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# Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry

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ABSTRACT

*Background:* Mitochondrial diseases belong to the most severe inherited metabolic disorders affecting pediatric population. Despite detailed knowledge of mtDNA mutations and progress in identification of affected nuclear genes, diagnostics of a substantial part of mitochondrial diseases relies on clinical symptoms and biochemical data from muscle biopsies and cultured fibroblasts.
*Methods:* To investigate manifestation of oxidative phosphorylation defects in isolated lymphocytes, digitonin-permeabilized cells from 48 children were analyzed by high resolution respirometry, cytofluorometric detection of mitochondrial membrane potential and immunodetection of respiratory chain proteins with SDS and Blue Native electrophoreses.
*Results:* Evaluation of individual respiratory complex activities, ATP synthesis, kinetic parameters of mitochondrial respiratory chain and the content and subunit composition of respiratory chain complexes enabled detection of inborn defects of respiratory complexes I, IV and V within 2 days. Low respiration with NADH-dependent substrates and increased respiration with glycerol-3-phosphate revealed complex I defects; changes in $p_{50}$ for oxygen and elevated uncoupling control ratio pointed to complex IV deficiency due to *SURF1* or *SCO2* mutation; high oligomycin sensitivity of state 3-ADP respiration, upregulated mitochondrial membrane potential and low content of complex V were found in lymphocytes with ATP synthase deficiency due to *TMEM70* mutations.
*Conclusion:* Based on our results, we propose the best biochemical parameters predictive for defects of respiratory complexes I, IV and V manifesting in peripheral blood lymphocytes.
*General significance:* The noninvasiveness, reliability and speed of an approach utilizing novel biochemical criteria demonstrate the high potential of isolated lymphocytes for diagnostics of oxidative phosphorylation disorders in pediatric patients.


## 1. Introduction

Mitochondrial diseases belong to most severe inherited metabolic diseases affecting pediatric population. They are caused by disorders of mitochondrial biogenesis or by mutations in the structural subunits of oxidative phosphorylation (OXPHOS) apparatus [1]. Genetics of mitochondrial OXPHOS disorders is quite unique: they can be caused either by mutations in nuclear genes or mutations in mitochondrial DNA (mtDNA), which encodes 13 of ~90 OXPHOS structural subunits. Mutations in the maternally-transmitted mtDNA are already well characterized. Several hundreds of them have been described to date (www.mitomap.com) and their detection and screening have become rather routine task [2]. However, according to current estimates, mtDNA mutations are responsible for only 25% of mitochondrial diseases. The remainder originates from mutations in nuclear genes,

*Abbreviations:* $\Delta\Psi_m$, mitochondrial membrane potential; AA, antimycin A; BNE, Blue Native PAGE; cI–cV, respiratory chain complexes I–V; COX, cytochrome *c* oxidase; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GP, glycerol-3-phosphate; GPDH, mitochondrial FAD-dependent glycerophosphate dehydrogenase; OXPHOS, oxidative phosphorylation; PAGE, polyacrylamide gel electrophoresis; s3, state 3-ADP; s3u, state 3-uncoupled; s4o, state 4-oligomycin; TMPD, tetramethylphenylenediamine; TMRM, tetramethylrhodamine methyl ester

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and their diagnostics still poses a significant challenge. Within the last couple of years pathogenic mutations resulting in a mitochondrial disease have been uncovered in ~110 nuclear genes [3] including numerous novel factors of mitochondrial biogenesis (e.g. TMEM70 [4,5], C12orf65 [6,7] C20orf7 [8], TACO1 [9], RMND1 [10], SDHAF1 [11], SDHAF2 [12]). Nevertheless, most of the disease-causing genes from the broad repertoire of more than thousand nuclear genes contributing to the mitochondrial proteome (www.mitocarta.com [13]) still remain to be identified. For example, among the very frequent defects of cytochrome *c* oxidase — complex IV (cIV), the terminal enzyme complex of the respiratory chain, the genetic basis underlying the disorder was found in less than 50% of the cases [14].

Diagnostics of a substantial part of mitochondrial diseases thus relies on clinical symptoms and biochemical analyses. Assessment of energetic function and content of individual mitochondrial proteins in patient tissues is mainly performed in bioptic samples of skeletal muscle and/or cell cultures of skin fibroblasts. Both approaches have significant drawbacks: muscle biopsy allows for an instant, single biochemical analysis of the mitochondrial quantity and function, but it is often complicated by muscle atrophy caused by mitochondrial disorders. Due to its invasive nature, it is also often refused by the parents of the patient, and the same holds true for the skin biopsy, necessary for establishing of fibroblast cell culture. While the cell culture allows for more systematic and repeated analysis of mitochondria, the diagnostic results are only obtained after a prolonged period, ranging from several weeks to months.

The analysis of mitochondrial energetic apparatus in peripheral blood lymphocytes, with the help of sensitive functional methods and detection of quantitative and qualitative changes in OXPHOS proteins, could become an attainable alternative method. Although it is practically noninvasive and easily repeatable, functional and protein analyses of mitochondrial OXPHOS system in lymphocytes or lymphoblasts have only been used sporadically for the study of mitochondrial diseases [15–25]. Even in this limited number of studies, lymphocytes were more often used in monitoring of tissue specific presentation of respective mutations than as a primary diagnostic tool.

To establish this approach as an alternative diagnostic tool, we adapted and optimized highly sensitive oxygraphy of digitonin-permeabilized cells for analysis of the function of mitochondrial respiratory chain in lymphocytes. Wherever feasible, we combined it with the analysis of mitochondrial membrane potential ($\Delta\Psi_m$) by cytofluorometry and with protein analysis of OXPHOS complexes by SDS-PAGE and BNE followed by specific immunodetection of structural subunits of mitochondrial respiratory complexes. Subsequent statistical analysis uncovered reliable and selective markers that can distinguish samples harboring deficiencies of complex I, complex IV caused by *SURF1* and *SCO2* mutations, and complex V on the basis of *TMEM70* mutations.

## 2. Materials and methods

### 2.1. Patients

Peripheral blood samples (3–7 ml depending on patients' age) were obtained from 48 children aged 1 month to 18 years. Out of them, 35 children with neurological disturbances and/or developmental delay were referred by their local pediatricians for metabolic screening and their parents agreed to take part in our study; seven children (P1, P4–P5 and P7–P9) had already known mitochondrial disorder confirmed at biochemical and/or molecular level and six disease free children served as the age-matched controls. Altogether, 37 children were analyzed once and eleven children were analyzed 2–3 times.

Mitochondrial patient P1 at the age of 11 years had severe encephalopathy and arrest of any mental and motor development due to deficiency of respiratory chain complex I of unknown nuclear origin without any mutation in the mtDNA. Patient P2 is a 13 years old girl with intrauterine growth retardation and repeated attacks of cephalea since the age of 6 years, dissociative psychiatric disease, progressive bilateral deafness and progressive visual impairment with bilateral concentric visual field loss. The parents did not approve of skin or muscle biopsy. Molecular analyses from blood and buccal smear did not detect any mtDNA mutation responsible for any of LHON, MELAS or MERRF syndromes. Patient P3 was born at the 32nd week of gestation with a birth weight of 1990 g and a length of 42 cm. Early postnatal adaptation was uneventful, but he developed pharmaco-resistant seizures during the third week of life. MRI of the brain revealed pachygyria and cortical dysplasia. The lactic acidosis was not present. The boy died at the age of 4 months and autopsy revealed polymicrogyria focalis gyri temporalis superioris l.sin. Patients P4 and P5 had progressive encephalopathy and Leigh disease since the age of 10 and 12 months respectively due to deficiency of respiratory chain complex IV and homozygous mutations in *SURF1* gene (c.845_846delCT and c.312_321del10insAT, respectively). Patient P6 presented since the age of 7 months with failure to thrive, progressive encephalomyopathy and hypertrophic cardiomyopathy. The activity of complex IV was low in lymphocytes and the consequent DNA analysis identified that the boy is compound heterozygote for mutations c.1A>G and c.418G>A in *SCO2* gene. Patients P7–P9 at the age of 17, 11 and 2.5 years have encephalocardiomyopathy with deficiency of respiratory chain complex V due to homozygous c.317-2A>G mutation in *TMEM70* gene (first two of them were already described as P9 and P11 in [4]).

All work involving human samples was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethical Committees of the participating institutions. The written informed consent was obtained from patients or patients' parents.

### 2.2. Lymphocytes

Within 1–2 h after collection, whole fraction of intact lymphocytes was isolated from EDTA-blood by density medium centrifugation at 4 °C, using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences) following a standard protocol. Briefly, blood sample was layered on top of an equal volume of Ficoll and centrifuged at 800 *g* for 20 min. Separated lymphocytes were carefully collected (~1 ml) and resuspended in 15 ml of erythrocyte lysing buffer (30 mM $NH_4Cl$, 2 mM $NH_4HCO_3$, 20 μM $Na_2EDTA$, pH 8) and incubated for 20 min on ice. Lymphocytes were pelleted by 800 *g* centrifugation for 20 min, weighed and resuspended in PBS supplemented with protease inhibitors (1:500 protease inhibitor cocktail, Sigma) for respiration measurements and SDS-PAGE analysis. For BNE dry pellet of lymphocytes was used (see Section 2.4). Protein concentration was determined by Bradford method [26] using BSA as standard.

### 2.3. High resolution respirometry

Oxygen consumption was measured at 30 °C as described before [27] using Oxygraph-2k (Oroboros). Freshly isolated lymphocytes (0.6 mg protein) were suspended in 2 ml of KCl medium (80 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM K-Pi, 10 mM Tris–HCl pH 7.4) and digitonin 0.05 g/g protein was used to permeabilize the plasma membrane. For measurements the following concentrations of substrates and inhibitors were used: 3 mM malate, 10 mM pyruvate, 10 mM glutamate, 10 mM succinate, 10 mM glycerol 3-phosphate (GP), 1 mM ADP, 5–200 nM oligomycin, 150–200 nM FCCP, 0.25 μM antimycin A, 2 mM ascorbate, 0.6 mM TMPD and 0.5 mM KCN. The oxygen consumption was expressed in pmol oxygen/s/mg protein.

$p_{50}$ value – the partial oxygen pressure ($pO_2$), at which the cellular respiratory rate is half-maximal – was determined as before [28]. Briefly, the volume-specific rate of oxygen consumption (oxygen flux) was calculated as the negative slope of oxygen concentration recorded at 1 s time intervals. The signal was deconvoluted with the exponential time constant of the oxygen sensor (3 to 5 s) and