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MPC2 variants disrupt mitochondrial pyruvate metabolism and cause an early-onset mitochondriopathy

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Pyruvate is an essential metabolite produced by glycolysis in the cytosol and must be transported across the inner mitochondrial membrane into the mitochondrial matrix, where it is oxidized to fuel mitochondrial respiration. Pyruvate import is performed by the mitochondrial pyruvate carrier (MPC), a hetero-oligomeric complex composed by interdependent subunits MPC1 and MPC2. Pathogenic variants in the MPC1 gene disrupt mitochondrial pyruvate uptake and oxidation and cause autosomal-recessive early-onset neurological dysfunction in humans. The present work describes the first pathogenic variants in MPC2 associated with human disease in four patients from two unrelated families. In the first family, patients presented with antenatal developmental abnormalities and harboured a homozygous c.148T>C (p.Trp50Arg) variant. In the second family, patients that presented with infantile encephalop-athy carried a missense c.2T>G (p.Met1?) variant disrupting the initiation codon. Patient-derived skin fibroblasts exhibit decreased pyruvate-driven oxygen consumption rates with normal activities of the pyruvate dehydrogenase complex and mitochondrial respiratory chain and no defects in mitochondrial content or morphology. Re-expression of wild-type MPC2 restored pyruvate-dependent respiration rates in patient-derived fibroblasts.

The discovery of pathogenic variants in MPC2 therefore broadens the clinical and genetic landscape associated with inborn errors in pyruvate metabolism.

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Introduction

Pyruvate is a key molecule required for aerobic metabolism. Pyruvate is produced by the cytosolic glycolytic pathway and must be shuttled across the inner mitochondrial membrane (IMM) where it will enter the tricarboxylic acid cycle (TCA cycle or Krebs cycle) to fuel the production of NADH and FADH₂, which serve as electron donors for the respiratory chain.¹ Forty years ago, before the molecular identification of the mitochondrial pyruvate carrier (MPC) complex, the first functional studies suggested a specific and active import of pyruvate into mitochondria.^{2,3} Twenty-five years later, the first patients with impaired mitochondrial pyruvate import were reported.⁴ These patients presented hypotonia, dysmorphia, periventricular cysts, marked metabolic acidosis and hyperlactacidaemia. The lactate/ pyruvate (L/P) molar ratios, mitochondrial respiratory chain and pyruvate dehydrogenase (PDH) activities were normal.⁴ It took a decade for the molecular identification of the MPC in eukaryote cells.5 The MPC in mammals is composed of two essential and interdependent subunits, MPC1 and MPC2, which must oligomerize in order to form a functional carrier for pyruvate transport across the IMM. Depletion of either subunit provokes the impairment of pyruvate oxidation and pyruvate-driven oxygen consumption.^{5,6} The central role of pyruvate leads to severe and multisystemic metabolic consequences when its transport is impaired.⁷ In rodents, MPC dysfunction contributes to cell proliferation in colorectal or prostate cancer^{8,9} as well as pathological cardiac hypertrophy 10,11 and diabetic nephropathy,¹² and it has also been shown that MPC is required for myocardial stress adaptation.¹³ In humans, loss-of-function MPC1 variants prevented MPC assembly and mitochondrial pyruvate uptake.^{5,14} To date, there are only 10 patients reported in the literature,¹⁴ with clinical presentations including hypotonia, encephalopathy and frequent early death.^{14,15} Typically, high lactate and pyruvate concentrations with a normal L/P ratio are found in fluids of patients, usually suggesting PDH deficiency. However, MPC impairment should be considered for similar cases. Here, we report the first pathogenic variants in the MPC2 in four patients from two unrelated consanguineous families with pyruvate transport deficiency.

Materials and methods

The full methodology is provided in the Supplementary material.

Data availability

There are no large datasets associated with the study.

Results

Clinical and genetic studies: overview of affected family members

Family 1 is a first-degree consanguineous family (Fig. 1A and Table 1) with two prematurely terminated pregnancies characterized by developmental abnormalities. The first foetus (Patient F1-II.1) was diagnosed prenatally at the second trimester by ultrasound examination with a diffuse subcutaneous oedema,

cardiomegaly, corpus callosum agenesis, ventriculomegaly and a hypoplasia of the cerebellum with hydramnios hyperechogenic punctiform elements in the cerebral parenchyma. The pregnancy was terminated at the 27th week. The second pregnancy resulted in the birth of a healthy daughter (Patient F1-II.2). For the third pregnancy, the foetus (Patient F1-II.3) was examined in the first trimester by ultrasound and presented identical abnormalities to those of the first foetus associated with choroid plexus cysts and domed philtrum, suggesting Fowler's disease (OMIM: 225790; Fig. 1B and C). The pregnancy was subsequently terminated. The karyotype and comparative genomic hybridization array generated from amniotic samples taken from both foetuses (Patients F1-II.1 and F1-II.3) were normal and Fowler's syndrome was ruled out by sequencing of the FLVCR2 gene.¹⁶ During the third pregnancy, an amniocentesis was performed; PDH activity in the amniotic cell and in the primary cultured fibroblast was in the normal range (Table 1 and Supplementary Table 1).

To pinpoint the genetic origin of the deficit, DNA from the second affected foetus (Patient F1-II.3) was subjected to next-generation sequencing (NGS) using a bespoke panel of 200 nuclear DNA-encoded genes and full mtDNA sequencing, supplemented by a subsequent whole-exome sequencing analysis (Supplementary Table 2). A homozygous variant [c.148T>C, p.(Trp50Arg)] in the MPC2 gene (NM_001143674.4) encoding one of the two subunits of the MPC was identified in this foetus. This variant was absent in ExAC, 1000G or gnomAD databases and modified highly conserved nucleotide and amino acid (Phastcons = 1; conserved amino acid from baker's yeast to human; Fig. 1E). It was predicted to be deleterious according to established algorithms (PolyPhen-2 v.2.2 score: 1.000, MutationTaster score: deleterious). This variant was also confirmed in the first affected foetus by Sanger sequencing in a homozygous state, whereas both parents and their healthy child were heterozygous carriers (Fig. 1A). Thus, the variant could be considered to be a likely pathogenic variant following the American College of Medical Genetics (ACMG) classification (PM1, PM2, PP1, PP3). Our functional studies then allow upgrade of the variant pathogenicity to class 5.

Family 2 is also a first-degree consanguineous family with no family history of a known or suspected genetic condition. The first child (Patient F2-II.1) is a 6-year-old girl. She was initially diagnosed at 8 months of age with an anoxo-ischaemic encephalopathy. This diagnosis was revised at the age of 21 months, when she presented with an isolated dyspnoea associated with elevated levels of lactate in the fasting [4.9 mM, reference values (RV): 0.6-2.2 mM] and postprandial (7.7 mM) states. Brain MRI with spectroscopy and the auditory-evoked potential were normal but the visual-evoked potential was abnormal, pointing to optical pathway impairment. She fed normally without any chewing or swallowing problems but had a severe delayed psychomotor development with no acquisition of sitting position and language. At the age of 5 years, redox cycle in blood showed elevated levels of lactate and pyruvate in the fasting (lactate 5.14 mM and pyruvate 0.239 mM, RV < 0.15 mM) and in postprandial (6.24 and 0.324 mM, respectively) states with normal ratio (15-18 mM, RV <20 mM). Chromatography of amino acids was normal and chromatography of organic acids showed high lactate level



Figure 1 Pedigree and imaging details of MPC2-affected familial individuals carrying deleterious variants. (A) Pedigree of Family 1 (F1) carrying the c.148T>C, p.(Trp50Arg) variant. (B) Cerebral ultrasonographic examination of 24 weeks foetus (Patient F1-II.3) showing important radial vascularization from peri callosal artery (PCA), also seen in Fowler's disease. (C) Normal brain vascularization in a control foetus at the same term, with basilary artery (BA); cerebral anterior artery (CAA); peri callosal artery (PCA); radial vascularization (RV); cerebral intern vein (CIV); vein of Galen (GV). (D) Pedigree of Family 2 (F2) carrying the c.2T>G, p.(Met1?) variant. (E) Locations and conservation analysis of the detected variants in MPC2 (in red), conservation of the mutated amino acids is indicated by the alignment of eight species. (F-I) MRI features associated with MPC2 variants in Patient F2-II.2 showing hypoplasia and partial agenesis of the corpus callosum with axial T₂-weighted images (F and H), axial FLAIR (fluid-attenuated inversion recovery) weighted image (G) and sagittal weighted image (I) showing bilateral T₂ and FLAIR hyperintensities of the thalamus as well as cortical, subcortical and corpus callosum attrophy (arrow).

in urine. A PDH deficiency was suspected but excluded after analysis of PDH enzymatic activity and sequencing of the PDHA1 gene, which failed to reveal any defects. The second affected child (Patient F2-II.2) is the younger brother of Patient F2-II.1. His pregnancy was uneventful and his birth was complicated by neonatal respiratory distress, which required 8 days of clinical attention in a neonatal intensive care department. The child presented with phototherapy-responsive neonatal jaundice. At the age of 3 months the parents consulted for hypotonia, excessive head lag and psychomotor retardation. Two months later, the boy acquired the smile response with language at the babbling stage but no motor acquisition was observed. A transfontanellar ultrasound showed dilatation of the third ventricle with aberrant visualization of the corpus callosum. At 7 months, clinical examination showed poor eye tracking, visual impairment, axial hypotonia with slight segmental hypotonia, weak osteotendinous reflexes in all four limbs, horizontal spontaneous nystagmus, mild coarse facial features with hirsutism and microcephaly (–2 to 3 SD). Brain MRI performed at 2 years showed cortical and subcortical atrophy, hypoplasia of the corpus callosum, T₂ and FLAIR hyperintensity of thalami (Fig. 1F–I). Abdominal and cardiac ultrasounds were normal. Electroneuromyography, visual- and auditory-evoked responses were also normal. Elevated lactate levels in the plasma (8.7 mM) and CSF (10.4 mM, RV: 0.6–2.2 mM) were detected along with high proteinorachia (0.84 g/l, RV < 0.40 g/l). Redox cycle showed

Table 1 Abridged clinical presentation and metabolic findings of patients with homozygous loss-of-function MPC2 variants

	Family 1		Family 2	
	Patient II.1	Patient II.3	Patient II.1	Patient II.2
Year	2012	2017	2016	2019
Sex of the affected individuals	ND	ND	Female	Male
Age at onset	Foetus	Foetus	8 Months	3 Months
Genetic change	$c.148T > C^{a}$		$c.2T > G^{a}$	
Allelic genotype	Homozygous		Homozygous	
Predicted protein change	p.(Trp50Arg)		p.(Met1?)	
Prenatal events	Subcutaneous oedema, agenesis, cerebellum ł	cardiomegaly, corpus callosum nypoplasia, ventriculomegaly	Uneventful	Foetal distress
Phenotype	Post-mortem	-	Encephalopathy,	Respiratory distress syndrome,
	examination: facial		dyspnoea, visual	hypotonia, nystagmus, facial
	dysmorphism,		impairment	dysmorphia, corpus callosum
	microcephaly cardiac failure		-	hypoplasia
Developmental	MTP 27 wg	MTP 26 wg	Absence of language,	Severe psychomotor retardation
delay	C	C C	inability to reach a seated posture	
Blood metabolic	_	-	Hyperlactacidaemia	Hyperlactacidaemia (5.14–
findings			(7.32–11.6 mM; NR: 0.79–	6.24 mM; NR: 0.79–1.74 mM)
-			1.74 mM)	Hyperpyruvicaemia (0.24–
			Hyperpyruvicaemia (0.48–	0.32 mM NR: 0.07–0.13 mM)
			0.62 mM NR: 0.07-	Normal L/P ratio
			0.13 mM)	
			Normal L/P ratio	
Tissue metabolic	-	Normal PDH activity	Normal PDH activity	-
findings		(amniocyte), decreased		
		pyruvate-stimulated oxygen		
		consumption (fibroblast)		

MTP = medical termination of pregnancy; ND = not determined; NR = normal range; wg = weeks of gestation.

^aReference transcript of MPC2 is GenBank: NM_001143674.4.

elevated levels of lactate and pyruvate in the fasting (lactate 7.32 mM and pyruvate 0.47 mM) and in postprandial (11.6 and 0.62 mM, respectively) states with normal ratio (15–19; Table 1 and Supplementary Table 1). He is now aged 4 years. He is hypotrophic [weight 10 kg (–3 SD) and height 85 cm (–2 SD)] and has microcephaly [head circumference 45 cm (–3.5 SD)]. His contact and ocular pursuit are poor with irritability and he also has severe generalized hypotonia with no acquisitions and week osteotendinous reflexes.

NGS analysis revealed a previously undescribed homozygous c.2T>G, p.(Met1?) variant in the MPC2 gene in both siblings (Supplementary Table 2). Each parent was found to be a heterozy-gous carrier of the variant. This variant, absent from public sequencing databases, is predicted to disrupt the initiation codon and was classified as pathogenic according to the ACMG classification criteria (PVS1, PM2 and PP3; Fig. 1D and E).

Molecular studies

To evaluate the impact of the genetic variants identified in MPC2, we analysed the expression of MPC subunits in patient-derived fibroblasts (from Patients F1-II.3 and F2-II.1). Immunoblot analysis revealed severe decreased expression of MPC2 (>90% in both MPC2^{Met1?} and MPC2^{Trp50Arg}) relative to the corresponding control cells obtained from unrelated healthy individuals (Fig. 2A and B). This was accompanied by a marked reduction in the steady-state levels of MPC1 in fibroblasts of both MPC2^{Trp50Arg} and MPC2^{Met1?} patients. These results are in accordance with the literature showing that MPC1 and MPC2 levels are interdependent: the ablation of one

subunit prevents the MPC complex assembly, resulting in the destabilization and subsequent degradation of the other subunit.^{5,6} Indeed, as shown with MPC1^{Arg97Trp}, which is less stable than MPC1^{Lys79His}, the loss of MPC1 leads to MPC2 degradation (Fig. 2A). We did not observe any differences in the steady-state levels of other IMM proteins such as succinate dehydrogenase (SDHB) or ATP5A (Fig. 2A), pointing to a specific defect in the MPC rather than a general decrease in mitochondrial content or IMM proteins. These data were further corroborated by confocal microscopy analysis of mitochondrial content of patient-derived fibroblasts (Supplementary Fig. 1E) labelled with TMRE (tetramethyl rhodamine, ethyl ester), which also did not reveal any obvious alterations in mitochondrial morphology (Supplementary Fig. 1F).

In silico predictions of the MPC2 variants that we identified point to a deleterious loss-of-function effect on MPC2. To prove whether defects in patient-derived fibroblasts are specifically caused by MPC2 dysfunction, we sought to functionally complement them by expressing wild-type MPC2 fused to a haemagglutinin (HA) tag. Stable expression of MPC2-HA at levels comparable to MPC2 levels of control cells was able to rescue MPC1 protein deficiency in MPC2^{Met1?} patient cells (Fig. 2A and B), indicative of a functional rescue of the MPC complex assembly defect.

To determine whether the decreased MPC2 protein levels in patient-derived fibroblasts were the result of reduced stability or synthesis of MPC2 mRNA, we quantified the relative abundance of human MPC1 and MPC2 transcripts by qRT-PCR (Fig. 2C). We detected normal levels of MPC2 transcripts in MPC2^{Met1?} cells in which



Figure 2 Analysis of MPC1 and MPC2 expression levels. (A) Representative immunoblot of equal amounts of lysates from control (Control 1 and 2), MPC2-deficient fibroblasts before (MPC2^{Trp50Arg} or MPC2^{Met1?}) and after functional complementation with human MPC2-HA (MPC2^{Met1}?+MPC2), and MPC1-deficient (MPC1^{Arg97Trp} or MPC1^{Lys79His}) patient-derived fibroblasts, separated by SDS-PAGE and immunoblotted with the indicated antibodies. An number sign denotes MPC2 native form, triangle denotes truncated MPC2; asterisk denotes MPC2-HA form. (B) Densitometric quantification of MPC2 and MPC1 protein levels in A relative to VINCULIN in patient-derived fibroblasts (n=2-4). (C) Relative MPC2 and MPC1 mRNA levels in patient-derived fibroblasts as compared with controls (n=4). (D and E) Pyruvate-dependent respiration of control (Control 1 and 2), MPC2-deficient fibroblasts (MPC2^{Trp50Arg} or MPC2^{Met1}?) before and after functional complementation with human MPC2-HA (MPC2^{Trp50Arg+NPC2} or MPC2^{Met1}?+MPC2</sup>), and MPC1-deficient (MPC1^{Arg97Trp} or MPC2^{Lys79His}) patient-derived fibroblasts measured by seahorse flux analyser. Basal oxygen consumption was measured in the incubation media without glucose and with pyruvate and maximal respiration was measured after injection of CCCP (carbonyl cyanide m-chloro ophenyl hydrazone). All data are presented as mean ± SEM and analysed by two-tailed unpaired Student's t-test with (**P ≤ 0.01, *P ≤ 0.05, ns = not significant).

MPC2 protein levels were reduced, suggesting that the MPC2^{Met1?} mutation destabilizes MPC2 protein, leading to its proteolytic removal. Interestingly, we observed elevated levels of MPC1 transcript levels in MPC2^{Met1?} patient-derived fibroblasts, suggesting a compensatory response to MPC complex dysfunction. For MPC2^{Trp50Arg} patient-derived fibroblasts, we observed markedly reduced levels of both MPC1 and MPC2 transcripts, correlating with reduced protein levels observed by immunoblot analyses. Unfortunately, due to poor growth of MPC2^{Trp50Arg} patient-derived fibroblasts, we were unable to perform in-depth characterization of MPC2^{Trp50Arg} patient-derived fibroblasts. Together, our data indicate that the reduced MPC2 levels observed in MPC2 patient-derived cells is the result of destabilization of mutant MPC2 protein rather than reduced MPC2 transcript levels.

Next, we assessed the pyruvate-dependent respiration of patient-derived fibroblast by measuring basal oxygen consumption in the presence of either pyruvate or glutamate. Glutamate is capable of compensating for mitochondrial pyruvate deficiency by entering the TCA cycle and supports normal respiration rates in MPC1 patient-derived fibroblasts.¹⁵ Yet in the presence of pyruvate (and absence of glutamate), mitochondrial respiration relies exclusively on the redox equivalents generated by intramitochondrial pyruvate pools that require MPC.

Under basal conditions when respiratory substrates are not rate-limiting (Fig. 2D), we did not observe defects in oxygen consumption rates in MPC2 patient-derived fibroblasts. However, upon uncoupler-mediated maximal respiration conditions in which substrates including those of the TCA become rate-limiting, we observed a decrease in pyruvate-dependent oxygen consumption rates in MPC2-deficient fibroblasts (Fig. 2D), as evidenced by the reduced ratio of maximal/basal oxygen consumption rates (Fig. 2E), which could be rescued by restoring MPC2 levels (Fig. 2E). Importantly, we observed no functional defects in glutamatedependent oxygen consumption rates under basal or uncouplermediated maximal respiration conditions (Supplementary Fig. 1G), highlighting a specific defect in pyruvate utilization caused by MPC2 variants identified in these patients. Indeed, cells from individuals with pathogenic variants in MPC1 exhibited impaired pyruvate-dependent respiration defects that could be partially rescued by re-expression of wild-type human MPC1.⁵ Thus, pyruvatedependent respiration measurements coupled with western blot analysis of MPC subunits represent rapid and effective tools to pinpoint functional defects in the MPC in patient-derived fibroblast.

Discussion

In mitochondria, pyruvate is critical to drive ATP production. Once imported into mitochondria by the MPC, its metabolism is tuned by mainly two enzymes: pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-coA, and pyruvate carboxylase (PC), which catalyses the conversion of pyruvate to oxaloacetate in fasting conditions. Numerous cases of pathogenic variants disrupting the activity of PDH or PC, and recently MPC1, have been described, 14,17,18 all leading to severe diseases characterized by various phenotypes including lactic acidosis, hypotonia, developmental delay, seizures and even premature death. Here, we describe the first pathogenic MPC2 variants associated with an equally devastating multisystemic dysfunction in children. Fibroblasts isolated from patients from both families in our study show normal rates of proliferation yet a decreased oxygen consumption rate in the presence of pyruvate but not in the presence of malate and glutamate. Supplementation of fibroblasts from MPC2 patients with the wild-type protein leads to

recovery of pyruvate transport and oxygen consumption (Fig. 2E), even if it remains lower than normal. Together, these data pinpoint the deficiency of the mitochondrial respiratory chain secondary to pyruvate transport impairment.

Our results show that MPC2 deficiency is comparable to that of MPC1 deficiency (Supplementary Table 3). Indeed, both MPC subunits are required to assemble the essential transporter of pyruvate across the IMM. Immunoblot analysis in fibroblasts derived from patients harbouring pathogenic variants in either MPC1 or MPC2 show reduced levels of both subunits of the MPC complex. As observed in Mpc2 mouse models,¹⁹ we show that hypomorphic MPC2 variants reduce the steady-state levels of MPC2 and thus promote the loss of MPC1, likely through turnover by mitochondrial proteases, which are charged with the task of turnover of mutant and unassembled proteins.²⁰ Inhibiting the turnover of mutant membrane proteins of the IMM has been shown to ameliorate respiratory chain assembly²¹ and confer benefits to preclinical models of neurodegenerative mitochondrial diseases.²² While this paradigm is untested for pyruvate transport deficiencies, future studies should identify the mitochondrial proteases responsible for the turnover of mutant MPC subunits and determine whether impairing this degradation may lead to amelioration in the assembly and activity of the MPC complex.

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Competing interests

The authors declare no competing interests.

Supplementary material

Supplementary material is available at Brain online.

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