	2-KGA, LA	NA	2-KGA, LA	LA, 2-KGA	NA	2-KGA, LA	2-KGA, LA, MMA	Normal

(continued on next page)

Table 1 (*continued*)

Data and symptom(s)	Patient KI (p)	Patient KW (b)	Patient SzO (p)	Patient BF (p)	Patient KBS (m)	Patient PM (p)	Patient GP (p)	Patient KG (p)	Patient PM (p)	Patient ZJ (p)	Patient PZ (p)
Patient no.	1	2	3	4	5	6	7	8	9	10	11
Family no.	1		2	3		4	5	6	7	8	9
pH	NA	NA	7.43	7.49	NA	7.46	NA	7.374	7,368	7.3	7.39
pO_2 [mmHg]	NA	NA	117	86.5	NA	83.5	NA	81.6	NA	121	92.3
pCO_2 [mmHg]	NA	NA	26,3	21.5	NA	26.7	NA	25.6	35.7	18.7	32.1
	Muscle biochemistry										
Complex I	NA	NA	NA	NA	NA	10	NA	12.2	11.6	<3.0	5.9
Complex II	NA	NA	NA	NA	NA	7.6	NA	6.5	3.5	7.3	3.4
Complex II + III	NA	NA	NA	NA	NA	4.7	NA	4	5.5	<3.0	3.6
Complex III	NA	NA	NA	NA	NA	44.7	NA	69.2	47.5	71.9	72
Complex IV	NA	NA	NA	NA	NA	10	NA	6.1	19.5	5.2	3.9
SC	NA	NA	NA	NA	NA	275	NA	211.4	122.6	513.8	268.2
E1α [% of mean reference]	NA	NA	NA	52.5	NA	0	NA	38.5	NA	20.9	59.7
	Muscle histology and histochemistry										
Variability of fiber size	No	ND	ND	ND	ND	Yes	ND	Yes	ND	No	Yes
Lipid accumulation	No	ND	ND	ND	ND	Yes	ND	No	Yes	Yes	No
Predominance of fibers type I	No	ND	ND	ND	ND	No	ND	Yes	ND	Yes	Yes
	Clinical diagnosis										
Clinical diagnosis prior to genetic testing	Guillain-Barre syndrome	Family study	Family study	PDHc deficiency	Ataxia	PDHc deficiency	Leigh syndrome	Mitochondrial encephalopathy	Mitochondrial encephalomyopathy	PDHc deficiency	Leigh syndrome
Nijmegen score	4	3	5	3	2	3	4	3	6	3	6
Type of PDHc	PDH E1α subunit	PDH E1α subunit	PDH E1α subunit	PDH E1α subunit	PDH E1α subunit	PDH E1α subunit	PDH E1α subunit	PDH E1α subunit	PDH E1α subunit	PDH E1α subunit	PDH E3 subunit
Neurological phenotype (final assigning)											
Neonatal encephalopathy with lactic acidosis							**x**	**x**	**x**	**x**	
Basal ganglia abnormalities (Leigh-like)			**x**								**x**
Chronic/progressive neurologic deterioration	**x**	**x**			**x**						
Intermittent ataxia				**x**		**x**					
	Molecular genetics										
DNA source (tissue)	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Muscle	Muscle	Muscle	Muscle
Molecular test	WES	Sanger	Sanger	Sanger	Sanger	Sanger	WES	Sanger	WES	Sanger	WES
Mutated gene	*PDHA1*	*PDHA1*	*PDHA1*	*PDHA1*	*PDHA1*	*PDHA1*	*PDHA1*	*PDHA1*	*PDHA1*	*PDHA1*	*DLD*
Nucleotide exchange	c.262C > T	c.262C > T	c.464T > C	c.787C > G	c.787C > G	c.787C > G	c.856_859dupACTT	c.904C > T	c.933_935delAAG	c. 934_940delAGTAAGA	c.1123G > A
Amino acid exchange	p.R88C	p.R88C	p.M155 T	p.R263G	p.R263G	p.R263G	p.Arg288Leufs*10	p.R302C	p.Arg311del	p.S312Vfs*12	p.E375K
Localization of mutation	Exon 3	Exon 3	Exon 5	Exon 8	Exon 8	Exon 8	Exon 9	Exon 10	Exon 10	Exon 10	Exon 11
Mutation type	Missense	Missense	Missense	Missense	Missense	Missense	Frameshift	Missense	Inframe deletion	Frameshift	Missense
Mutation status	Known	Known	Novel	Known	Known	Known	Novel	Known	Known	Known	Known
Mutation inheritance	Maternal	Maternal	Maternal	Maternal	Na	Na	*De novo*	*De novo*	*De novo*	Na	Parental
	Familial history										
Affected relative	Brother	Brother	No	Mother, uncle	Son, brother	NA	No	No	No	NA	No

p - proband; b - brother; m - mother; y - year; m - month; 2-KGA - ketoglutaric acid; LA - lactic acid; MMA - methylmalonic acid, NA - not analysed; ND - no data; PA - pyruvic acid, Sanger - Sanger sequencing; VH - vermis hypoplasia; WES – whole-exome sequencing.

blotting of the PDHc E1α subunit in muscle and of fasting/postprandial lactate/pyruvate ratio specificity in the recognition of PDHc defect when pyruvate oxidation in fresh muscle cannot be measured [3].

2. Methods

2.1. Patients

Muscle specimens of 86 patients with suspicion of mitochondrial disorder (MD) were selected from 570 muscle biopsies performed at our nation-wide referral center (CMHI) in the period from 1996 to 2015. Open muscle biopsy was done and histochemical and spectrophotometric techniques were applied for assessment of oxidative phosphorylation system function (OXPHOS) as described previously [4].

The inclusion criteria were: (1) OXPHOS activity normal or inconclusive, (2) chronic/progressive encephalopathy with lactic acidosis, brain malformations, and Leigh syndrome, (3) lactate elevation in response to glucose/carbohydrate load, (4) negative screening for common mtDNA mutations (m.8993T > C and m.8993T > G in *MTATP6*, m.3243A > G in *MTTL1*, m.8344A > G in *MTTK*) and nDNA mutations (c.311_312insAT312_321del10 and c.845_846delCT in *SURF1*) responsible for LS (5), muscle sample and/or DNA availability, (6) informed consent of the patient's parents or guardians.

2.2. Western blot analysis

Tissue lysates (30 μg protein) from muscle biopsies (patients and healthy controls) were separated electrophoretically in 10% SDS polyacrylamide gels and transferred onto PVDF membranes (BioRad). The membranes were blocked and proteins were detected using *anti*-subunit E1α pyruvate dehydrogenase (PDH) WB Antibody (Abcam) followed by appropriate secondary AP-conjugated antibodies (1:1000, Bio-Rad). The level of the E1α subunit of the pyruvate dehydrogenase complex was analyzed densitometrically and the levels of individual subunits were calculated as ratios to the corresponding subunit in control tissue. Reference values were established in 27 muscle samples from patients with other (non-mitochondrial) diseases. The deficiency threshold was arbitrarily assumed as below of 50% of the reference mean. When important inclusion criteria coexisted (LS, X-linked inheritance, normal lactate/pyruvate ratio), a higher threshold was applied (about 60%).

2.3. Molecular analysis

Genomic DNA was isolated from different tissues (blood, muscles, fibroblasts) using standard phenol-chloroform or automatic DNA extraction (MagNA Pure LC 2.0, Roche).

Scanning of *PDHA1* molecular variants was performed by Sanger sequencing and long-range polymerase chain amplification as a gene deletion assay. The sizes of wild-type or mutant (shorter) PCR fragments were determined by agarose (1%)-gel electrophoresis. In *PDHA1*-unsolved patients, whole-exome sequencing (WES) was further conducted.

WES was performed on a HiSeq 1500 using an Exome Enrichment Kit (Illumina) according to a published protocol [5]. The average read depth was 120 with >90% of the targeted regions covered at least 20-fold. Generated reads were aligned to the hg19 reference human genome. Alignments were viewed with Integrative Genomics Viewer v.2.3.40. The detected variants were annotated using Annovar and converted to MS Access format for final manual analysis. Variants were filtered to exclude changes with an average frequency higher than 0.01 (for AR inheritance model) and 0.001 (for AD inheritance model) in different exome sequencing project databases (e.g. project ESP 6500, ExAC 60,706), and POL 400 (in-house-project of 400 exomes of Polish individuals with unrelated diseases). Molecular variants were assessed by pathogenicity prediction tools (CADD, MetaSMV, PolyPhen2 HDIV and HVAR, Mutation Assessor, LRT, MetaLR, SIFT, FATHMM and MutationTaster softwares). The websites were simultaneously consulted using dedicated Alamut Interactive Biosoftware. The nomenclature of molecular variants follows the Human Genome Variation Society guidelines (HGVS, www.hgvs.org/mutnomen) and referral to the cDNA sequences follows the Human Gene Mutation Database (HGMD, www.hgmd.cf.ac.uk).

Molecular analysis was performed after obtaining informed consent from the patients' parents or legal guardians.

2.4. Biochemical analysis

Lactate response to glucose loading was assessed during a routine intravenous glucose tolerance test (IVGTT) in cases with suspicion of PDHc defect. The plasma lactate level was measured at 0′, 15′, 30′, 60′, 90′, and 120′ and the pyruvate level, at 0′, 60′, and 120′. Control values were obtained from 25 non-mitochondrial patients in whom IVGTT was performed during differential diagnosis. The reference group included 8 patients with various molecularly confirmed mitochondrial disorders not related to PDHc defect (*ACAD9*, *SCO2*, *SURF1* and *DGUOK* pathogenic changes).

3. Results

3.1. Western blot findings

In the first step of the study, the amount of E1α was assessed in 86 muscle specimens. Its content ranged from 0 to 272% of the mean reference. In 33 of 83 muscle bioptates, low amounts of E1α (below 50% of the mean reference value) were found, and in another 6 patients, amounts below 60% were determined.

3.2. Molecular findings

In the second step of the study, Sanger sequencing of *PDHA1* was performed in a total of 21 unrelated patients with low muscle E1α. Pathogenic variants in *PDHA1* were revealed in four of these probands (19%). In one of them (P4 in Table 1), with a number of data pointing to a PDHc defect, the E1α amount was 52.5% (above 50% of the reference value).

In the third step of the study, WES was performed in 7 patients with negative results of *PDHA1* sequencing. Pathogenic variants were found only in not PDHc-related genes (*FBXL4*, *MTND1*, *MTND5*, *PGAP2*) in four of seven patients with muscle E1α amounts below 50% of the mean reference value. One PDHc-related defect detected by WES was a homozygous *DLD* variant found in the patient (P11 in Table 1) with LS brain changes seen on MRI (another inclusion criterion).

Molecular characteristics conducted together for all PDHc defects diagnosed in Poland encompassed five patients identified under the study protocol and four others diagnosed outside the protocol. In the latter patients, muscle biopsy was not performed nor was the amount of E1α assessed. They were identified by WES carried out due to general MD suspicion (3 cases). One patient was properly diagnosed through a positive family history and pathogenic variant was identified in *PDHA1* by Sanger sequencing.

Molecular analysis revealed pathogenic variants in two PDHc-related genes in nine probands and two relatives. Two novel changes [c.464T > C (p.Met155Thr), c.856_859dupACTT (p.Arg288Leufs*10)] and five known variants [c.787C > G (p.Arg263Gly), c.904C > T (p.Arg302Cys), c.934_940delAGTAAGA (p.Ser312Valfs*12), c.262C > T (p.Arg88Cys), c.933_935delAAG (p.Arg311del)] in *PDHA1*, as well as one homozygous known mutation, c.1123G > A (p.Glu375Lys), in *DLD* were identified (Fig. 1, Table 1). Long-range PCR of the entire *PDHA1* gene excluded major intragenic rearrangements, e.g., large deletions or insertions, in patients without a pathogenic variant.

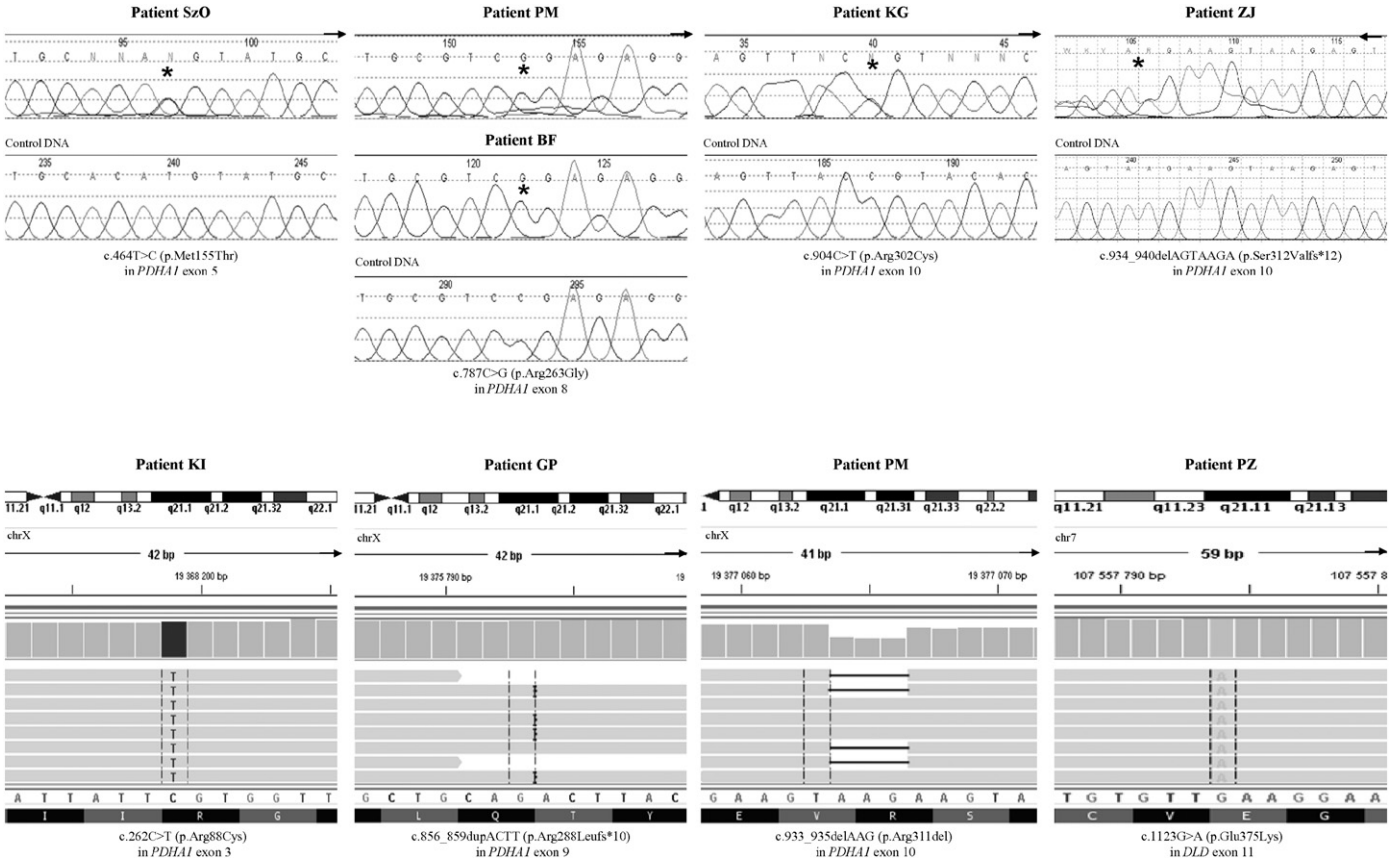


Fig. 1. The results of genetic analysis showing novel variants in *PDHA1* and *DLD* identified by Sanger (A) and whole-exome (B) sequencing.

Available parents and family members were also investigated. Carrier status of the *PDHA1* variant was confirmed in two mothers (one of them symptomatic) and in one affected brother, while two remaining changes occurred *de novo* (Table 1). Both parents and one sister of the patient with the homozygous *DLD* variant were found to be asymptomatic carriers.

3.3. Clinical findings of PDHA1 patients

Detailed clinical, biochemical and molecular characteristics of ten patients (P1–P10) with *PDHA1* defects are summarized in Table 1. In all patients the course of disease corresponds to a major extent to the literature data [3]. The neurological phenotype was: neonatal encephalopathy with lactic acidosis in four patients, basal ganglia abnormalities in two, chronic progressive neurologic deterioration in three, and intermittent ataxia in two patients (Table 1). The onset of symptoms ranged from the neonatal period to 2 years. All patients survived; the oldest symptomatic carrier is the affected mother (P5 in Table 1), now aged 34 years.

Vitamin B_1 (thiamine) responsiveness is considered in five patients (from four families) only on the basis of subjective parents' observation. It is not, however, evidence based. We tried a loading test to objectively verify the responsiveness by use of lactate response for i.v. glucose loading and successfully demonstrated it in one affected individual.

The test was assessed 8 times in four *PDHA1*-mutated patients and in the reference group of eight non PDHc-related mitochondrial disease cases (Fig. 2). The plasma lactate response to the glucose bolus was antithetical in the compared groups. The mean lactate concentration increased by 23% in the *PDHA1* group and decreased by 27% in the non PDHc-related mitochondrial disease group (Fig. 2, panel A). The difference was statistically significant ($P = 0.0354$).

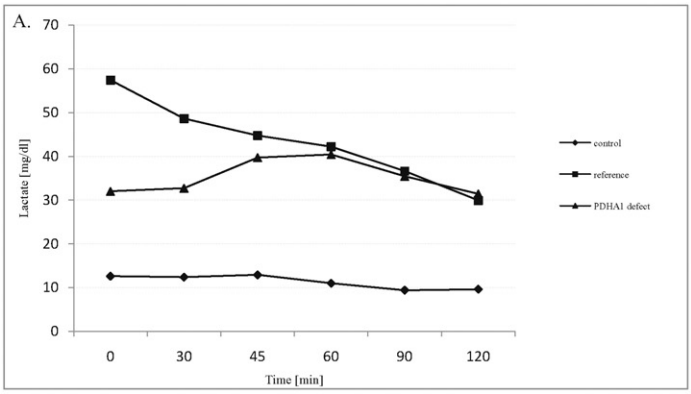
In the above-mentioned *PDHA1*-affected patient (bearing the p.Arg263Gly variant), the test performed before and during vitamin B_1 supplementation at the maximum dose of 300 mg/day showed complete normalization of the lactate response to glucose loading (Fig. 2, panel D).

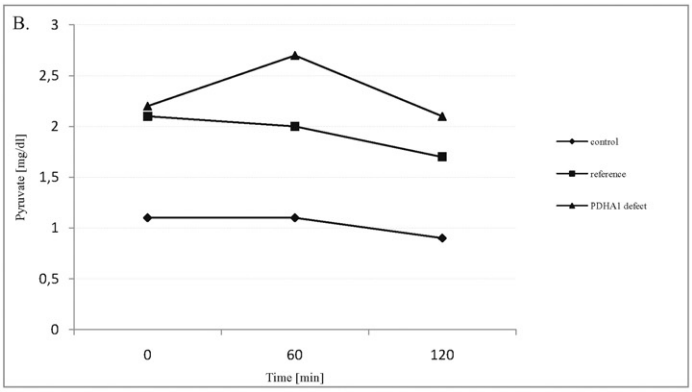
3.4. Clinical course of the DLD patient

In our lipoamide dehydrogenase-deficient patient with the *DLD* mutation, the expression of PDHc E2/E3BP and E1α subunits was reduced (45.2% and 59.7%, respectively) (Table 1). The patient with the *DLD* mutation responsible for dihydrolipoamide dehydrogenase deficiency (subunit E3 of PDHc) was the second child with Leigh syndrome in this study. This girl was born by cesarean section with a birth weight of 3250 g and Apgar score of 9 points. Her psychomotor development in infancy was moderately delayed. At the age of 12 months ptosis and involuntary movements developed. Markedly elevated lactate concentrations in plasma and cerebrospinal fluid were found (104 and 58.8 mg/dl) and basal ganglia changes were revealed in brain MRI scans. Muscle biopsy showed a generalized OXPHOS defect. At the age of 6 years her condition was stable. Liver function was never impaired. The girl is not able to walk, but her physical growth is otherwise normal, mental development is mildly impaired, and plasma lactate remains high borderline.

4. Discussion

Molecular analysis revealed PDHc-causing molecular variants in eleven patients from nine unrelated families. Seven different variants were identified in *PDHA1* and one homozygous mutation affected *DLD*. Six of these changes were reported previously and two novel heterozygous variants in *PDHA1* were found in female patients.





C.

Study group	Plasma lactate [mg/dl]						Plasma pyruvate [mg/dl]		
	0	30 min	45 min	60 min	90 min	120 min	0	60 min	120 min
Control	12.6 ± 7.8	12.4 ± 6.7	12.9 ± 5.6	11.0 ± 4.4	9.4 ± 4.4	9.6±4.0	1.1 ± 0.6	1.1 ± 0.5	0.9 ± 0.4
Reference	57.4 ± 30.8	48.6 ± 33.1	44.7 ± 28.7	42.2 ± 20.1	36.6 ± 19.5	29.9 ± 15.4	2.1 ± 0.6	2.0 ± 0.9	1.7 ± 0.8
PDHA1 defect	32.0 ± 17.8	32.7 ± 17.8	39.7 ± 18.9	40.4 ± 23.3	35.4 ± 23.6	31.4 ± 21.4	2.2 ± 0.9	2.7 ± 1.1	2.1 ± 1.1

Control, group of 25 patients after exclusion of mitochondrial and carbohydrate disorders; Reference, group of 8 patients with mitochondrial disorder not related to PDHc defect: *SURF1 (2x)*, *SCO2 (4x)*, *ACAD9* and *DGUOK* mutated cases; *PDHA1* defect, group of 5 patients (8 tests) with *PDHA1* defect.

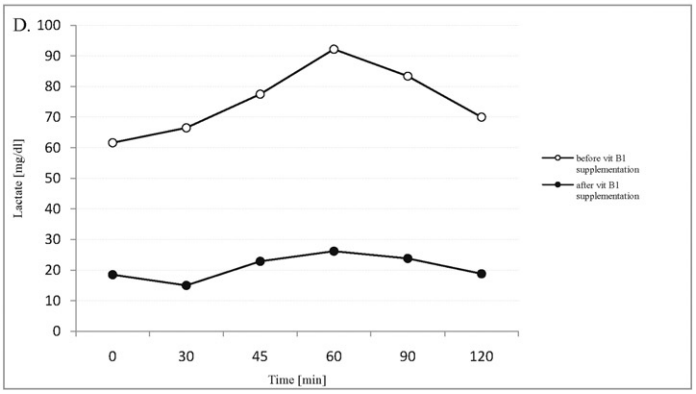


Fig. 2. The results of response to intravenous glucose loading in PDHc-affected patients and reference groups. Mean value of lactate concentration (A), pyruvate concentration (B), mean value of lactate and pyruvate concentrations with standard deviation (C), effect of vitamin B_1 supplementation (300 mg/day) in patient PM (D).

The first novel variant is a missense substitution c.464T > C (p.Met155Thr) in exon 5. Although there is only a moderate physicochemical difference between hydrophobic methionine and polar, uncharged threonine, this change is predicted to be disease causing by Mutation Taster and SIFT software. Methionine 155 is a very highly conserved residue across species (Table 2), and the change to threonine at this residue is predicted to be deleterious also by the Grantham score, which categorizes codon replacements into classes of increasing chemical dissimilarity (Grantham score = 81, moderately radical change). This variant was not found in the ClinVar, dbSNP, LOVD, and PubMed databases, or in the collection of POL 400, indicating its rarity.

The second novel pathogenic variant, c.856_859dupACTT (p.Arg288Leufs*10), is a *de novo* duplication of 4 bps in exon 9 that creates a frameshift starting at codon Arg288 and results in premature termination nine positions downstream. The mRNA product is probably targeted for nonsense-mediated decay. A different duplication, but resulting in the same translation effect, has been reported previously [6].

Worldwide, at least 337 patients with *PDHA1* mutations [3,7] and >160 molecular variants have been reported [8]. Most of them are missense changes, similar to the findings in this study. Among them, the c.787C > G (p.Arg263Gly) variant is considered the most frequent mutation in *PDHA1* since it is repeated in 19 published cases [2,8]. The recurrent p.Arg263Gly variant (as well as the second, p.Arg88Cys) was reported to occur in vitamin B1 responders [9,10].

The p.Arg263Gly variant was also found in our molecularly confirmed *PDHA1* group: in two unrelated boys and in the mother of one of them. The woman has presented symptoms of mild neuropathy since the age of 3 years. Leigh syndrome described in association with this substitution [2] was not observed in any of our p.Arg263Gly mutated cases. The families observed vitamin B_1 efficiency in doses of 50–150 mg per day, but this should be treated with caution. The appropriate way of determining vitamin B_1 responsiveness has not yet been described in the literature. In one of the patients with the p.Arg263Gly variant we managed to demonstrate that supplementation with vitamin B_1 at a dose of 300 mg per day not only improved his clinical condition, but resulted in a decreased fasting lactate concentration, as well as in a

Table 2
Protein alignment of ten species shows the methionine 155 residue is evolutionary conserved.

Species	Match	Gene	AA	Alignment																					
Patient SzO	Not conserved		155	G	C	A	K	G	K	G	G	S	M	H	T	Y	A	K	N	F	Y	G	G	N	G	I
Human		ENST00000422285	155	G	C	A	K	G	K	G	G	S	M	H	M	Y	A	K	N	F	Y	G	G	N	G	I
P.troglodytes	All identical	ENSPTRG00000021717	155	G	C	A	K	G	K	G	G	S	M	H	M	Y	A	K	N	F	Y	G	G	N	G	I
F.catus	All identical	ENSFCAG00000012903	155	G	C	A	K	G	K	G	G	S	M	H	M	Y	A									
M.musculus	All identical	ENSMUSG00000031299	155	G	C	A	K	G	K	G	G	S	M	H	M	Y	A	K	N	F	Y	G	G	N	G	I
G.gallus	All identical	ENSGALG00000016430	162	G	C	A	K	G	K	G	G	S	M	H	M	Y	T	K	N	F	Y	G	G	N	G	I
T.rubripes	All identical	ENSTRUG00000016917	163	G	I	A	K	G	K	G	G	S	M	H	M	Y	C	K	H	F	Y	G	G	N	G	I
D.rerio	All identical	ENSDARG00000012387	158	G	I	A	K	G	K	G	G	S	M	H	M	Y	T	K	H	F	Y	G	G	N	G	I
D. melanogaster	All identical	FBgn0028325	203	G	C	A	R	G	K	G	G	S	M	H	M	Y	A	P	N	F	Y	G	G	N	G	I
C.elegans	All identical	T05H10.6	166								G	S	M	H	M	Y	T	K	N	F	Y	G	G	N	G	I
X.tropicalis	All identical	ENSXETG00000006212	184									S	M	H	M	Y	A	K	N	F	Y	G	G	N	G	I

AA: Amino acid number corresponds to the mutated position p.Met155Thr of *PDHA1* gene. Data obtained from http://www.mutationtaster.org

decrease in lactate/pyruvate response to glucose load (Fig. 2, panel D). We speculate that measuring lactate during an IVGTT test may be a useful way to assess vitamin B_1 responsiveness in PDHc defect. We have used the glucose loading test in the differential diagnostics of PDHc defect in practice. The intravenous, not oral, glucose load was arbitrarily chosen to simplify the interpretation. We began to use the test in the 1990s when access to molecular methodology was limited in Poland. We took advantage of the pioneering studies of Fernandes on glucose homeostasis in GSD type I and GSD type III/VI/IX and applied them to MD diagnostics. We assumed that the PDHc defect would behave like GSD type III/VI/IX, whereas the other mitochondrial defects would be more like GSD type I [11]. The results proved in our hands to be extremely useful and we continue to apply the test even today at the bedside. We believe that the use of this test to assess responsiveness of the *PDHA1* defect to vitamin B_1 may find wider application. This assumption requires future confirmation, however.

Two small deletions and one small duplication were identified in our group of *PDHA1* pathogenic variants. These defects of the *PDHA1* gene are rare (20% of cases) and mainly associated with a frameshift effect that leads to premature termination of translation [1,8]. The literature also contains descriptions of gross rearrangements (22 cases) that may be masked by the presence of the wild-type allele. Long-range polymerase chain amplification of the entire *PDHA1* gene [12] revealed no major intragenic rearrangements in our female patients.

Lipoamide dehydrogenase deficiency (E3 subunit defect) found in one of our patients is a very rare cause of PDHc deficiency that has been reported to date in only 29 cases [3]. The phenotype of the disorder is poorly characterized, and ranges from neonatal distress to paroxysmal myoglobinuria and recurrent liver failure [13]. The patient presented in this study (homozygous for c.1123G > A (p.Glu375Lys) in *DLD*) shows a clear Leigh syndrome phenotype, as once reported earlier [14].

It should be noted that all of the patients with *PDHA1* deficiency reported in this study survived and more than half of them seem to be vitamin B_1 responders, whereas in previously described cohorts nearly half of the patients died in infancy or early childhood [15] and the percentage of vitamin B_1 responsiveness did not exceed 10% [3]. This may, in part, be due to the children with a severe, fatal course dying without a diagnosis of PDHc deficiency because specific diagnostic methods were unavailable in Poland for a long time. Another explanation can be better selection of patients suspected of PDHc deficit in our material due to routine inclusion of lactate response to i.v. glucose loading in differential diagnostics.

Our results indicate limited usefulness of E1α expression assessed in muscle homogenate for the recognition of PDHc deficiency. Unexpectedly, among 21 cases with reduced E1α expression we confirmed only four patients with a *PDHA1* pathogenic variant. On the other hand, exome sequencing revealed a significant number of genetic defects (including mitochondrial) in this group. Admittedly, secondary PDHc deficiency was described in single cases of such disorders as MELAS or MERRF [16,17], but definitive confirmation of such a relationship or its absence requires more data and observations.

The paper describes a study on archival muscle bioptates stored when *PDHA1* sequencing was not available in our lab. At present we recommend starting from *PDHA1* sequencing in each patient with clinical and biochemical features of PDHc defect (including normal lactate/pyruvate ratio, abnormal response of lactate to i.v. glucose load, vit B1 responsiveness, typical MRI changes, normal OXPHOS, low E1α in muscle, X-linked inheritance). In our opinion, the next step is WES which enables detection of variants in a vast range of known genes as well as in candidates.

5. Conclusion

The results of the study suggest that a specific lactate/pyruvate response to glucose load may provide a marker of responsiveness of *PDHA1* deficiency to vitamin B_1 administration that is dependent on the type of *PDHA1* pathogenic variant.

Assessment of E1α expression using Western blotting may contribute to the differential diagnosis of PDHc only to a small extent. It should be stressed that this method reveals not only primary PDHc defects caused by mutations in genes coding for individual subunits of the complex, but also may reflect its secondary dysfunction that is not yet sufficiently recognized.

Both methods can be useful in diagnostics, but whole-exome sequencing definitely prevails in mitochondrial diagnostics and is becoming the method of choice also for the diagnostics of PDHc defects.

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