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Case Report

Mitochondrial dysfunction in methylmalonic acidemia: A pilot study using Seahorse technology in peripheral blood

Sinziana Stanescu [a,*], Olatz Villate [b], Fernando Andrade [c], Domingo Gonzalez-Lamuño [d], Amaya Bélanger-Quintana [a], Francisco Arrieta [a], Maria-Luz Couce [e], Alfonso Muriel [f], Luis Aldamiz-Echevarria [g]

[a] Metabolic Diseases Unit, MetabERN, Pediatric Department, University Hospital Ramón y Cajal, Madrid, Spain
[b] Olatz Villate, Pediatric Oncology Group, Biobizkaia Health Research Institute, 48903 Barakaldo, Bizkaia, Spain
[c] Metabolomics Platform, Biobizkaia Health Research Institute, 48903 Barakaldo, Bizkaia, Spain
[d] Pediatric Division, Valdecilla Health Research Institute-University Hospital Marqués de Valdecilla and Universidad de Cantabria, Santander, Spain
[e] Diagnosis and Treatment of Congenital Metabolic Diseases, University Clinical Hospital of Santiago de Compostela, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela University, CIBERER, RICORS, MetabERN, Spain
[f] Biostatistic Unit, University Hospital Ramón y Cajal, IRYCIS, CIBERESP, University of Alcala, Madrid, Spain
[g] Luis Aldamiz-Echevarria, Health Research Institute of Santiago de Compostela (IDIS), Spain

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ABSTRACT

Introduction: Isolated methylmalonic acidemia (MMA) is an inborn error of metabolism due to the deficiency of the methylmalonic mutase enzyme. Many patients develop chronic complications such as basal ganglia lesions or kidney impairment. A growing body of evidence supports secondary mitochondrial dysfunction as the main cause for the development of these long-term complications, even in patients with good metabolic control. Currently, available methods to study mitochondrial function are often invasive, such as muscular or skin biopsy.
Objectives: This pilot study is aimed to develop a safe, non-invasive method to assess mitochondrial and glycolytic function in isolated MMA patients using lymphocytes.
Materials and methods: Mitochondrial bioenergetics and glycolysis were evaluated in lymphocytes from two mut^0 MMA patients and two age- and sex-matched controls using Seahorse technology. *In vitro* treatments with triheptanoin, citrate, and resveratrol were performed.
Results: MMA lymphocytes showed significant impairment in mitochondrial respiration and glycolysis compared to healthy controls. Triheptanoin exposure improved ATP production and glycolytic flux (ECAR), but no significant changes were observed in oxygen consumption (OCR). Citrate and resveratrol had no measurable impact on bioenergetic parameters.
Conclusions: This exploratory study suggests that Seahorse technology can detect mitochondrial dysfunction in MMA lymphocytes. Further studies in larger cohorts are required to validate these findings and explore their clinical relevance.

1. Introduction

Isolated methylmalonic acidemia (MMA) is an inborn error of propionate metabolism secondary to deficiency of the methylmalonyl- CoA mutase enzyme. The functional consequence of MMA is the inability to catabolize methylmalonyl-CoA to succinyl-CoA, resulting in the accumulation of propionyl-CoA and other metabolic intermediates, such as propionylcarnitine (C3), 3-hydroxypropionic acid, 2-methylcitric acid (MCA), and methylmalonic acid [1,2]. Nowadays, treatment is mainly dietetic and consists of reducing the intake of natural proteins, usually adding free propiogenic amino acids (valine, isoleucine, methionine and threonine) mixtures [1]. In cobalamin responsive forms, supplementation with vitamin B12 is an important part of the treatment; therefor, patients are designated mut^0 *versus* mut^- subtype based on the partial

* Corresponding author.
E-mail addresses: sinziana.stanescu@salud.madrid.org (S. Stanescu), OLATZ.VILLATEBEJARANO@osakidetza.eus (O. Villate), fernando.andradelodeiro@biobizkaia.eus (F. Andrade), amaya.belanger@salud.madrid.org (A. Bélanger-Quintana), Maria.Luz.Couce.Pico@sergas.es (M.-L. Couce), alfonso.muriel@uah.es (A. Muriel).

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response to B12 in the latter [2].

The evolution of these patients, far from being satisfactory, is fraught with multiple episodes of serious metabolic decompensation, hyperammonemia and kidney failure. Hepatic and kidney transplantation have become frequent procedures to avoid or compensate these complications. However, other chronic complications such as optic nerve atrophy and basal ganglia alterations among many others are not always related to acute metabolic crises and can appear even in patients with good metabolic and dietetic control or after liver transplantation [1,2]. These long-term complications mark the prognosis in MMA patients and treatment options are very limited.

One of the hypotheses that has recently gained more relevance in the pathophysiology of MMA refers to cellular energy failure due to secondary mitochondrial dysfunction. Patients with MMA share clinical and metabolic features with monogenic primary mitochondrial diseases [2–4]. Moreover, MMA patients might have white matter alterations and/or suffer from globus pallidus infarcts like the ´metabolic strokes´ of basal ganglia seen in primary mitochondrial patients [5,6]. Along with central nervous system involvement, optic nerve atrophy has often been described [7–9]. Kidney impairment is highly prevalent in MMA [10] and has also been attributed to mitochondrial disfunction as shown by studies in animal models [11]. Other affected organs are pancreas (acute or chronic pancreatitis) or skeletal muscle (myopathy, rhabdomyolysis) [1,12]. Cardiomyopathy, with a high prevalence in patients with propionic acidemia (PA), is observed to a lesser extent in MMA [2]. Moreover, morphological changes have been described in organs of MMA patients or in animal models, such as megamitochondria, abnormal cristae or mitochondrial inclusions [11,13–15].

The extent and mechanism of the mitochondrial dysfunction is still debated, though several aberrant metabolic pathways converge and may be responsible. The mutase deficiency alters a main anaplerotic pathway *via* propionyl CoA, as it cannot be metabolized to succinyl CoA that eventually fuels the Krebs cycle. Moreover, the excess of propionyl CoA sequesters oxaloacetate to form the highly toxic MCA, that further depletes de Krebs cycle causing a deficient flux of intermediates such as citrate or alfa-ketoglutarate, as shown by the low levels of plasma glutamine [16–19]. The accumulation of MCA also leads to inhibition of pyruvate dehydrogenase (PDH) [2,20,21]. This finally impairs mitochondrial function causing an OXPHOS deficiency and an altered redox homeostasis [22–25].

The accumulation of toxic metabolites inhibiting mitochondrial enzymes induces oxidative stress in propionate inborn errors of metabolism. Low levels of antioxidants together with increased high reactive species of oxygen (RSO) have been documented in MMA and PA patients, contributing presumably to the secondary mitochondrial dysfunction [26,27].

In view of new therapies, as well as to evaluate the response to different options of treatment, there is an urgent need of new biomarkers [27]. Changes in primary metabolites such as MCA, the MCA:citric acid ratio, or the oxidation of 13C-propionate [28,29], methylmalonic acid and C3 have demonstrated clinical relevance in patients MMA, especially at diagnosis. Other potential biomarkers include secondary metabolites, such as ammonium. Mitochondrial markers, such as the fibroblast growth factor 21 (FGF 21) or growth differentiation factor 15 (GDF 15) are also used [28,30,31].

The assessment of mitochondrial function based on functional studies could provide answers regarding the pathophysiology of MMA and help identify those patients at high risk for the development of chronic complications. However, until now, most studies of mitochondrial function usually require a skin or muscle biopsy [32], are expensive, and cannot be translated into clinical practice. The aim of this work was to develop a safe and noninvasive *in vivo* method to assess the glycolytic pathway and mitochondrial function parameters using lymphocytes from patients with MMA.

2. Materials and methods

2.1. Patients

We selected two unrelated patients with *mut*^{0}, based on genetic diagnosis,. Informed consent to be included in the study was provided by the patients 'parents. The parameters for the mitochondrial function and glycolysis were compared with sex and age-related healthy controls. Both patients were receiving CoQ10 supplementation during the study, which may have influenced mitochondrial bioenergetic parameters and represents a potential confounder. The study was approved by the Ethical Committee of the Cruces University Hospital, Bilbao, Spain (PI2020021).

2.2. Mononuclear cell (PBMCs) extraction from peripheral blood

Extraction of 5–10 ml of blood in EDTA tubes was performed for each patient and healthy controls. Samples were centrifugated at 1800 rpm for 15 min at 20 °C without centrifuge brake and then plasma was removed and RPMI medium 1:1 was added. Sample was added to a tube with Ficoll and centrifugated at 328*g* for 30–40 min at 20 °C without brake. PBMCs were collected and lymphocytes were separated from monocytes.

2.3. Seahorse analysis

Lymphocytes were thawed and maintained in culture 24 h before the experiment. After the culture, they were washed and resuspended in Seahorse XF DMEM Medium (pH 7.4, Agilent Technologies) supplemented with 10 mM glucose, 2 mM glutamine and 1 mM pyruvate. 96 well microplates were treated with Cell-Tak (Corning) and cells were plated into six replicates at 60,000 cells/well. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were determined using a Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies) from the Centro Achucarro- Basque Center for Neuroscience (UPV-Leioa, Spain). Seahorse XF Cell Mito Stress Test (Agilent Technologies) was used following manufacturer's indications, using 1, 1 and 0.5 μM of oligomycin, FCCP and rotenone/antimycin A, respectively. Data were analyzed with Seahorse Wave Desktop software v.2.6 (Agilent Technologies).

The selected concentrations of triheptanoin (10 mM), resveratrol (70 mM), and citrate (30 mM) were based on previous *in vitro* studies that utilized supra-physiological levels to elicit detectable bioenergetic responses in cell models of metabolic diseases [17,33,34]. Although these concentrations exceed physiological norms, their use in this pilot study was justified as a proof-of-concept approach to ensure measurable bioenergetic modulation within the limited duration of the Seahorse assay. Future studies should explore more physiologically relevant concentrations. Triheptanoin was used at 10 mM and lymphocytes were maintained in this medium for 24 h before measuring mitochondrial parameters [35].

2.4. Statistical evaluation

Due to the extremely limited sample size ($n = 2$ patients), statistical results must be interpreted as descriptive and exploratory. To analyze the association between variables, a Generalized Estimating Equations (GEE) model with an autoregressive structure of order 1 (AR-1) was employed, as multiple measurements at different times were taken from the same patient. All analyses were performed using Stata version 18.

3. Results

3.1. Patients

Two MMA *mut*^{0} patients were included in the study. The genetic

analysis identified compound heterozygosity for previously described *MMUT* pathogenic variants: NM_000255.4:c.[313 T > C]/ [2150G > T] in the case of patient 1 and NM_000255.4:c.[1277G > A]/[682C > T] for patient 2. Both patients, aged 8 and 5 years old at the time of enrollment, presented neonatal onset of isolated MMA with recurrent metabolic decompensation crises characterized by hyperammonemia during the first years of life; systemic treatment with vitamin B12 did not show any clinical or metabolic benefit in any of them. At the time of the study, both patients exhibited normal renal function, with no other organic complications detected, see Supplementary Table 1S. The 5-year-old patient (patient 2) displayed a more unstable clinical course with frequent hospital admissions, prompting the addition of carglumic acid to the treatment. Biochemically, this patient demonstrated persistent metabolic acidosis, elevated biomarkers in plasma or urine and high levels of FGF21, see Supplementary Table 1S.

3.2. Mitochondrial function

The Seahorse data detected an important significant reduction in oxygen consumption rate (OCR) and ATP production, as well as glycolisis (extracellular acidification rate, ECAR) in both MMA patients compared with healthy controls, see Fig. 1 and Table 1. Interestingly, when triheptanoin was added to patients´ lymphocytes, we detected an improvement in both ATP production and ECAR, but not in OCR, see Fig. 2 and Table 2. The high concentrations of resveratrol and citrate used did not produce detectable bioenergetic changes. The observed improvement in ATP production and ECAR after triheptanoin exposure must be interpreted with caution, as the increase in glycolysis may not necessarily reflect a full recovery of mitochondrial function. Although triheptanoin exposure increased ATP production and ECAR in patient-derived lymphocytes, the lack of OCR improvement suggests the effect may predominantly reflect enhanced glycolysis rather than a true restoration of mitochondrial oxidative phosphorylation.

Table 1
Mitochondrial function in lymphocytes of 2 *mut*^{0}patients compared with healthy controls. ATP production (pmol/min); OCR: oxygen consumption rate (pmol/min); ECAR: extracellular acidification rate (mpH/min).

Variable	Coefficient	95 % conf interval	*p*-value
OCR	−21.1	−35.6; −6.5	0.004
ECAR	−6.0	−11.2; −0.8	0.023
ATP production	−11.1	−15.3; −6.8	< 0.0001
Basal respiration	−13.5	−17.5; −9.5	< 0.0001
Spare respiratory capacity	−9.7	−15.6; −3.7	0.001

4. Discussion

This pilot study suggests that Seahorse technology can detect mitochondrial dysfunction in peripheral lymphocytes from MMA patients. This provides a novel proof-of-concept for a noninvasive approach in MMA, taking in consideration the unmet need for functional and clinically feasible biomarkers in view of the new therapies such as gene therapy or mRNA.

Although triheptanoin demonstrated partial bioenergetic improvement, its effect appears to preferentially stimulate glycolysis rather than oxidative phosphorylation; moreover, the potential risk of propionyl-CoA accumulation precludes its clinical application in MMA without further safety evaluation. There is increasing evidence regarding the altered energy metabolism in the propionate inborn of metabolism [2,36–38]. It is our understanding that impaired anaplerosis of the Krebs cycle in MMA patients has been sufficiently demonstrated, both using *in vivo* and *in vitro* studies [19,21]. The deficient refilling the tricarboxylic acids (TCA) cycle through propionate pathways together with high oxidative stress and low levels of antioxidants have a major impact on the energetic metabolism, and it complements the toxicity associated with the accumulation of abnormal chemical intermediates due of the primary enzymatic deficiency.

Triheptanoin, an importante anaplerotic molecule, is a medium odd-chain triglyceride that undergoes complete liver metabolism to form

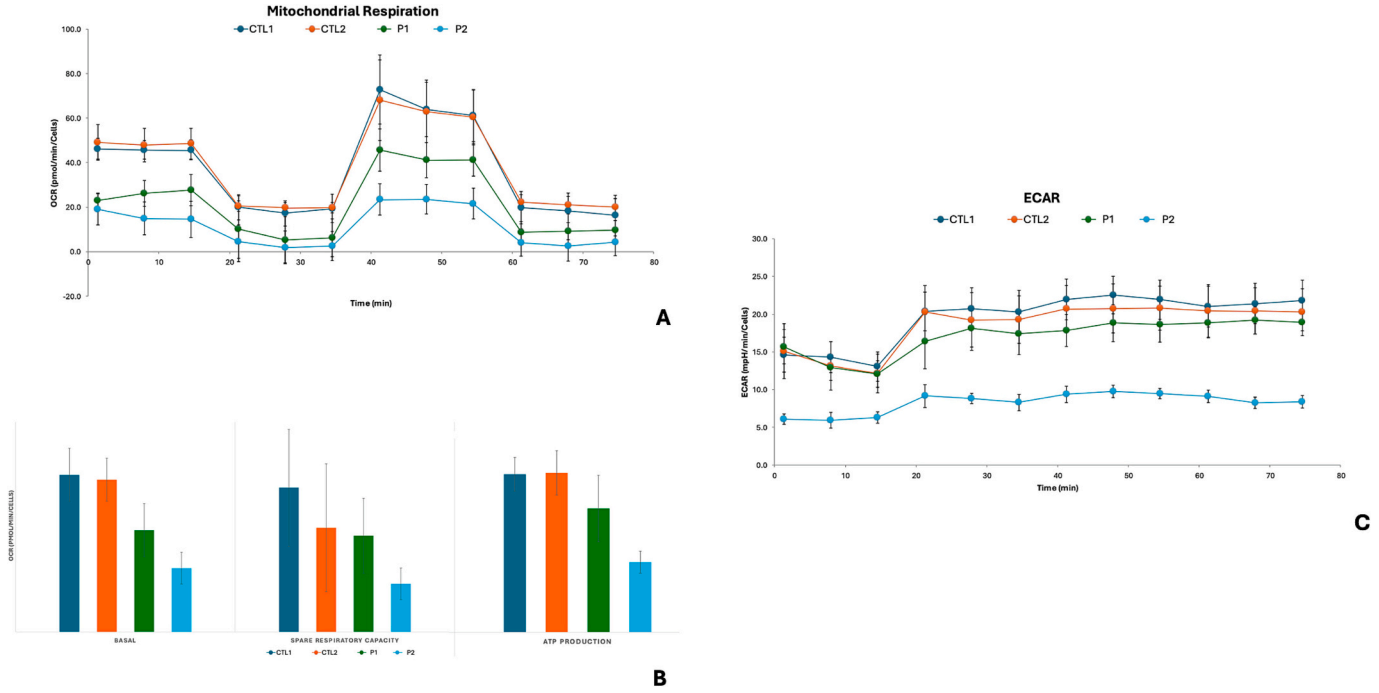

Fig. 1. Description of mitochondrial bioenergetics in lymphocytes of MMA patients compared with healthy controls. Fig. 1A: mitochondrial respiration reflected by oxygen consumption rate (OCR) at different times; Fig. 1B: mitochondrial function parameters as shown by basal respiration, spare respiratory capacity and ATP production; Fig. 1C: Extracellular acidification rate (ECAR) reflecting glycolysis at different times; CTL 1 and CTL 2 are the healthy controls; P1: patient 1; P2 patient 2.

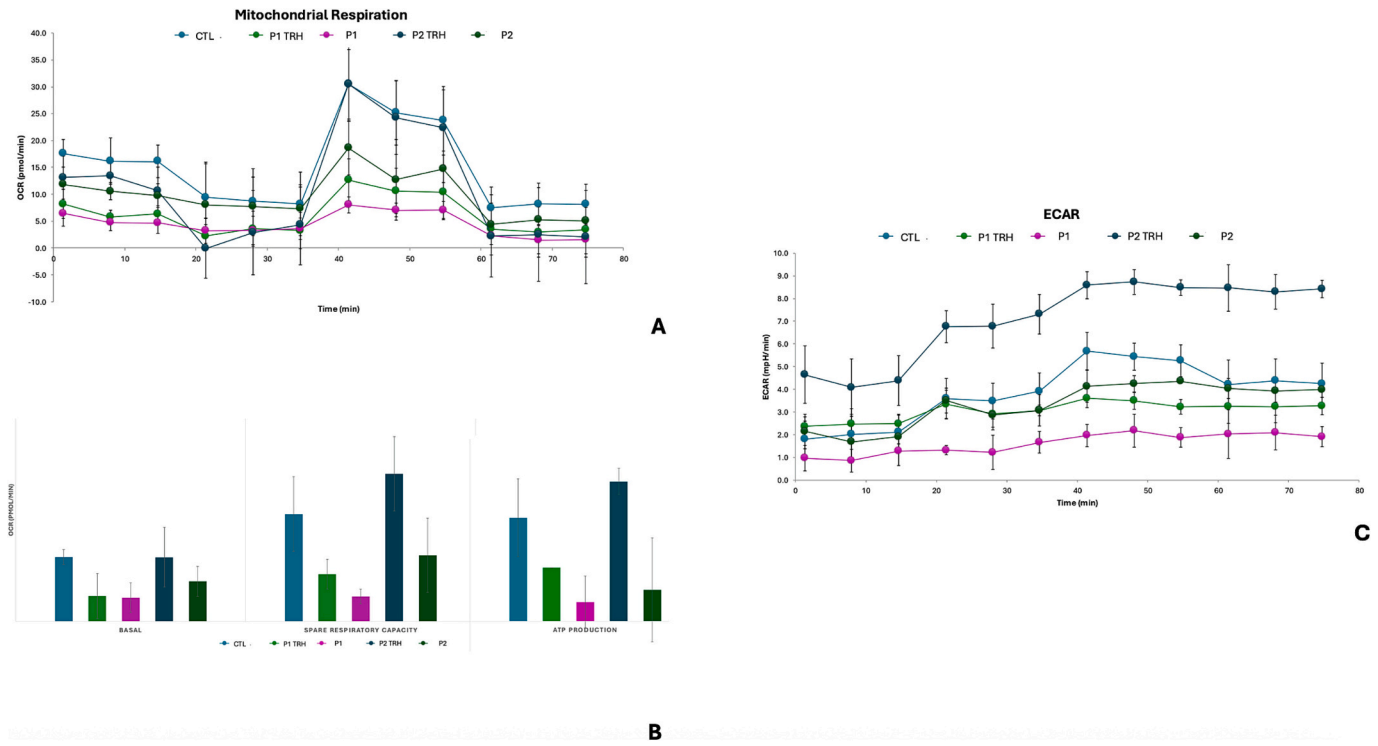

Fig. 2. Descriptive measurements of triheptanoin exposure for mitochondrial function; patients 'lymphocytes were compared before and after intervention and with a healthy control (CTL). Fig. 2A: mitochondrial respiration reflected by oxygen consumption rate (OCR) at different times; Fig. 2B: mitochondrial function parameters as shown by basal respiration, spare respiratory capacity and ATP production; Fig. 2C: Extracellular acidification rate (ECAR) reflecting glycolysis. CTL: healthy control; P1, P2: patients 1 and 2 respectively before intervention; P1 TRH, P2 TRH: patients 1 and 2 respectively after exposure to triheptanoin.

Table 2
Effect of exposure of patients' lymphocytes to triheptanoin in mitochondrial function in lymphocytes of 2 *mut*^{0}patients. ATP production (pmol/min); OCR: oxygen consumption rate (pmol/min); ECAR: extracellular acidification rate (mpH/min).

Variable	Coefficient	95 % conf interval	*p*-value
OCR	1.3	−1.9; 4.5	n.s.
ECAR	2.6	2.0; 3.1	< 0.0001
ATP production	5.4	2.9; 8.0	< 0.0001
Basal respiration	1.7	−0.8; 4.3	n.s.
Spare respiratory capacity	6.9	3.0; 10.8	< 0.0001

heptanoate (C7), which is then converted to C5 ketone bodies (beta-hydroxypentanoate and beta-ketopentanoate). These metabolites can theoretically fuel the Krebs cycle by providing acetyl-CoA and propionyl-CoA [39]. Anaplerotic therapy with triheptanoin has shown significant benefits in various inborn errors of metabolism, albeit the necessity for unimpaired propionate metabolic pathways, contra-indicating its use in MMA patients due to potential damaging mechanisms of excess propionyl CoA [34,39,40].

Although triheptanoin increased ATP production and ECAR, it did not modify OCR. This pattern suggests a preferential stimulation of glycolysis rather than a direct improvement in oxidative phosphorylation.

Another attempt to modulate the mitochondrial function in propionate inborn errors of metabolism is the supplementation with antioxidants molecules, considering the high oxidative stress and the deficiency of antioxidant levels in these patients [22,24,41–45]. In one of our previous studies, we compared metabolic markers in 7 patients with PA that received ubiquinol supplementation for 6 months and observed an improvement of urinary excretion of Krebs cycle intermediate citrate and the citrate/methylcitrate ratio [45]. In animal models of MMA, a therapeutic regimen, directed at reducing oxidant injury with CoQ_{10} and vitamin E, ameliorated the loss of glomerular filtration rate [27]. In this regard, we investigated the effect of resveratrol, a natural antioxidant [33], on the mitochondrial parameters. Supplementation with resveratrol has been studied in various metabolic disorders, including MMA [46], but evidence supporting its efficacy is limited and it is unlikely to restore TCA cycle function. Thus, we could not demonstrate any benefit of the mitochondrial function with the resveratrol supplements. The supra-physiological concentrations of resveratrol and citrate likely limited their translational relevance. Although these concentrations were selected to ensure measurable responses in this experimental setting, future studies should adopt more clinically relevant dosing.

Finally, it should be noted that we also observed glycolysis deficiency as shown by lower ECAR in MMA patients compared with controls. This could have important clinical implications, considering that many of these patients use industrially processed low-protein foods, which contain a large amount of carbohydrates [47–49]. The connection between MMA and a potential defect in glycolysis is not as direct and well-defined as the primary defect in propionate metabolism. Nevertheless, several hypotheses suggest how MMA might influence the glycolysis pathway. In the earliest descriptions of the MMA pathophysiology, it has been proposed that methylmalonyl-CoA could compete with acetyl-CoA for the allosteric activating site of pyruvate carboxylase (PC) and inhibit its activity converting pyruvate to oxaloacetate [50,51]; in rat hepatocytes, methylmalonic acid also inhibits malate oxidation [52], offering another possible explanation for the hypoglycemia observed in metabolic crisis [3]. Moreover, the excess of propionyl CoA inhibits pyruvate dehydrogenase, directly affecting glucose metabolism [53,54]. Notably, low plasma alanine and glutamine levels as markers of deficient anaplerosis in muscle might further impair hepatic and renal gluconeogenesis [16,36]. This may have direct clinical implications in patients with metabolic disorders such as MMA that necessitate a tailored nutritional approach aimed to optimize energy metabolism and supporting metabolic health.

Our study has important limitations. First, the small sample size and limited correlation with other metabolic or clinical biomarkers are significant constraints and force us to be cautious in our conclusions. Given the small sample size and the lack of direct measurements of propionyl-CoA or related toxic metabolites, no clinical recommendations regarding triheptanoin use can be made based on this study. As stated before, the failure of assessment possible toxicity owing the lack of direct biomarkers precludes the use of triheptanoin in clinical practice. Moreover, while glycolysis is a crucial energy-producing pathway, higher ECAR may also reflect metabolic stress or inefficiency. It is evident that we need to increase the number of patients to categorize the severity and therefore the degree and intensity of the intervention. Future studies should incorporate measurements of intracellular metabolites, such as propionyl-CoA, methylcitrate, and citrate, to evaluate potential metabolic imbalances induced by triheptanoin.

Limitations: The main limitations of this study are the small sample size, the absence of metabolite toxicity assessment, potential confounding by CoQ10 supplementation, and the use of pharmacologically high concentrations of resveratrol and citrate. These factors limit the generalizability and clinical applicability of our findings.

5. Conclusions

This exploratory pilot study demonstrates that Seahorse technology can effectively assess mitochondrial and glycolytic function in peripheral lymphocytes from MMA patients. Although the observed improvements following triheptanoin exposure are of interest, the absence of toxicity assessments and the extremely limited sample size preclude clinical extrapolation. Larger, longitudinal studies incorporating metabolic safety biomarkers are required to validate these preliminary findings and evaluate their clinical relevance.

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CRediT authorship contribution statement

Sinziana Stanescu: Writing – review & editing, Writing – original draft, Data curation. **Olatz Villate:** Methodology, Investigation. **Fernando Andrade:** Methodology, Investigation. **Domingo Gonzalez-Lamuño:** Writing – review & editing, Data curation, Conceptualization. **Amaya Bélanger-Quintana:** Writing – review & editing. **Francisco Arrieta:** Writing – review & editing. **Maria Luz Couce:** Writing – review & editing, Conceptualization. **Alfonso Muriel:** Software. **Luis Aldamiz Echevarria:** Writing – review & editing, Formal analysis, Data curation, Conceptualization.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee of the Cruces University Hospital, Bilbao, Spain.

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Declaration of competing interest

The authors declare no conflicts of interest.

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Data availability statement

Complete data is available provided by the authors, previous request.

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