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Genetické příčiny deficitu cytochrom c oxidázy u dětí

Genetic causes of cytochrome c oxidase deficiency in children

PhD Thesis

Supervisor: Ing. Markéta Tesařová, Ph.D.

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ABSTRAKT

Mitochondrie jsou hlavním, nepostradatelným zdrojem ATP, který je produkován především systémem oxidativní fosforylace (OXPHOS). Mutace v genech podmiňujících správnou funkci OXPHOS způsobují mitochondriální onemocnění, jejichž incidence je odhadována na 1:5000 živých narozených dětí. Cytochrom c oxidáza (COX) je klíčovým enzymem dýchacího řetězce, který katalyzuje přenos elektronů na kyslík za vzniku molekuly vody. Izolované nebo kombinované poruchy aktivity COX jsou spolu s deficitem komplexu I nejčastějším typem mitochondriální poruchy u dětí. Způsobeny jsou mutacemi v mitochondriálních nebo jaderných genech kódujících strukturní podjednotky a asemblační faktory jednotlivých komplexů OXPHOS. Přesná genetická podstata poruchy aktivity COX však zůstává u mnoha pacientů neobjasněna navzdory vzrůstajícímu počtu nově charakterizovaných genů.

Cílem dizertační práce bylo popsat genetickou příčinu mitochondriálního onemocnění u skupiny 60 nepříbuzných dětí z České republiky s biochemicky potvrzenou poruchou COX. Optimalizovanou metodikou high-resolution melting byly identifikovány čtyři heterozygotní varianty v exonech genů *COX4I2*, *COX5A*, *COX7A1* a *COX10*, které byly klasifikovány jako patologické, a proto jsou vhodnými kandidáty pro provedení cílené mutační analýzy u dětí s deficitem COX. Pomocí SNP DNA mikročipu byly nalezeny patologické rozsáhlé delece genů *TYMP*, *SCO2* a *PUS1* u 4/16 dětí. Tyto delece byly u 2 pacientů kombinovány s missense mutacemi v genech *TYMP* a *SCO2*. Předběžné výsledky mutačního skríningu mitochondriálního exomu provedeného na genomové DNA u 25/57 pacientů přispěly k nalezení kauzálních mutací u 5/25 pacientů v genech *AARS2*, *TSFM*, *TK2*, *AIFM1* a *MGME1*. U dalších 5/25 pacientů NGS technologie umožnila výběr kandidátních sekvenčních variant v genech *ACOX2*, *UQCRH*, *QARS*, *SUCLG2* a *ACBD3*, jejichž patogenita však ještě musí být experimentálně potvrzena. V průběhu studie se podařilo objasnit genetickou podstatu poruchy COX u 9 nemocných dětí.

Klíčová slova: mitochondrie, mitochondriální poruchy, laboratorní diagnostika, dědičnost, deficit cytochrom c oxidázy (COX)

ABSTRACT

Mitochondria are the key source of vital ATP molecules, which are largely produced within cells by a system of oxidative phosphorylation (OXPHOS). Genetic defects affecting any of the components of the oxidative phosphorylation system or the structure and function of mitochondria lead to mitochondrial disorders, which occur at an incidence rate of 1 in 5000 live births. Cytochrome c oxidase (COX) is the terminal enzyme and electron acceptor of a respiratory chain that catalyses oxygen to produce a water molecule. In addition to complex I deficiency, isolated or combined COX deficiency is the most common respiratory chain defect in paediatric patients, and it can arise from mutations located either in mitochondrial DNA or in nuclear genes encoding the structural subunits or corresponding assembly factors of the enzyme complex. However, the molecular basis of COX deficiency remains elusive in many patients despite advances in the identification of an increasing number of mutations and genes involved in the disease.

This thesis focuses on the identification of the genetic causes of mitochondrial diseases in a cohort of 60 unrelated Czech children with clinically and laboratory confirmed COX-deficiency. With the use of a high-resolution melting analysis mutation screen, four heterozygous sequence variants, located in *COX4I2*, *COX5A*, *COX7A1* and *COX10*, were found to be pathogenic and are suggested as candidate variants for future targeted-mutation screening in Czech COX-deficient children. The application of a DNA microarray SNP chip enabled the identification of rarely occurring but pathological large deletions in 4/16 patients affecting the *TYMP*, *SCO2* and *PUS1* genes, which were combined with causal missense mutations in *TYMP* and *SCO2*. The genomic DNA of 25/57 patients was analysed using next-generation sequencing targeted to the mitochondrial exome. The preliminary data analysis enabled the identification of pathological sequence variants in 5/25 patients, which affected the *AARS2*, *TSFM*, *TK2*, *AIFM1* and *MGME1* genes. Additional suspected disease-candidate variants were found in the *ACOX2*, *UQCRH*, *QARS*, *SUCLG2* and *ACBD3* genes of 5/25 patients, but their pathogenicity has yet to be confirmed experimentally. In conclusion, the genetic bases of COX deficiency have been clarified in nine paediatric patients.

Key words: mitochondria, mitochondrial disorders, laboratory diagnostics, inheritance, cytochrome c oxidase (COX) deficiency

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Last but certainly not least, I must express my sincere gratitude to my parents for their continued support during my studies.

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Abbreviations

Δp_m	proton motive force
$\Delta \Psi_m$	mitochondrial membrane potential
ADP	adenosine diphosphate
ANT	adenine nucleotide translocase
AR	autosomal recessive
ARMS	amplification-refractory mutation system
ASO	allele-specific oligonucleotide
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	F_1F_0-ATP synthase
BAEP	brain stem auditory evoked potential
BN-PAGE	blue native - polyacrylamide gel electrophoresis
cAMP	cyclic adenosine monophosphate
Chr.	chromosome
CJ	cristae junction
CM	cristae membrane
CNV(s)	copy number variation(s)
CS	citrate synthase
CSF	cerebrospinal fluid
DGGE	denaturing gradient gel electrophoresis
DGV	database of genomic variants
dHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EEG	electroencephalography
ECG	electrocardiography
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
ETC	electron transport chain
FADH2	reduced form of flavin adenine dinucleotide
gDNA	genomic DNA
GLUT1	glucose transporter 1

HRM	high-resolution melting analysis
IBM	inner boundary membrane
IGV	integrative genomics viewer
IM	inner mitochondrial membrane
IMS	intermembrane space
MELAS	mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes
MINOS	mitochondrial inner membrane organising system
MitOS	mitochondrial organising structure
MICOS	mitochondrial contact site
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA
$NADH+H^+$	reduced form of nicotinamide adenine dinucleotide
NGS	next-generation sequencing
OM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
ΔpH	ΔpH gradient across the inner mitochondrial membrane
P	patient
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PGD	preimplantation genetic diagnosis
PKC	protein kinase C
PMF	proton motive force
PND	prenatal diagnosis
PPARs	peroxisome proliferator-activated receptors
R123	rhodamine 123
rCRS	revised Cambridge reference sequence
RFLP	restriction fragment length polymorphism
RRFs	ragged red fibres
RNAi	RNA interference
ROS	reactive oxygen species
rRNA(s)	ribosomal ribonucleic acid(s)
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SNP(s)	single nucleotide polymorphism(s)

SSCP	single-strand conformation polymorphism
TCA	citrate acid cycle
TGGE	temperature gradient gel electrophoresis
TMPD	tetramethyl-phenylene-diamine
TMRE	tetramethylrhodamine ethyl ester
TMRM	tetramethylrhodamine methyl ester
tRNA(s)	transfer ribonucleic acid(s)
TTGE	temporal temperature gradient gel electrophoresis
UCP	uncoupling protein
VEP	visual evoked potential
VDAC	voltage-dependent anion channel

1 Introduction

The mammalian cytochrome c oxidase (COX, Complex IV, EC 1.9.3.1) is a multimeric copper haem A metalloenzyme embedded in the inner mitochondrial membrane; its function is to transport electrons from cytochrome c to molecular oxygen, which is then reduced to water. COX consists of 14 polypeptide subunits, of which the 3 largest are encoded by the mitochondrial genome (*MTCO*1, *MTCO2* and *MTCO3*); the other 11 small peripheral subunits are encoded by the nuclear genome [1,2]. COX activity is tissue specific due to the different ontogenic development and metabolic needs and is subjected to regulation by molecules including hormones, membrane lipids and second messengers. In humans, four of the nuclear-encoded subunits, COX4I, COX6A, COX6B, and COX7A, have tissue specific isoforms that reflect differences in the energetic demands of the particular tissues. For COX6A and COX7A, two isoforms of each are known: the heart (H) type, which is present in skeletal and cardiac muscle, and the liver (L) type, which is present in non-muscle tissues. The testis-specific *COX6B2* and lung-specific *COX4I2* isoforms have also been identified [3].

COX deficiency is a clinically heterogeneous group of disorders that predominantly affect tissues with high-energy demand. The disorders range from isolated myopathy to severe multi-system disease and exhibit onset from infancy to adulthood; they are caused by mutations located in mtDNA and in the nuclear genes required for mitochondrial function. The incidence of COX deficiency has been estimated at 1:35000 births in the Slavonic population, where the majority of detected mutations are located in two genes, *SCO2* and *SURF1* [3,4]. The defects can be biochemically isolated or combined with deficiencies of any other components of the respiratory chain [5]. Rare disease-related mutations have been described for all 3 of the mitochondrial DNA-encoded COX subunits. The majority of COX defects originate from mutations in nuclear genes involved in the assembly and maintenance of the holoenzyme complex, including *SURF1*, *SCO1*, *SCO2*, *COX10*, *COX14*, *COX15*, *TACO1*, *LRPPRC*, *COA5* and *COX20*. Until now, no mutations have been detected in nuclear genes for other assembly factors, such as *COX11*, *COX16*, *COX17*, *COX18*, *COX19*, which are thought to be required to prevent COX deficiency [6-8]. However, the first mutations were only recently characterised in nuclear genes coding for structural subunits COX4I2, COX6B1, COX7B, NDUFA4 [9-12]. Despite advances in

the identification of an increasing number of mutations and genes involved in the disease, the molecular basis of COX deficiency remains elusive in many patients, which leads to difficulties in genetic counselling.

2 Aims of the study

Mitochondrial diseases represent one of the most common groups of inherited metabolic disorders affecting adults and children [13]. Because of the dual genetic control of mitochondria, dysfunction of mitochondrial processes can be caused by mutations in the mitochondrial (mtDNA) or nuclear genome. The inheritance of mitochondrial disorders is either maternal (mtDNA) or Mendelian (nuclear encoding genes) and can have autosomal recessive, autosomal dominant or X-linked genetic traits. To date, mutations in 1500 proteins are thought to be potential causes of mitochondrial disorders [14], although pathological mutations have only been identified in a small fraction of them despite advances in applied research methodologies [15]. Thus, the genetic basis of mitochondrial disorder remains unexplained in a large number of patients manifesting clinical symptoms and biochemical properties of the disease. This lack of explanation implies that determining the complexity of the COX defect is exceptionally challenging, perhaps due to the genotype- phenotype variability and the overlap of disease phenotypes in patients with cytochrome c oxidase deficiency, similar to other types of mitochondrial disease [16].

The Laboratory for the Study of Mitochondrial Diseases in the Department of Paediatrics and Adolescent Medicine of the First Faculty of Medicine in Prague has dealt with the clinical diagnostics of mitochondrial dysfunction for over 20 years. In this laboratory, data were collected from a group of 60 unrelated Czech paediatric patients with clinically and biochemically confirmed isolated or combined COX deficiency resulting from an unclear genetic cause. The aim of this study is concentrated on determining the genetic causes of COX deficiency in the selected cohort of patients to illuminate the pathogenic mechanisms behind their phenotype.

The specific aims of the thesis were:

1) To optimise and perform a mutation screening methodology involving high-resolution melting analysis for genes encoding COX structural subunits and selected COX assembly factors

2) To analyse copy-number variations in 16 patients with COX deficiency

3) To apply targeted sequencing of the mitochondrial exome in a group of 25 children with COX deficiency and to prioritise candidate disease variants

3 Review of the literature

The human body is composed of approximately 200 cells types totalling 10^{12}-10^{18} cells whose directed differentiation ensures full body function [17]. The proper function of a cell as a basic self-sufficient organised unit of life is generally conditioned by concerted mechanisms of energy production. The key energy-carrying molecule in human cells is ATP, and 95% of its mass-production occurs at the inner membranes of mitochondria during the OXPHOS process [18]. With the exception of aerobic production of ATP, mitochondria are needed for many other cellular processes; they contain hundreds of enzymes involved in the metabolic pathways of amino acids, fatty acids, haem, organic acids; in the tricarboxylic acid cycle (TCA); in part of the urea cycle; and in the biosynthesis of steroid hormones, porphyrins, purine and pyrimidine nucleotides, phosphocreatine, acetyl-CoA, gluconeogenesis precursors and FeS clusters. Mitochondria also participate in the production of free radicals, regulation of cytoplasmic and mitochondrial matrix calcium, apoptotic signalling and signalling that modulates the movement of whole organelles inside cells [19-23]. Thus, many primary mitochondrial defects affect multiple organ systems, demonstrating the indispensability of energy generated via OXPHOS [24,25].

3.1 From mitochondrial molecular ultrastructure to function

The ultrastructure and function of human mitochondria have been extensively studied since the late 1940s [26,27]. mtDNA was discovered in 1963 and was characterised in more detail in 1967 [28]. The complete sequence of mtDNA was identified in 1981 and subsequently corrected in 1999, but detailed knowledge of mtDNA replication, transcription, translation and maintenance remain lacking [20]. Circular double-stranded mtDNA consists of 16568 bp coding 37 genes: 2 RNAs, 22 tRNAs and 13 structural proteins of the OXPHOS system. Each mitochondrion contains 2-10 molecules of mtDNA; thus, one nucleated cell can have 10^3-10^4 copies of mtDNA, depending on the specific cell type [19,29,30]. Within a single cell, sequences of the mtDNA molecules may be identical (homoplasmic) or distinct (heteroplasmic). Identical mtDNA molecules are packed into clusters (nucleoids) tethered to the inner mitochondrial membrane; this clustering is thought to facilitate mtDNA segregation [31]. Notably, it seems that nucleoids rarely change and/or share genetic material [32,33].

Mitochondria have several functionally different microcompartments: the mitochondrial outer membrane (OM), inner membrane (IM), intermembrane space (IMS), matrix, cristae membrane (CM), intra-cristal space and inner boundary membrane (IBM). The IM forms disk-like structures that protrude into the matrix (cristae) and extend the IM surface; the remaining portion of the IM is called the IBM (Fig. 1) [34].

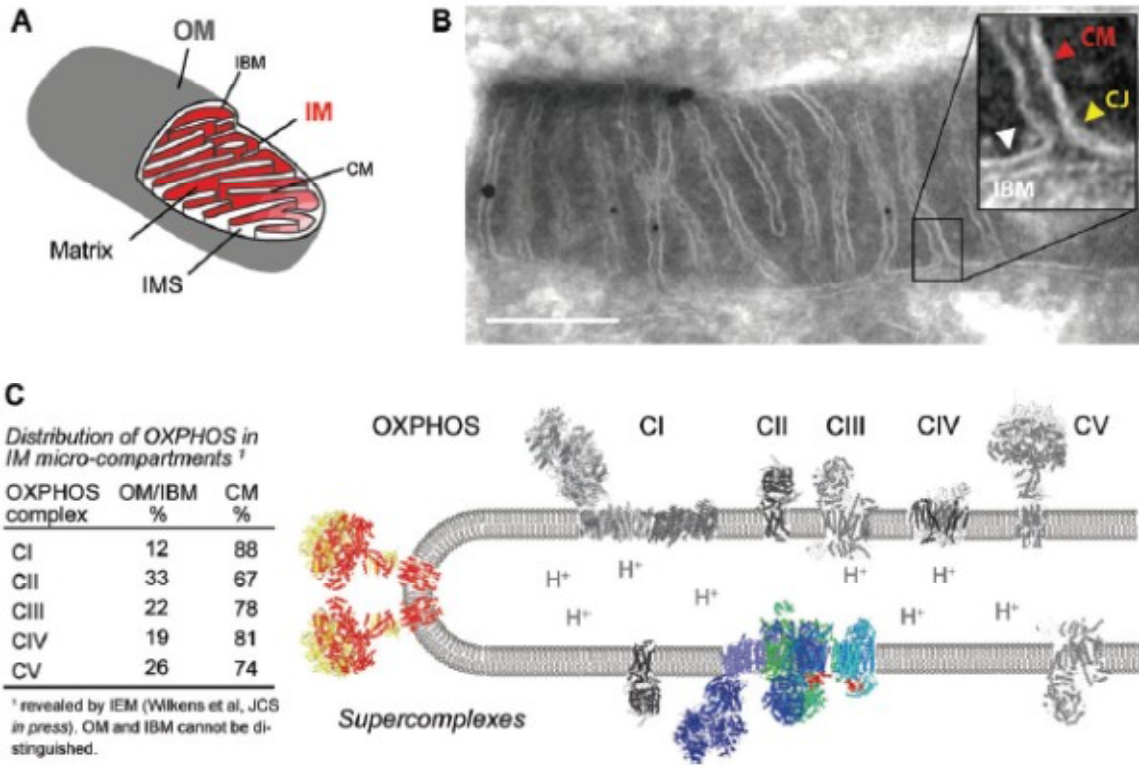

Fig. 1 The structure and composition of mitochondrial microcompartments

(A) A schematic view of a mitochondria with its main compartments: OM = outer membrane; IM = inner membrane; IMS = intermembrane space; CM = cristae membrane; CJ = cristae juntion. (B) Electron micrograph from a cryo cut mitochondrion with antibody probing of OXPHOS complexes. The localization in the cristae membrane is obvious. Inset: detailed view of the two IM microcompartments CM and IBM connected by the CJ. Scale bar: 150 nm. (C) Localization of OXPHOS complexes and supercomplexes in the CM [34].

Studies have shown the presence of tiny tubular junctions between cristae and the IBM, which further supports the hypothesis of a specific cristae microcompartment with a different pH value [34]. These microcompartments most likely differ in their membrane composition, especially in their lipid and protein contents. In general, the lipid fluid mosaic of membranes can drive membrane protein redistribution and/or

structural changes of lipid-binding domains. Cardiolipin, located exclusively in the inner mitochondrial membrane of eukaryotic cells, has been suggested as a stabilising factor of respiratory supercomplexes (all respiratory chain complexes and ATPase) although it has no adverse effect on their individual function and can be replaced by phospholipids with similar biochemical and structural characteristics [35,36]. Nonetheless, the mutation of *TAZ*, a nuclear gene involved in the formation of the mitochondrial membrane lipid milieu, has been described as an evident destabilisation factor for OXPHOS supercomplex assembly [37,38].

In addition to the foundational subunits that comprise every respiratory complex, several other nuclear-encoded ancillary scaffolding proteins that participate in their appropriate assembly processes are required. However, they do not belong to the structural constituent elements of OXPHOS. Complexes of the OXPHOS system, containing functionally indispensable prosthetic groups and metals in reactive enzyme centres, associate within the IM to form a higher-level of organised clusters called respirasomes, which enhance the effectiveness of the electron transport chain (ETC) [35]. Such respirasomes have been observed in many organisms, including fungi, plants and mammals [39]. The most frequent types of supercomplex assemblies are clusters of complexes I-III-IV, which have variable stoichiometries, and the dimeric or oligomeric forms of ATPase [18,40,41]. In addition, clusters comprising complex I and ATPase have been observed [42]. These supermolecular assemblies enable more efficient functioning of the respiratory complexes, leading to better substrate channelling, an increase in overall stability and a decrease in ROS formation. Recently, the *Rcf1* (human homolog *HIG2A*) and *Rcf2* genes, necessary for supercomplex assembly and stability, have been identified in yeast and shown to support the assembly and activity of the COX enzyme [43,44].

Although the complexes of the OXPHOS system are distributed over the entire IM, the majority of them are situated in the CM (Figure 1C). Nevertheless, the mobility of supercomplexes between the CM and the IBM is apparently rare due to spatial constraints and/or specific conditions related to the microcompartments. The extent that OXPHOS supercomplexes of the IBM differ in their composition and/or posttranslational modifications from those embedded in the CM can be hypothesised, in addition to predictions of whether the changes are significant enough to influence the local IBM potential and/or import of proteins or other substances into the mitochondria. In addition, the possibility of heterogeneous values of PMF within particular cristae of

single mitochondria needs to be confirmed [34].

The structure of cristae junctions is thought to support formation of a diffusion barrier between the intracristal space and the IMS; functionality of the barrier could be maintained by complex proteins such as MINOS/MitOS/MICOS, OPA1 or dynamin [45,46]. Additionally, it is clear that proteins involved in mitochondrial fusion and fission (*OPA1*, mitofilin, dynamin) and the spatial arrangement of cristae (ATPase, MINOS/MitOS/MICOS) determine not only the mitochondrial ultrastructure but also the conditions of mitochondrial homeostasis, depending on the specific metabolic needs of the cell [47-50] (Fig. 2).

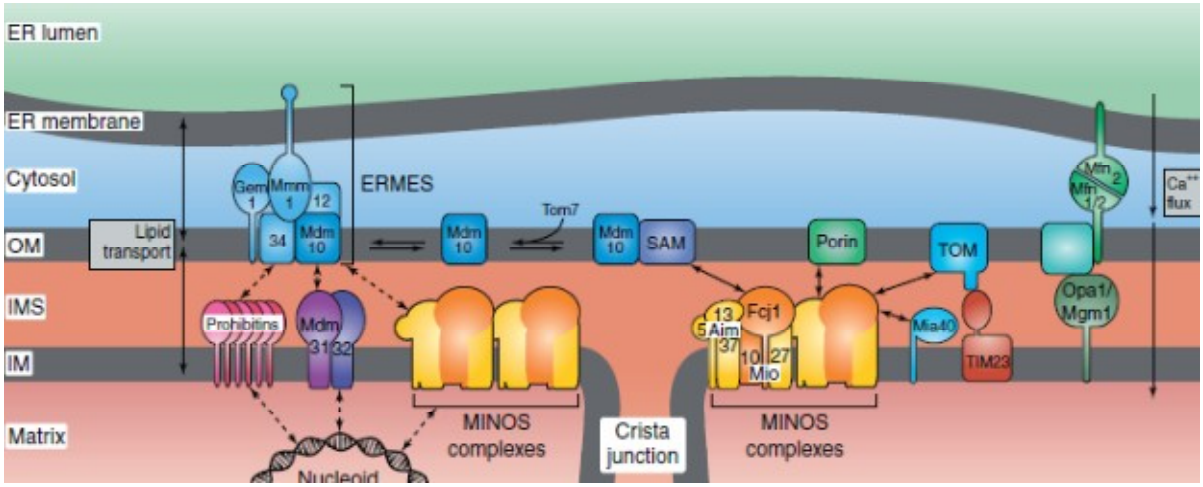

Fig. 2 Organisation of the endoplasmic reticulum (ER) – mitochondria network in yeast
The ER and the OM, as well as the OM and IM are connected by protein-protein interactions. This network of interactions promotes the transport of lipids and calcium ions between the compartments. The figure shows a schematic view of protein complexes that are involved in membrane contact sites. The ER – mitochondria organizing network comprises a branched chain of physical and genetic interactions that structurally and functionally link three intracellular membranes and the adjacent aqueous compartments from the mitochondrial matrix to the lumen of ER. Key players in this network are the membrane-bridging (ERMES) and the mitochondrial inner-membrane organizing system (MINOS) complexes. The ERMES physically links the OM with the ER membrane. The MINOS is involved in connecting the two subdomains of the IM, the IBM and the cristae. MINOS deficiency leads to detachment of cristae from the IBM and loss of cristae junctions. Additionally, MINOS components are engaged in multiple interactions with the OM [47].

The intracristal compartment plays a role in an early stage of apoptosis because it serves as a pool of cytochrome c, which is released to the IMS after cristae

remodelling. The cristae remodelling event and regulation of the cytochrome c efflux are complicated mechanisms that involve many mitochondrial proteins, such as OPA1, rhomboid PARL and prohibitin. RNAi-mediated deficiency was documented to change typical vesicle- and tubular-like cristae to onion-like structures similar to those found in *OPA1*- and prohibitin-deficient cells. Mitofilin-deficiency leads to a cytochrome c release to the IMS, loss of cristae junctions in mitochondria and fragmentation of the mitochondrial network [51-53]. Three years ago, the first mutation in nuclear gene *AIFM1*, a mitochondrial FAH-dependent NADH oxidase required for proper OXPHOS function and mitochondrial programmed cell death, was characterised [54]. AIFM1 is an interacting partner of OPA1, which is required for the assembly and stability of respiratory complexes I and IV, fusion of the mitochondrial network and sequestration of cytochrome c [55]. These data emphasise that in addition to the functions of individual mitochondrial proteins, important interactions with other molecules taking place in the mitochondria.

Despite the highly organised bioenergetic microcompartments of the ETC, the efficiency of the OXPHOS process is reduce by interactions between the electrons and inappropriate organic or inorganic electron acceptors, proton leaks, reverse electron transport, motility and solute transport [34]. The assembly of OXPHOS complexes is dependent on the membrane potential (ATPase dimerisation) and is fixed by inhibitory and regulatory factors [56]. The membrane potential also participates in the establishment of cristae shape; it is also influenced by the physical and chemical properties of the inner mitochondrial membrane, which confer an electrical charge on the membrane surface. The potential is especially influenced by the presence of negatively charged acidic groups [34].

In summary, the proper function of the bioenergetic compartments is modulated by many factors, such as the activities of energy-converting supercomplexes, physical and chemical properties of the membrane, the kinetic and electrostatic barrier formed by presence of a water layer in close proximity to the membrane, proper shape of the mitochondrial cristae decreasing proton losses, heterogeneous mosaic composition of the membrane phospholipid bilayer and variation in proteins or substrates diffusing to the membrane as a response to the demands of the cell. In addition, the maintenance of mtDNA and mitochondrial function is regulated by many other molecular factors that are responsible for mtDNA replication, transcription, signalling pathways, fusion and fission, protein assembly, biogenesis, renewal of the counter-balanced mitochondrial

nucleoside pool and transport of solutes, metabolites and proteins [34].

Mitochondrial dysfunction associated with impaired mitochondrial structure and function is increasingly reported as a primary or attendant phenomenon in the pathology of many human diseases affecting muscles, brain, liver, kidneys, vision, hearing or the cardiovascular system; for example, mitochondrial diseases, diabetes, cancer, neurodegenerative disorders, ageing and others [25,57]. As mtDNA is in close proximity to respiratory chain reactions, it is more susceptible to oxidative damage because of the lack of histones and presence of less effective DNA repair mechanisms; thus, the increased levels of ROS can induce mutagenesis in mtDNA.

3.2 OXPHOS system

The OXPHOS system is composed of organised multimeric protein enzyme centres and consists of an ATPase, F_1F_0-ATP synthase, complex V, and four electron transport chain complexes: complex I is an NADH:ubiquinone oxidoreductase; complex II is a succinate dehydrogenase; complex III is a ubiquinol cytochrome c reductase (bc1 complex) and complex IV is a cytochrome c oxidase (COX). These complexes are tightly coordinated and manage the direct connection of electron and proton transport, which results in the generation of ATP (Fig. 3) [34,39,58].

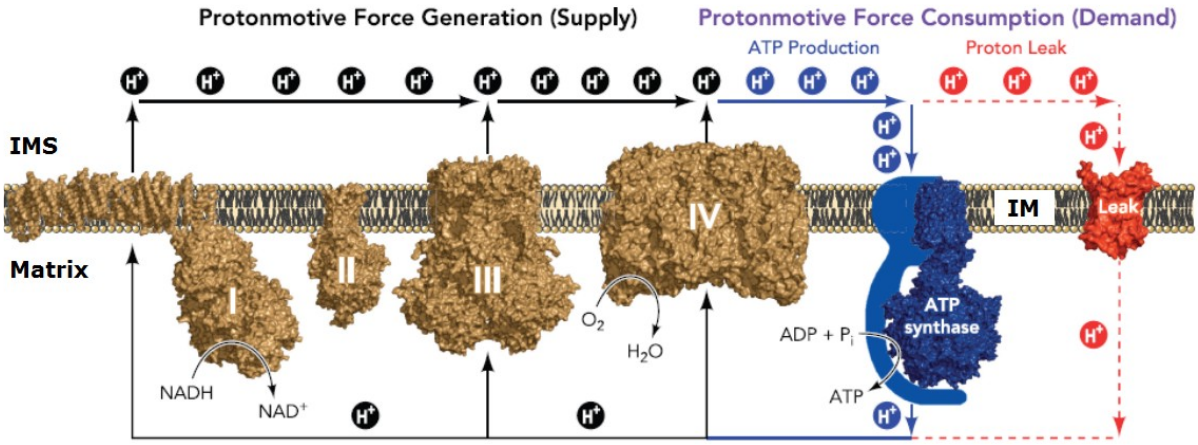

Fig. 3 Generation and consumption of the mitochondrial proton motive force

Electrons harvested from oxidizable substrates are passed through the respiratory chain in an exergonic process that drives proton pumping by respiratory complexes I, III and IV. The resulting electrochemical proton gradient across the inner membrane can be dissipated in two ways: (1) through ATPsynthase, where relieving the proton motive force drives ADP phosphorylation and 2) via proton leak pathways that do not generate ATP but regulate physiological process including nonshivering thermogenesis and perhaps glucose-stimulated

insulin secretion and protection from oxidative damage. Proton leak pathways are stucturally represented by the adenine nucleotide translocase (ANT), which can mediate both basal and inducible proton conductance [58].

The enzyme complexes of OXPHOS accept electrons from several electron carriers that are either intrinsic parts of the OXPHOS system, such as cytochrome c and ubiquinone, or extrinsic electron donors, such as FADH2 and $NADH^{+}+H^{+}$. $NADH^{+}+H^{+}$ produced in the cytosol is delivered to the mitochondria by the glycerol-phosphate and malate-aspartate shuttles. Once delivered, the reduced coenzymes proceed to either respiratory complex I ($NADH^{+}+H^{+}$) or complex II (FADH2), where the electrons are transferred to the mobile electron carrier coenzyme Q. Coenzyme Q also accepts electrons from other flavoprotein-linked dehydrogenases that are participating in the oxidation of fatty acids and branched-chain amino acids. Complex I couples the transfer of electrons from $NADH^{+}+H^{+}$ to ubiquinone with the ejection of protons from the matrix to the IMS. Complex II oxidises FADH2 to FAD to form ubiquinone and ensures the transfer of protons from the matrix to the IMS. Reduced coenzyme Q is oxidised by complex III, and the mobile carrier cytochrome c accepts the obtained electrons, which will then be transferred to complex IV. Thus, reduced ubiquinol supplies electrons from complexes I and II to complex III, which further transfers them to complex IV via the mobile carrier cytochrome c. Complex III also ensures reduction of ubiquinone into ubiquinol with an associated uptake of protons from the matrix to the IMS, as well as oxidation of ubiquinol via semiquinone to ubiquinone. Complex IV is the terminal electron acceptor and catalyses the reduction of oxygen, yielding a water molecule. Complex IV uses electrons from cytochrome c to reduce the molecular oxygen and produce water, which is also accompanied with consumption of protons. At the same time, the transport of other protons via complex IV occurs from the matrix to the IMS; this transport recovers the proton electrochemical gradient, as it is affected by ATP production. ATPase catalyses the spontaneous formation of ATP, which is subsequently released to the IMS due to conformation changes of ATPase, through utilisation of Δp_m energy provided via proton translocation to the matrix [59,60].

3.2.1 Regulation of OXPHOS system

Three OXPHOS complexes, I, III and IV, couple electron transport with proton pumping from the mitochondria matrix into the IMS. This proton transport generates an electrochemical gradient, also called the mitochondrial proton motive force (PMF,

Δp_m), and the positive charge of the IM facing the IMS. The generated Δp_m is used in ATP synthesis and in the transport of chemical agents from inside or outside the mitochondria, for example, metabolites, proteins and ions. The electrochemical gradient consists of two interdependent contributions: an electrical component $\Delta\Psi_m$ (the mitochondrial membrane potential) and a chemical component ΔpH (the pH across the IM). A hypothesis determining three zones of $\Delta\Psi_m$ has been postulated [60]. Normal $\Delta\Psi_m$ values measured in intact cells or intact organs can range from 80-160 mV. In general, the $\Delta\Psi_m$ value of maximal ATPase activity is approximately 100-120 mV, but the normal range is approximately 100-140 mV depending on the cell type. In conditions of cellular stress, when proton pumps are greatly dephosphorylated, the activity of OXPHOS complexes can be maximised to generate $\Delta\Psi_m$ values greater than 200 mV. This value has an inhibitory effect on further proton pumping and also plays a role in type II apoptosis. A $\Delta\Psi_m$ value greater than 150 mV leads to structural changes in complexes I, III and IV and inhibition of proton pumping, which hampers the excessive formation of free radicals. In addition, $\Delta\Psi_m$ is physiologically influenced by the metabolic state of a specific tissue (ATP/ADP), variable protein composition, inverse correlation to increasing age and the specific experimental conditions used during its measurement; for example, isolated mitochondria compared with intact cells [61,62].

The function of mitochondrial OXPHOS is driven by a controlled flow of electrons that are derived from nutrients or energy stores of the organism. It functions to synthesise ATP from ADP and inorganic phosphate by generating and utilising a chemiosmotic gradient of H^+ coupled with a reduction of oxygen to water. The proton gradient facilitates metabolite and proton transport across the IM. The basic principles of OXPHOS system function were proposed by Mitchell`s chemiosmotic theory, which connects electron transport and ATP synthesis with endergonic proton pumping activities of respiratory complexes. This connection is imperfect due to spontaneous proton leaks, which lower the Δp_m value, irrespective of ATPase activity. Because of the resulting Δp_m decrease, a proton leak is supposed as a protective factor against free radical formation that does not require substantial impairment of ATP synthesis [58]. The precise mechanism that ensures proton leakage is not known, but several agents are considered, including uncoupling proteins (UCPs), nucleotide transporters (ANT) and lipids of the membrane bilayer. Proton leaks are physiologically controlled at multiple levels and include biochemical (Ca^{2+} cycling, proteins, fatty acids, thyroid hormones,

cytokines, retinoid acid), transcriptional (PPARs, MyoD, SREBP-1c, Sirt1), translational (glutamine) and proteolytic events. The dissipation of Δp_m is used by UCPs to maintain thermogenesis, thus the membrane potential can be adjusted in this way [63,64]. Deficiency of UCPs has been identified in obesity, type 2 diabetes mellitus, cancer, cardiovascular diseases, pathologic immune responses related to disturbances of apoptotic signal pathways and age-related diseases caused by oxidative stress [58,65]. Other processes are proposed to affect proton leakage, for example, electron slipping in OXPHOS system when the concomitant transport of protons does not occur [39,61].

Higher values of $\Delta\Psi_m$ support the production of reactive oxygen species, induce accrual of mtDNA mutations (often multiple deletions) and accelerate ageing. Mitochondrial function usually deteriorates with age, which is associated with ETC defects [24]. However, a straightforward causal relation between ROS formation and ageing has not been indisputably confirmed [66]. Although some sources of mitochondrial ROS have been identified, many questions remain, such as the precise site of mitochondrial ROS production and the mechanisms that maintain physiological levels of ROS within the mitochondria and in close proximity to them [67]. The contribution of ROS to onset of pathological processes is a widely discussed topic [68,69].

Mechanisms regulating OXPHOS affect the availability of substrates, including $NADH^{+}+H^{+}$, ADP, and phosphate, as well as the generation of PMF. However, $NADH^{+}+H^{+}$ is the least limiting factor of OXPHOS function because other metabolic pathways can supply it. Oxygen levels are strongly limiting when tissue hypoxia occurs. The efficiency of the OXPHOS system is controlled not only by particular assembly factors but also by mitochondrial transporter proteins, uncoupler proteins and inhibitory agents and the ratio of ATP produced to oxygen consumed per number of transported electrons [59].

The proper function of the OXPHOS system is provided by PMF; supercomplex formation, such as tissue specific isoforms of particular subunits of ETC; cell signalling pathways, such as reversible phosphorylation; ATP/ADP allosteric inhibition; and regulatory mechanisms, such as posttranslational modifications, including the acetylation and oxidation of methionines and the competitive inhibition of cytochrome c oxidase by nitric oxide. cAMP and Src signalling mediate the phosphorylation of OXPHOS components, and a Ca^{2+}- dependent mechanism takes part in their dephosphorylation. Calcium is one of the most valuable signals for mitochondrial

activation. At the beginning of type II apoptosis, transitional membrane hyperpolarisation occurs repeatedly to cause higher production of free radicals, which are assumed to be major triggers of the apoptotic process. The IM depolarisation results in a lack of protons for ATP synthesis, which is needed to maintain a balanced energy state in the cell. This phenomenon indicates that the phosphorylation state of respiration complexes is the main mechanism determining OXPHOS activity, while $\Delta\Psi_m$ regulates only the function of proton pumps [60].

In addition to the OXPHOS system, which is the primary source of ATP, other metabolic pathways are able to supply cells with the molecule; these pathways include the citric acid cycle, β-oxidation of fatty acids, pyruvate oxidation and glycolysis. The key molecule of the TCA cycle is acetyl-CoA, which is supplied by oxidative decarboxylation of pyruvate and catalysed by PDH. The amino acid glutamine can serve as an alternative substrate for the TCA cycle following its conversion into glutamate and α-ketoglutarate, a TCA cycle intermediate, in the mitochondrion [70]. The TCA cycle is the final metabolic pathway for glucose, fatty acids and some amino acids, and it yields FADH2 and $NADH^{+}+H^{+}$ that can be processed by the OXPHOS system. Increased mitochondrial levels of Ca^{2+} stimulates TCA cycle dehydrogenases and activity of PDH and ATPase. The levels of mitochondrial and cytoplasmic Ca^{2+} are regulated by many factors, including hormones, growth factors and electrical signals, and they are maintained by Ca^{2+} protein transporters [59,70].

3.3 Mitochondrial disorders

The combined incidence of inherited metabolic disorders is more than 1 in 500 live births, and mitochondrial disorders are among the most abundant of these [71]. In a strict sense, mitochondrial disorders are caused by defects of genes encoding the components directly involved in ATP synthesis and the electron transport chain, medically known as a primary deficiency of the affected enzyme complex. Causal genetic disturbances in any other genes involved in the maintenance of mitochondrial homeostasis are termed secondary mitochondrial disorders or secondary respiratory chain dysfunctions [72]. Isolated and combined defects of respiratory chain enzymes are also signs of primary mitochondrial disorders [73,74]. The genetic basis of mitochondrial disorders is still unexplained in a large number of patients despite advances in applied research methodology [3]. To date, approximately 1500 proteins have been identified as potential causes of mitochondrial disorders; pathologic

mutations were identified in approximately 300 of the corresponding mitochondrial and nuclear encoded genes [75,76].

3.3.1 Clinical presentation of mitochondrial disease

Mitochondrial disease can present at any age, affect any tissue and manifest any symptom [77]. Mitochondrial disorders predominantly affect tissues with higher energy demands, such as the heart, skeletal muscles and central nervous system, although other organs are often affected, for example, the retinas, kidneys, liver, gastrointestinal tract, bone marrow, and endocrine system [72,78-80]. Neuromuscular manifestation is the most common and typical clinical sign of mitochondrial disorders [30,81-83]. Manifested clinical symptoms and commonly affected organs in patients with mitochondrial disorders are summarised in Table 1. These symptoms and organs show that mitochondrial disorders represent a very heterogeneous group that presents poor phenotype-genotype correlation, which is often observed even in patients within the same family (*POLG1*, *OPA1*) [31,84,85]. One mutation may present a variable spectrum of symptoms and, conversely, the same signs of a particular mitochondrial disorder can be caused by mutations in diverse genes, including *TYMP*, *RRM2B*, *MT-TL1*, *MTTV* and *POLG1* [86-89]. For this reason, clinical symptoms and findings of metabolic and functional assays must be considered when a mitochondrial disorder is suspected [90].

Both nuclear and mitochondrial genetic backgrounds contribute to the phenotype of patients suffering from a mitochondrial disorder [91-93]. Thus, many genetic factors can impair diagnosis of mitochondrial disorders; for example, the tissue specificity of a disorder (tissue specific expression of genes, somatic mosaicism), variability in gene copy number affecting the genomic neighbourhood, identification of sequence variants with unclear effects on gene expression and/or function, potential roles of epigenetic factors, possibility of variable penetrance, expression of a disease-gene being caused by modifier genes or a rarely occurring reversible disease phenotype. At present, the resulting clinical effect of these genetic factors is difficult to determine by routine diagnostic procedures [94] and suggests why the diagnosis of mitochondrial disorders is challenging. However, several investigation flowcharts regarding the clinical and laboratory diagnosis of mitochondrial disorders have been proposed [24,95-99], although none are used as a general compulsory guideline. Despite these difficulties,

some pieces of basic information regarding the management, diagnosis and therapy of mitochondrial disorders are available online at www.mitosoc.org [100].

Table 1 The most common clinical symptoms of mitochondrial disorders

Syndromes and symptoms of patients with mitochondrial diseases adapted from [24,72,101]

Red-flag findings
Neurologic: stroke-like episodes, encephalopathy (recurrent or with low/moderate dosing valproate), epilepsia partialis continua, myoclonus, MRI findindings consistent with Leigh disease, characteristic MRS peaks (lactate, succinate)
Cardiovascular: hypertophic cardiomyopathy, dilated cardiomyopathy with muscle weakness, Wolf-Parkinson-White syndrome
Opthalmologic: pigmentary retinal degeneration, opthalmoplegia, ptosis, sudden- or insidious-onset optic neuropathy/atrophy
Gastroenterologic: unexplained or valproate-induced liver failure, severe dysmotility, pseudo-obstructive episodes
Laboratory findings: 3-methylglutaconic aciduria (mainly with neutropenia and hyperammonemia), lactic aciduria, excretion of Krebs cycle intermediates, level of lactic acid in blood/CSF, level of serum and CSF alanine
Others: a newborn/infant/young child with unexplained hypotonia/weakness/failure to thrive, myopathy, exercise intolerance
Nonspecific findings
Constitutional: short stature, intrauterine growth retardation, microcephaly, muscle wasting, brain malformation
Neurologic: hypotonia, infantile spasms, intractable epilepsy, unexplained movement disorder, ataxia, hearing loss (sensorineural), axonal neuropathy, status epilepticus with an additional red-flag or nonspecific feature, episodic coma, dystonia, pyramidal signs, hemiparesis, chorea, spasticity
Opthalmologic: cataract
Gastroenterologic: chronic or cyclic vomiting
Dermatologic: symmetric lipomatosis, hypertrichosis
Endocrine: diabetes mellitus, hypothyreoidism
Imaging: unexplained central nervous system atrophy (cerebral, cerebellar), unexplained leukodystrophy
Family history: multigenerational maternal inheritance pattern of migraine headaches/depression/anxiety disorder
Others: congenital nephrotic syndrome, renal tubulopathy, pancytopenia or failure of specific blood cell lines (sideroblastic anemia)

3.3.2 Causative mutations, inheritance, prevalence

Mitochondrial disease can be classified according to several criteria regarding the phenotypic, genetic, functional and/or diagnostic properties. Considering functional and genetic information, primary and secondary mitochondrial diseases can be grouped as follows [16,22,28,102]:

- ♣ Mutations in mtDNA genes encoding:
  - structural subunits of the OXPHOS system
  - mitochondrial tRNAs and rRNAs
- ♣ Mutations in nuclear genes encoding:
  - proteins important for formation of the inner mitochondrial membrane lipid milieu, genetic factors driving the maintenance (disorders of intergenomic communication) and expression (dNTP pool maintenance, replication, transcription, translation) of mtDNA, mitochondrial proteins with functions indirectly linked to OXPHOS, genetic factors responsible for mitochondrial protein import, synthesis and metabolism (mitochondrial proteases etc.) and for mitochondrial dynamics (fusion, fission, mobility)
  - structural subunits, assembly and/or ancillary factors of the OXPHOS system, enzymes of metabolic pathways and other genetic or signalling factors that have effects inside the mitochondria and/or indirectly influence OXPHOS function

Because of the dual genetic control of mitochondria, the dysfunction of mitochondrial processes can be caused by mutations in mtDNA or the nuclear genome. These possibilities imply that the inheritance of mitochondrial disorders can be either maternal, at the level of mtDNA, or Mendelian, at the level of nuclear genes presenting as autosomal recessive, autosomal dominant or X-linked inborn mutations. Disorders of the OXPHOS system, including both primary mitochondrial and nuclear defects, are considered to occur at an incidence rate of 1 in 5000 live births [103]. In paediatric patients, mtDNA mutations give rise to only 10-25% of defects of the OXPHOS system [82,104]. As opposed to mutation in mtDNA, mutations of nuclear genes are more frequent, typically fatal and can occasionally lead to physical abnormalities [83,105,106]. OXPHOS defects caused by mutations in nuclear genes usually present autosomal recessive inheritance [98,107]. The first mutation in a nuclear encoded gene (*ANT1)* that severely deteriorated mitochondrial function was characterised in 2000 [108].

Epidemiological studies are absolutely dependent on the accurate diagnosis and classification of findings. Currently, canonical criteria applicable to the diagnosis of mitochondrial disease combine both laboratory and clinical findings, but their overriding shortcoming lies in the scarce application of clinical studies, as their

diagnostic trustworthiness cannot be determined [104,109]. Additionally, the concluding prevalence data could be influenced by the disunited application of diagnostic criteria for primary mitochondrial disease in adults and children, especially when studies have enrolled individuals without clearly identified pathogenic mutations [81,82,110]. This limitation means that some patients present with few discernible symptoms, so that the representative clinical criteria of mitochondrial disorders are not fulfilled thoroughly, leading to doubt about the primary cause of their disease [99,104,109]. For these reasons, well-designed longitudinal studies monitoring the health status of patients with suggested mitochondrial disease are needed to show the specificity of the criteria for primary mitochondrial disorders, as illustrated by Morava and co-workers [109].

3.3.3 mtDNA focus

Until recently, the prevalence of mtDNA mutations was difficult to estimate because of clinically asymptomatic carriers in the worldwide population. Based on recent findings, the de novo mtDNA mutation rate, determined by the detection of ten frequent mtDNA pathogenic mutations in the general population, was assessed to be 1:1000 in neonates. At least one in 200 healthy individuals of the background population carries a pathogenic mtDNA mutation that may potentially cause disease in the offspring of female carriers [111]. This finding supports the results of another recent study, which used a next-generation parallel sequencing method to detect a very low level of heteroplasmy in DNA samples from both blood and skeletal muscle in all tested healthy individuals [40]. These studies are important because further clinical monitoring of the investigated individuals would make the known prevalence of mitochondrial disorders more precise. In addition, the effect of mtDNA mutations may be modulated by frequent and closely related mtDNA polymorphisms (haplogroups) whose precise role in mitopathy has not been conclusively explained [24,111-113]. Over 100 pathogenic point mutations and 200 rearrangements (deletions and/or insertions) of mtDNA have been characterised since the first mtDNA mutations were described in 1988 [72]. The prevalence of mtDNA mutations causing a disease phenotype was suggested to be approximately 1:10900-14300 in adults carrying mutations in mitochondrial genes encoding mitochondrial tRNAs in approximately 50% of cases [112,114-116].

The maternal mode of inheritance is characterised by transmission of a mutation

from an affected mother to her children and not from affected fathers to their children. The second typical feature of maternal inheritance is a mitochondrial bottleneck, which explains the random shift of mutational load that occurs in the course of segregation of mtDNA copies during oocyte development, in foetal tissues and/or anytime in adulthood. By this mechanism, an asymptomatic mother with low heteroplasmy levels could give birth to a clinically affected child with a higher copy number of mutated mtDNA molecules. The mitochondrial bottleneck theory clarifies the probable mechanism of a mutation threshold, as well as tissue specificity that can be associated with mutations in mtDNA. The mutation threshold depends on the type of mutation, for example mtDNA rearrangements or point mutations [31]. Large-scale mtDNA deletions are usually sporadic and invariably heteroplasmic, but they can also be secondary with respect to a primary nuclear gene defect. In contrast, mtDNA point mutations are usually maternally inherited and may be homoplasmic or heteroplasmic [30]. Nonetheless, the majority of mtDNA mutations are heteroplasmic [117]. The disease-indicative level of mutated mtDNA is predicted to be approximately 60-90% [20], but heteroplasmic mtDNA mutations may also present an unusual dominant disease phenotype at 25% of the mutation load in affected tissues [118]. Additionally, a higher mtDNA mutation load corresponds to a more severe phenotype of adversely affected tissue. This correlation explains why heteroplasmic mtDNA mutations confer more heterogeneous clinical manifestations than homoplasmic mutations [30,119,120]. Thus, assessment of the proportion of mutated mtDNA and characterisation of mtDNA deletion breakpoints is highly desirable [20,75]. Pathogenicity criteria for homoplasmic and heteroplasmic mtDNA mutations have been proposed by several authors [20,119,121,122] and should be considered to avoid incorrect assessment of SNPs, as illustrated by the mitochondrial variant m.8296A>G [123,124].

The effect of mtDNA mutations may be diluted or even disappear as a growing number of cultured cell passages are used for diagnosis of mitochondrial disorders, as substantiated in fibroblasts [106]. Mitochondrial defects can sometimes be absent in patient muscle tissue samples or in cultured cells (fibroblasts, muscle) despite a proven mutation and frequent involvement of the specific tissue in disease pathology; thus, absence cannot rule out a primary mitochondrial deficiency as in some cases the examined tissue could not have an OXPHOS defect [75,83,98,125]. Therefore, both genetic factors and the patient phenotype should be considered if pathogenicity of any identified mitochondrial and/or nuclear sequence variant is suggested [106].

3.3.3.1 Genetic counselling

Several issues affect the genetic counselling of families with mitochondrial disorders. Specifically, the mutation load of mtDNA present by amniocytes or chorionic villi can be completely different from that in other foetal tissues and may be startlingly variable due to random mitotic segregation [49,119]. Consequently, routine genetic counselling of mitochondrial disorders is currently restricted to known nuclear gene defects and/or clearly quantifiable OXPHOS deficiency in examined tissue samples because pathologic mutations could be excluded from mtDNA [126,127]. This scenario implies that conventional prenatal diagnosis (PND) relating to the transmission of mtDNA mutations from an affected mother to her progeny is problematic and primarily designated for *de novo* mtDNA disease or carriers with a low mtDNA mutation load. However, it would be highly effective to perform preimplantation genetic diagnosis (PGD), a contemporary laboratory technique, in carriers with a higher mtDNA mutation load because in many cases they are able to ensure the birth of healthy offspring [41,45]. Several pioneering case studies with successful preimplantation diagnosis have been published [46,128-130]. In a recent study, an affected mother with low mutation heteroplasmy in her muscle that was absent in her blood had a very low probability of passing the disease phenotype to her child [130]. Additionally, an asymptomatic child was born after a preimplantation genetic diagnosis confirmed a low mtDNA mutation load of m.3243A>G in the blastocyst [129].

However, PND and PGD methods cannot be used to prevent the transmission of homoplasmic mutations [35]. This limitation could be overcome by methodologies using the replacement of mutated mtDNA with wild-type mtDNA, such as germinal vesicle transfer, metaphase chromosome transfer, pronuclear transfer and ooplasmic transfer. Hopefully, further research exploring laboratory methods to prevent the spread of mitochondrial disease will show new trends and refinements for the preimplantation procedure, as well as for genetic counselling [42].

3.4 Laboratory diagnostics of mitochondrial disorders

The close interdisciplinary collaboration of medical specialists is needed to correctly diagnose mitochondrial dysfunction. Therefore, knowledge of family history, clinical evaluation of manifested symptoms and careful consideration of laboratory results is vital [24]. If they are available, it is important that laboratory assays are performed on affected tissue samples [127]. The outcomes of these assays can confirm

suspicion of mitochondrial aetiology of disease, if deficiency of one or more enzymes of the energy transport system is detected. Notably, a combined dysfunction of the OXPHOS system is more common [131]. If the affected tissue samples are not available or reduced efficiency of the OXPHOS system is not accompanied by a specific enzyme defect, mitochondrial disorder cannot be completely excluded. However, findings can be distorted by the poor clinical status of a patient, mtDNA depletion (cut off: <50% of age matched control) or omission of a secondary mitochondrial dysfunction because of other types of primary disorder, such as muscular dystrophy [75,101].

None of the laboratory investigations alone can provide results to inquiring clinicians because particular biochemical markers, e.g., lactate, can be normal or borderline in patient samples and can fluctuate noticeably during metabolic crises. Conceivably, wrong conclusions can be drawn from any borderline values, especially when appraising low or mildly reduced activities of respiratory enzymes [127,132,133]. This is an unfortunate implication of the clinical heterogeneity of mitochondrial diseases.

3.4.1 Basis of biochemical and follow-up investigations

Using fresh (preferably) or frozen bioptic patient tissues, including muscle and liver, or cultured cells such as fibroblasts and myoblasts, biochemical methods are used to determine the function of the OXPHOS system or the rate of oxidation of specific substrates (malate, pyruvate, glutamate, succinate, ascorbate, TMPD), ATP production and oxygen consumption, as well as the activity of individual respiration complexes of the OXPHOS system. Functional assays of PDH, enzymes of the TCA cycle and β–oxidation, coenzymes and transmembrane carriers can be helpful to identify the type of mitochondrial dysfunction [98,101]. Notably, measurements of respiratory enzyme activities performed on frozen tissue samples should be assessed with caution [134]. Total cellular ATP content is a very poor marker of mitochondrial dysfunction; thus, determining the cause of variable ATP levels requires further experiments [61].

The key metabolic hallmarks of mitochondrial disorders are elevation of lactate (blood/serum, CSF or urine) and pyruvate and low or high levels of acetone, ketones and the lactate/pyruvate ratio, as well as other metabolites [72]. Lactate acidosis, originating from $NADH^{+}+H^{+}$ accumulation, and less effective pyruvate utilisation cause overall fluctuations in cellular pH, which can increase the production of ketone bodies due to changes in the lactate/pyruvate and $NADH^{+}/NAD^{+}$ ratios, as well as alterations in

the concentration of TCA cycle intermediates. Such metabolic disturbances support the conversion of pyruvate to alanine, mediated by alanine aminotransferase, whose elevated levels are frequently detected in patients with defects in the OXPHOS system [23,135,136]. Mitochondrial disease can also be indicated by worsened β-oxidation, which is frequently signalled by an increase in the plasma acylcarnitine level [24].

In a few patients with mitochondrial disorders, increased concentrations of lactate could affect the oxidation of proline, leading to its elevation [137]. Glycine and sarcosine may also be increased in mitochondrial dysfunction [98]. Redox imbalance has been detected in the white blood cells of patients with mitochondrial disease and organic acidaemias manifesting hypocitrullinaemia and glutathione deficiency [138]. Moreover, diabetes is a common feature in adult patients with OXPHOS disorders and reflects impaired glucose signalling and cellular ATP homeostasis including GLUT1, phosphofructokinase, pyruvate kinase, ATP/AMP ratio or AMP-activated protein kinases [70]. Disturbances of the creatine-phosphocreatine-creatine kinase system, which functions in intracellular ATP homeostasis, can accompany mitochondrial deficiency when muscle or brain tissues are affected [139].

All the biomarkers mentioned, including lactate and pyruvate, should be considered simply as signs of respiratory chain dysfunctions that lack significant sensitivity and specificity to primary or secondary mitochondrial diseases [21,101,133]. For mitochondrial dysfunctions, the relative ratios of enzyme activities and/or biochemical analyses can be informative, for example, the ratios of lactate/pyruvate, alanine/lysine, alanine/phenylalanine + tyrosine, miscellaneous organic acids/creatinine, proportion of certain acyl-carnitine esters, some neurometabolites/creatine and phosphocreatine/inorganic phosphate [75,98]. Thus, altered concentration values of additional mitochondria-related biomarkers strengthen the diagnosis of mitochondrial disorders, although attention must be paid to the selection of compared markers, as they do not have to correlate at all, as exemplified by blood lactate/plasma creatine or respiratory chain activities/plasma creatine [139].

Proton circuit (PMF) and respiration rate measurements are the most employed techniques used to monitor mitochondrial function in isolated mitochondria and in intact cells. $\Delta\Psi_m$ comprises the majority of PMF, and its absolute value can be monitored as a change in the distribution of membrane-permeable cations with the use of isolated mitochondria. The measurement of relative $\Delta\Psi_m$ values is easier because of the use of living cells and voltage-dependent fluorescent probes accumulating in mitochondria

[61]. As documented, $\Delta\Psi_m$ depends on the activity of charged or electroneutral chemical substances transported across the IM [60]. The detection of $\Delta\Psi_m$ provides additional information on mitochondrial function and structure, as well as on the metabolic state of an intact cell, which is commonly monitored by fluorescent probes such as TMRM, TMRE and R123. Additionally, this fluorescent technique also enables detection of $\Delta\Psi_m$ generated by particular respiratory chain complexes [61,76,140].

The activity of respiratory enzymes is routinely measured by spectrophotometric analyses that usually use citrate synthase (CS) as an internal control of mitochondrial mass [141]. The activity of OXPHOS enzymes is usually indicated as a percentage of the mean control value of a particular respiratory complex/CS. Major and minor criteria that can be used to determine the severity of OXPHOS deficiency have been published [142]. Another approach uses a polarographic assay to measure the ability to oxidise radioactively labelled substrates; however, monitoring of the cellular energy state is possible via additional modalities [61,98,143]. Without the need for mitochondria isolation from tissue, the activity of COX and complex I can be detected by enzyme immunocapture assays using human cultured fibroblasts, whole blood or cheek swabs, which requires only very small sample size [144,145].

The measured activities and assembly profiles of respiratory complexes can be misrepresented due to compensatory mechanisms that can hide the OXPHOS deficiency, as shown in a patient with Kearns Sayre syndrome and in some patients with autosomal dominant optic atrophy [146,147]. This compensatory effect may be indicated via increased activity of CS, but some of the compensatory mechanisms do not conform to this rule [148]. Additionally, the normalisation of respiratory enzyme activities to the total protein content allows the identification of patients with mtDNA depletion [149]. In addition, most tissue-specific spectrophotometric OXPHOS assays provide resultant activity values that cannot be compared inter-laboratory easily due to the lack of standardised physiological ranges and obligatory guidelines [127,142].

Recently, several multi-centre studies investigating standardisation processes and/or the conditions of spectrophotometric OXPHOS activity assays have been published [132,150,151], and the necessity of a standardised protocol with regard to uniform costs, laboratory equipment, reagents and quality control management is being debated. Although some contradictory statements relating to the significance of changeable analytical factors (buffer composition, sample handling) persist, establishing a routine protocol would ensure collection of homogeneous and cohesive data that

would be advantageous for the meta-analysis of rarely occurring cases, as well as for updating the list of diagnostic criteria for mitochondrial disorders. In addition, the reproducibility of such a unified protocol would be improved. Consequently, several findings of these recent studies could be applied to routine laboratory procedures, specifically regarding the appraisal of borderline respiratory chain activities in clinically suspicious patients, normalisation of measured respiratory enzyme activities, inclusion of proprietary reference samples in inter-laboratory studies, ensuring the linearity of assays and the buffer types used [132,149-151].

Laboratories diagnosing mitochondrial disorders currently have to collect their own set of positive and negative control samples, which ideally should be the same age as the patients. Thus, many research groups have made immense efforts to find infallible and widely applicable markers of mitochondrial defects. One candidate is FGF21, an endocrine-acting metabolic hormone that has been identified as a significant marker for primary neuromuscular respiratory chain deficiencies in adults and children [152,153]. However, the sensitivity and specificity of FGF21 for various mitochondrial disorders should be tested in an another large-scale study that include patients with different phenotypes and genotypes because FGF21 has a broad range of physiological roles in the human body, and its elevated serum levels have been observed in other diseases [154-157]. Such an FGF21 study is likely to be swiftly accompanied by certified, commercially affordable ELISA kits. Another promising approach is the metabolic profiling of affected tissues derived from genetically modified animals or media from cultured cell lines [133,158]. By this methodology, a retrospective study of commonly measured blood metabolites revealed potentially significant markers of primary mitochondrial respiratory chain disease, including ratios of branched amino acids/glutamate, glycine/glutamate and alanine/glutamate, but these data have not yet been validated in further studies [159]. Thus, identification of a steady “housekeeping” biomarker closely linked to mitochondrial energy metabolism, whose values would be comparable to presently known biomarkers, could be another approach. For instance, higher levels of alanine and glutamate were detected in one patient carrying causal mutations in the *AARS2* gene [13].

With the use of electrophoretic techniques, analysis of the respiratory chain assembly profile can be performed by immunoblotting coupled with SDS-PAGE, BN-PAGE or clear-native PAGE; certain mitochondrial disorders can display a representative assembly pattern [160,161]. However, this approach requires verification

of a large group of patients with the same type of mutation. Defective mitochondrial protein synthesis can be observed with the use of radioactive labelling (^{35}S-methionine) of mitochondrial translation products in cultured cells, which are subsequently visualised on pulse-chase SDS-PAGE gradient gels [162,163].

Regarding specific tissue defects, the performance of further assays can be required; for example, MRI (brain, muscle), EEG, ECG, EMG, assessment of the retina, optic nerve or hearing (BAEP or VEP), exercise tests, liver function tests, tests for diabetes mellitus, measurements of the dNTP pool and membrane potential and evaluation of the urinary profile of organic acids [24,72,75,164]. Other modalities can also be used to determine the basis of mitochondrial dysfunction [98].

3.4.2 Genetic analyses

Although the outcomes of biochemical investigation can reveal the probable causes of mitochondrial disorders, further analyses are required to identify causative mutations and to confirm the effects of the specific mutations. If the symptoms present in an affected patient are characteristic, Sanger sequencing or PCR-RFLP and ASO analysis can be used to perform targeted molecular analyses of specific nuclear genes (white blood cell DNA) or the most frequent large deletions and point mutations in mitochondrial genes (white blood cell DNA and/or affected tissue). The Sanger sequencing approach has flaws involving the inappropriate detection of large deletions and mutation load (cut off: 20%), as summarised elsewhere [75]. Excluding a few recognisable mitochondrial phenotypes, clinical and laboratory data are frequently not satisfactory enough to identify causative genes and only provide insight into the most likely location of genetic defects [121]. There are several more accurate screening methods that can be used to consider a broad spectrum of genes: ARMS, SSCP, DGGE, TGGE, TTGE, dHPLC, ASO hybridisation method, DNA arrays, HRM analysis and targeted exome sequencing [165,166]. Quantitative Southern blot analysis or real-time quantitative PCR can be used to assess the amount of mitochondrial DNA in muscle or liver samples if the expression of nuclear genes, mitochondrial depletion or content of mutated mtDNA (homoplasmy, heteroplasmy) are being analysed [31,167,168]. In addition, large-scale mtDNA rearrangements can be identified by long-range PCR [169-171]. Some of these methods have been superseded, but they can still be useful in routine mutation testing, especially because advanced technologies are only available in specialist research centres at present.

If clear results are not obtained from the fundamental screening approaches, DNA arrays and next-generation sequencing (NGS) are frequently used to investigate genes and their expression levels. DNA arrays are principally based on the findings of many genome-wide association studies [172-174]. MitoCarta is a list of over one thousand candidate genes that are localised to mitochondria and have recorded defects that induce mitochondrial dysfunction [175]. However, this list should be expanded for use in targeted NGS analysis because novel genes involved in mitochondrial biology are still being characterised. NGS technology is advantageous to Sanger sequencing because a small sample can be used for the fine detection of mutation load, and the method allows the analysis of a large quantity of genes; however, the employment of NGS technologies (whole exome or genome sequencing) cannot guarantee identification of causal mutations because of evident limitations [15]. Genetic factors such as the type of inheritance, occurrence of de novo mutations and/or cause of tissue-specific phenotype manifestations must be considered [173,176,177]. This requirement implies that there are many additional genetic factors, such as SNPs, CNVs and new sequence variants with unclear significance, which are capable of modifying the effect of causative mutations and generating huge phenotypic variability among afflicted patients, which hinders diagnosis and genetic counselling. Studies using CNV have clearly shown that CNVs are significant genetic factors that can affect the apparent phenotype of every individual. Typically, CNVs are structural variants of human DNA of at least 1 kb or greater in length [178,179].

The significance of any new mutation or a mutation located in a newly characterised gene should be proved in a cell model system such as patient-derived fibroblasts or myoblasts, using methodologies that include cybrids, viral complementation or bacterial systems with inserted plasmid vectors. Complementation techniques using patients cells with viral vectors and/or plasmids expressing the wild-type candidate gene are of special relevance to confirm the effect of newly identified mutations or suspected sequence variants [57,106]. Transmitochondrial cybrids are experimental model cells that enable the study of mtDNA sequence variants. Cybrids are constructed by combining the mtDNA of interest with mtDNA-less rh^0 cells; the disease phenotype can show improvement after complementation with wild-type RNAs. However, this complex combination of polymorphic mtDNA and its twofold genetic control can cloud the analysis due to changeable penetrance of homoplasmic mutations, threshold effects of heteroplasmic mutations or differences in the global genetic

background of the cell; these factors can modulate the effect of a pathogenic mutation by compensatory and/or epigenetic mechanisms [30,121,180]. Thus, genetic counselling for mtDNA diseases is still difficult despite several guidelines for the diagnosis of mitochondrial disorders.

3.4.3 In silico methods

As the interpretation of laboratory findings and clinical symptoms remains ambiguous, functional studies using computational methods, such as evolutionary comparison, population databases, and structural modelling, can reveal the effect of suspected sequence variants [181,182]. However, these approaches are not sufficient to overcome our incomplete knowledge of mitochondrial physiology and pathology [24].

Briefly, *in silico* tools represent free online software that can predict the effect of identified mitochondrial or nuclear sequence variants and score them as neutral, possibly pathogenic or pathogenic [183]. A large quantity of software packages enable structural 3D-analysis of unknown protein sequences of interest; however, a characterised crystal structure of the wild-type protein and/or of a protein derived from the same family with high sequence identity are needed for this type of analysis [184].

3.4.4 Imaging methods

Skin and muscle biopsies, as well as (less frequently) liver biopsies, are widely used as samples for morphological evaluation and/or for monitoring of specific protein expression [78]. Light or electron microscopy techniques typically show pathologic mitochondrial ultrastructure, which manifests as changes in the number, shape and size of mitochondria (enlarged, swollen, onion-like with reduced cristae), the presence of pathologic inclusions or as a disintegrated mitochondrial network in affected cells, especially in muscles (Fig. 4). These morphological ultrastructural pathologies are general characteristics of mitochondrial disorders rather than specific indicators of particular types of mitochondrial dysfunction [83,185,186].

In preparations for light microscopy, subsarcolemmal accumulation of abnormal mitochondria may be seen as red granular deposits called “ragged red fibres” (RRFs) with the use of histological staining of muscle cells (Gomori trichrome), where activity of cytochrome c oxidase may be decreased. RRFs are markers of faulty protein synthesis that results in variable muscle fibre atrophy [30,186]. However, RRFs are found in only a small minority of children with respiratory chain defects, where they can be detected as a mild subsarcolemmal increase of proliferating mitochondria

[75,98]. RRFs can also be observed in individuals without mitochondrial disease, particularly in association with ageing and other types of muscle disorders [98,187].

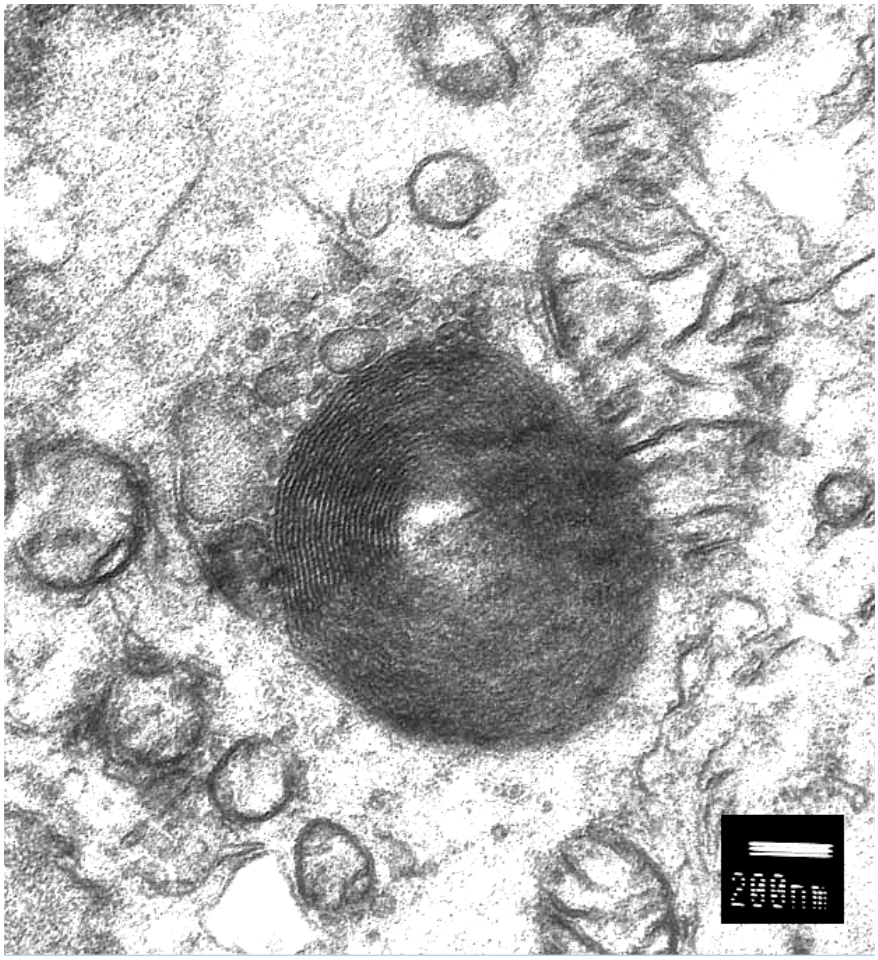

Fig. 4 Ultrastructure of mitochondria

Electron microscopy of mitochondria was performed on patient-cultured myoblasts. An onion-like mitochondrion with concentric cristae, which is located in the centre of the picture, is surrounded by a set of normal patient mitochondria in the section. The picture was kindly provided by RNDr. Jana Sládková, CSc.

If Gomori staining is combined with succinate dehydrogenase (ragged-blue fibres) and COX staining, the resulting abnormal fibres are specific to mitochondrial defects if more than 0,5% of abnormal fibres are coupled with the absence of another primary neuromuscular disease. However, the presence of only a small proportion of RRFs and COX-deficient fibres does not exclude the mitochondrial aetiology of a disease [187]. Accordingly, muscle biopsy is widely used in microscopic studies as the

gold standard procedure to differentiate mitochondrial dysfunction from other neuromuscular disorders [185].

3.5 Cytochrome c oxidase: structure, function, biogenesis and regulation

The function, biogenesis and regulation of COX have been explored in more detail than the other OXPHOS complexes. Knowledge of human COX structure is based on studies performed in model organisms such as bacteria, mammals and, especially, yeasts. Accordingly, the nomenclature of human orthologous COX subunits reflects the designation of COX subunits in yeast, where they were initially identified [3,188,189].

Recently, *NDUFA4* was revised and suggested to be the 14th COX structural subunit, with a role in COX enzyme function and biogenesis and whose mutations cause COX deficiency; however, its exact role and location in the COX structure remain to be elucidated [2,11,65]. Mammalian cytochrome c oxidase is composed of 14 subunits including 2 haems, cytochrome aa_3 and 2 copper centres, which become 3 redox centres: CuA, haem a and haem a_3-CuB. The enzyme catalytic core is formed by 3 mtDNA-encoded subunits, COXI, COXII and COXIII. Subunit I binds the haem a and a_3 prosthetic groups to form the CuB redox centre, which is close to the cytochrome c binding site. Subunit II binds the CuA centre. Subunit III provides electron transport and proton pumping [59,190,191]. In COX, the key role of ligands is coupled with either histidine residues of COXI or with an amino acid sequence of two cysteine-methionine and glutamate residues located in COXII. Seven nuclear-encoded subunits are transmembrane proteins that interact with other nuclear or mitochondrial subunits. Three peripheral subunits are present on the matrix side or on the IMS side [65]. The remaining 11 characterised nuclear encoded structural subunits and their isoforms (COX4I1, COX4I2, COX5A, COX5B, COX6A1, COX6A2, COX6B1, COX6B2, COX6C, COX7A1, COX7A2, COX7B, COX7C, COX8A, NDUFA4) are thought to be responsible for the stability, regulation and/or tissue-specific activity of the mammalian COX enzyme and for protection of the catalytic core subunits from oxidative damage, although their precise function is not yet determined [11,188]. Each nuclear gene encoding a COX subunit and/or specific isoform is located in a different chromosomal location. In addition, there are accessory proteins that take part in COX assembly and biogenesis [192-194].

The regulation of COX activity is driven by the ratio of several nuclear-encoded tissue-specific COX isoforms (COX4, COX6A, COX6B, COX7A) and by the stage of tissue development, which is accompanied with switches in the expression of COX isoforms. The remaining subunits are expressed ubiquitously. Expression of muscle isoforms COX6AH and COX7AH is regulated by muscle-specific elements e.g., MyoD. Expression of L-isoforms is primarily regulated at the posttranscriptional level. In general, a liver-type COX present in tissues with a lower mitochondrial density (liver, brain) shows a higher activity, whereas a heart-type COX (heart and skeletal muscle) has a high mitochondrial capacity [60]. For example, lung COX has approximately 2.5 times more activity than liver COX, which results in increased electron flux through the ETC and fewer free electrons accessible for ROS generation. The expression of tissue specific isoforms is variable and dependent on the degree of tissue maturation, which is dissimilar in foetuses and adults. Many promoters of the COX genes contain binding site for the SP1, NRF2 and NRF2 regulatory proteins, which together with thyroid and adrenal steroid hormones further modulate COX biogenesis [59].

The COX complex is active as a dimer, with the COX6A and COX6B subunits linking the two monomers. The stability of the COX dimer is also provided by the adenine nucleotides located between COX6AH and subunit I. Thus, interactions with the COX6AH subunit are influenced by ATP [60]. Electrons are carried to the COX complex by reduced cytochrome c, which binds at the IMS side of the enzyme involving residues of subunits II, VIA and VIB [59]. The electrons are transferred via the CuA centre to the haem a and subsequently to the a_3-CuB centre, where the reduction of oxygen occurs. In a monomeric and dimeric state, COX is able to perform transfer of electrons and reduction of oxygen; however, the dimeric form alone is sufficient for proton pumping. The effect of slipping protons was documented in COX, when the $\Delta\Psi_m$, ΔpH or ATP/ADP ratio was increased. Notably, the variability in H^+/e^- stoichiometry was not present in complex III, even in a wide range of measured $\Delta\Psi_m$ values. In contrast, the efficiency of proton pumping in COX decreases with higher values of Δp_m and/or $\Delta\Psi_m$ and fully disappears at maximal rates of electron transport (at high ΔpH) [65].

The phosphorylation of individual OXPHOS components (COXI, COXII, COX4, COX5A, COX5B, COX6A, cytochrome c and the β subunit of ATPase) represents a mechanism that changes the activity of affected molecules. Moreover, phosphorylated sites may be tissue-specific as phosphorylation of cytochrome c oxidase

is specific to liver and heart [60,195]. Active phosphorylated COX permits its allosteric regulation through ATP by its binding to subunits COX4 and COX6A. Thus, the dephosphorylation of COX regulates respiration via the ATP/ADP ratio. In the mitochondria and/or in the cytosol, the balanced state of ATP and ADP is regulated by ANT, in cooperation with VDAC and tissue-specific creatine kinase. The correct function of ANT stimulates the influx of protons into the mitochondrial matrix, resulting in a lower pH. Starvation initiates the cAMP-signalling pathway in liver tissue, which leads to the phosphorylation of COXI; there is no evidence of this mechanism in heart tissue. In addition, hypoxia triggers PKC-dependent phosphorylation of COX4 and increases COX activity. This phenomenon implies that both the metabolic state of the cell and tissue-specific properties modulate the activity of COX. When the COX enzyme is phosphorylated at its regulation sites, proton slipping does not cause excessive ROS production, as documented in COX6A [60].

The allosteric regulation of cytochrome c and COX activity is possible via their binding sites for adenine nucleotides, which inhibit respiration by binding to cytochrome c or a specific subunit of cytochrome c oxidase, COX6A. In contrast, binding of ATP to COX4 leads to higher COX activity. Nucleotide binding to COX subunits at an increased ATP/ADP ratio induces a drop in respiration and proton pumping, as well as in Δp_m and $\Delta\Psi_m$. This allosteric ATP inhibition of COX, which leads to uncoupled respiration, can be ceased by binding of 3,5-diiodothyronine to COX5A, dephosphorylation of the COX enzyme and low levels of cardiolipin connecting the COX monomers [36]. The allosteric inhibition of COX that occurs at a high intramitochondrial ATP/ADP ratio is performed by cAMP-dependent phosphorylation (mitochondrial protein kinases) of COX subunits, which is supported by the mitochondrial uptake of Ca^{2+} and leads to lowered $\Delta\Psi_m$ via proton slipping. This inhibition via ATP is abolished by hormone signals (3,5-diiodothyronine) or Ca^{2+}-dependent dephosphorylation (Ca^{2+}-activated protein phosphatases), which subsequently leads to an increase in $\Delta\Psi_m$, respiration and ATP synthesis, as well as proton slipping and the production of heat. A high nutrient uptake may also release ATP inhibition due to raised $NADH^+ + H^+$ production in the TCA cycle [73,196].

In addition, other mechanisms control COX function, such as divalent cation binding sites, fatty acids and NO. Mitochondrial NO synthase interacts with COX5A to result in reversible inhibition of COX due to NO competition with oxygen for the binuclear haem a_3-CuB binding site. With regard to the NO inhibitory effect, its

increased production is thought to be a cause of neurodegenerative disorders [60,197]. Membrane lipid composition has been found to influence the stability and activity of COX in addition to affecting the overall respiration rate [65].

3.6 Cytochrome c oxidase deficiency

As shown by histological studies, presence of COX-deficient fibres is more frequent than ragged-red and ragged-blue fibres in both patient and control groups [187]. In muscle tissues, mosaic expression of COX activity is indicative of a heteroplasmic mtDNA mutation, while global COX-deficiency suggests mutation in nuclear genes important for COX function and/or biogenesis. However, there are several exceptions, including some homoplasmic tRNA mutations, and this classification also depends on an equal distribution of COX positivity in affected tissue, as observed in MELAS [98]. Thus, the presence of COX-deficient fibres is not a definite marker of a causal mutation in COX-related genes [198]. COX-deficient fibres can be in excess of RRFs in paediatric patients with other neuromuscular disorders, and both types of fibres can be fully missed despite clearly deteriorated mitochondrial ultrastructure [199,200]. Analysis of the COX assembly profile is more helpful for the diagnosis of COX deficiency, as patients with *SURF1*, *SCO1*, *COX10* and *COX15* defects express distinct disturbances of COX biogenesis [22].

Together with complex I deficiency, isolated or combined COX deficiency is one of the most common respiratory chain defects in paediatric patients. It can arise from mutations located in either mitochondrial genes or in nuclear genes encoding the structural subunits or corresponding assembly factors of the enzyme complex [22,77,201,202]. Importantly, COX deficiency manifests as a fatal infantile form or as a rarely occurring benign reversible infantile form (homoplasmic m.14674T>C or T>G mutations in $tRNA^{Glu}$, pathogenic mutations in nuclear gene *TRMU*) that can be improved when treated effectively [203-208]. As a complicating factor, mutations in COX-related genes can give rise to secondary impairment of other respiratory chain complexes. Primary deficiencies of respiratory complexes I and III were documented to have secondary effects on the activity of COX, which impedes correct interpretation of laboratory findings [209,210].

Supplementary table 1 lists genetic defects leading to clinically and biochemically confirmed primary and/or secondary COX deficiency (see the Attachment section). These collected genetic data illustrate that isolated or combined

COX deficiency can arise from mutations of genes involved in the biogenesis of any OXPHOS complex, in mitochondrial DNA synthesis and maintenance, apoptosis, cytochrome c sequestration, mitochondrial ultrastructure maintenance, mitochondria networking and the metabolism of various chemical substances present in mitochondria, in addition to the genes relating to mitochondrial protein synthesis, whose defects often lead to combined OXPHOS deficiency. However, a decrease in COX activity was observed in only a few of the mitochondrial patients with tabulated causal mutations, and in some cases, a secondary COX defect was only displayed with concomitant faulty assembly or stability of the COX enzyme [38,211,212]. Novel nuclear genes involved in COX biogenesis are incessantly characterised; therefore, the contemporary COX-related genetic spectrum will be probably enriched with other functional categories that relate to mitochondrial homeostasis.

4 Material and methods

4.1 Group of patients

A group of 60 Czech unrelated paediatric patients without a known genetic cause of COX deficiency was included in this study. Seventeen of these patients were diagnosed with an isolated and 43 with a combined COX defect. The onset of "mitochondriopathy" was observed in 35 neonates: 14 during the first year of life, 6 at the age of 1 – 5 years, 2 at the age of 5 – 10 years and 3 at the age of 10 – 15 years. The patients included in this study presented with the following symptoms: failure to thrive (30/60), delay of psychomotor development (29/60), encephalopathy (28/60), hypotonia (26/60), visual impairment (25/60), myopathy (19/60), dysmorphia (15/60), cardiomyopathy (14/60), hepatomegaly (14/60), intrauterine growth retardation (12/60), spasticity (10/60), hearing impairment (9/60), epilepsy (7/60), dystrophy (7/60), microcephaly (7/60), nephropathy (6/60) and diabetes mellitus type 2 (2/60). Routine metabolic workup showed lactate acidosis (23/60), anaemia (23/60) and hepatopathy (22/60). Thirty patients died prior to the beginning of this study; their survival ranged from 4 days to 13 years, with a median of 1.1 years.

4.2 Samples

Genomic DNA (gDNA) was extracted from the peripheral blood lymphocytes of the patients and/or their parents, patients' cultivated fibroblast cells or patients' muscle biopsies and used for subsequent genetic analysis.

4.3 Ethics

All the biochemical and genetic analysis was approved by the Ethics Committee of the General University Hospital in Prague. All samples were analysed with the informed consent of the patients or their parents.

4.4 Sanger sequencing

Prior to the start of HRM mutation screening, the mtDNA of all 60 patients was sequenced. Briefly, the whole mtDNA molecule was amplified from muscle or fibroblast total DNA by PCR in 34 overlapping fragments. All fragments were sequenced in both direction on an ABI PRISM 3100/3100-Avant Genetic Analyser (Applied Biosystems), and the obtained sequences were compared with the Revised Cambridge Reference Sequence (rCRS) of the Human Mitochondrial DNA (NC_012920, http://www.mitomap.org/bin/view.pl/MITOMAP/HumanMitoSeq).

Based on the clinical phenotypes manifested by the patients and the results of the completed genetic analyses, sequencing of suspected candidate disease-genes covering exons and their flanking intronic regions of *SCO2* (NG_016235.1), *TYMP* (NG_011860.1), *PUS1* (NG_013039.1), *TSFM* (NG_016971.1), *AARS2* (NG_031952.1), *TK2* (NG_016862.1) was performed according to the standardised internal laboratory procedure (Table 2).

Table 2 Primers used to confirm findings of targeted exome sequencing

Type of amplicon	Forward primer (5´→3´) Reverse primers (5´→3´)	Length of amplicon	PCR mixture	PCR conditions
SCO2[a] exon 2	CAGGAAACAGCTATGACCCTGACATCTGC CCAGACGAG AATACGACTCACTATAGGGCCGCTGGTAC AGATCACAC	477 bp	1x CPM, 6% DMSO, 0.8 µM primers	95°C 2 min; 33x: 95°C 30sec, 64.3°C 30 sec, 72°C 1 min; 95°C 1 min; 64.3 °C 30sec; 72°C 3 min
TYMP exon3	TGCCCCACCGCTGTGGGCTG TGCGGTATAGGCTCCCGTCT	293 bp	1x CPM, 0.8 µM primers	95°C 2 min; 40x: 95°C 30sec, 68°C 10 sec, 72 °C 40sec; 72°C 7 min
PUS1[a] gDNA	TGGCCTGATTTTTCCTAGGTT ACAAAGGGTTCCTCGCAGTA	488 bp	1x PM, 0.8 µM primers	95°C 2min, 35x: 95°C 20 sec, 60°C 10 sec, 72°C 50 sec; 72°C 7 min
cDNA	*GTGCTGCTCATGGCCTATTC* *GATGATGGTGGGGTAGATGTG*	840 bp		95°C 2 min, 35x: 95°C 20 sec, 60°C 20sec, 72°C 1 min; 72°C 7 min
TSFM exon 4	TTTCCGTTGAGTCTGTAGCTTG ACGGGGGAAGGGTAATTCTA	389 bp		95°C 2 min; 35x: 95°C 30 sec, 20 sec (exon 4 - 55.5.°C, exons 6 and 16 - 61.8°C, exon 13 - 64.6°C), 72°C 40 sec; 72°C 7 min
exon 6	CAAACTGGGCCTCTTCTGTG CTCGGTCTGAAGAGGTTTGG	587 bp		
AARS2 exon 13	GCAGGGCAAGAGGTGAGTC AGTGCCCAGTTCAGCAGGT	394 bp		
exon 16	GAAGCCCTTTTGCTGGAGA TAAGGGCTGATGGCTCCAA	291 bp		
TK2[a] econ 3	ATACGACTCACTATAGGGCTGACATTCCCC TGGGTGCTT GAAACAGCTATGACCATGATTATCCCATC AAGCTTTCT	221 bp	1x CPM, 0.8 µM primers	95°C; 35x: 95°C 30sec, 54°C 25 sec, 72°C 40 sec; 72°C 7 min
exon 6	ATACGACTCACTATAGGGCCTCCTTTTCCC CTGAGTTAG GAAACAGCTATGACCATGTACTCCATATCT GTCAATCG	248 bp		

Abbreviations : PM = PPP Master Mix; CPM = Combi PPP Master Mix; gDNA = genomic DNA
[a] Mutation analysis of *PUS1* gene were kindly performed by Ing. Kamila Beránková.
[b] PCR products covering *SCO2* and *TK2* gene were amplified and sequenced with the unitary accessory sequences for forward or reverse primers.

RNA was isolated from patient cultured skin fibroblasts (P8, P12, P17, P29) by TRI REAGENT® (Molecular Research Center, Inc.) and transcribed to cDNA as

described previously [128]. cDNA and promoter sequence analysis of the *COX4I2, COX5A, COX7A1* and *COX10* genes were performed in patients P8, P12, P17 and P29 with the use of an Expand Long Template PCR System, according to manufacturer's protocol (Roche). The used PCR primers are summarised in Table 3.

Table 3 Primers used to confirm sequence variations in COX-related genes

Type of amplicon	Forward primer (5´→3´) Reverse primers (5´→3´)	Length of amplicon	Cycles	PCR mixture[a] Annealing temperature
COX4I2 *cDNA*	*GAGCTTGGTGCTGAGGAAAG* *GGAGACAGCTGGGGATGC*	517 bp	40	1x CPM, 1mM $MgCl_2$, 6% DMSO, 1.6 µM primers; 66.8°C
promoter	GTGGGAGAAACAGCAAGGAG CCCTCTAAGACAGGGACCAC	455 bp	35	1x PM, 6% DMSO, 0.8 µM primers; 64.6°C
COX5A *cDNA*	*GGCTTCTCTCTGTCCTCAGC* *TCAATAAATCCTTGGGGAAGC*	528 bp		1x PlM, 6% DMSO, 0.8 µM primers; 61.8°C
promoter	CTCTGCCTCCTGGGTTCC CGCTGAGGACAAACTGTTAGC	801 bp		1x CPM, 8% DMSO, 0.8 µM primers; 64.6°C
promoter	GGCTTCTCTCTGTCCTCAGC CGAAGCGTTCCTATGCTTGT	835 bp		1x CPM, 1.5 mM MgCl2, 8% DMSO, 0.68 µM primers; 58.4°C
COX7A1 *cDNA*	*CAGAATGCAGGCCCTTCG* *CCCCCAGGCTTCTTGGTC*	362 bp		1x PM, 2% DMSO, 0.8 µM primers; 61.8°C
promoter	GAGGCTGCAGTGAGCTATGA GTTCCAACTCCCTGTTCTGTCT	395 bp		1x CPM, 8% DMSO, 0.68 µM primers; 58.4°C
promoter	CGGAGAAGGGAGGTGACTC CCACCTGGAAGAGCTTCTGT	784 bp		1x PM, 6% DMSO, 0.8 µM primers; 64.6°C
promoter	ACTTCAGGACACCCCTCCAG AGCCAAGGGAGTACAAGCTG	806 bp		1x CPM, 6% DMSO, 0.64 µM primers; 64.6°C
COX10[b] *cDNA_1*	*CAGGAAACAGCTATGACATGGCCGCATCTCC* *AATACGACTCACTATAGCACCGCTTTTCCTCTTTT*	462 bp		1x PlM, 0.8 µM primers; 61.8°C
cDNA_2	*CAGGAAACAGCTATGACTGTCCAGAAAGCCAAATGAA* *AATACGACTCACTATAGACAGCACAACAAGTGGCAAA*	443 bp		1x PlCM, 0.8 µ primers; 68.4°C
cDNA_3	*CAGGAAACAGCTATGACTGCTGCCAACTCCATCAA* *AATACGACTCACTATAGCTGCGGACAGCACGA*	540 bp		1x PlCM, 0.8 µ primers; 68.4°C
cDNA_4	*CAGGAAACAGCTATGACTCCTGGCAGTTTCCTCATTT* *AATACGACTCACTATAGTCAGCTGGGAGGGGG*	409 bp		1x PlCM, 0.8 µ primers; 64.6°C

Abbreviations: PM = PPP Master Mix; CPM = Combi PPP Master Mix; PlM = Plain PP Master Mix; PlCM = Plain Combi PP Master Mix

[a] PCR steps were identical for all amplicons: 95°C 2 min; cycling: 95°C 30 sec, annealing temperature 30 sec, 72°C 1 min; 72°C 7 min

[b] cDNA of *COX10* gene was analysed as four overlapping sequential fragments.

4.5 High-resolution melting analysis

Primers were designed, using the software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), to amplify the coding regions of *COX4I1, COX4I2, COX5A, COX5B, COX6A1, COX6A2, COX6B1, COX6C, COX7A1, COX7A2, COX7B, COX7C, COX8A, COX10* and *COX15*. Genomic

DNA was amplified by PCR in the presence of LCGreen Plus Melting Dye (Idaho Technology Inc.).

For genetic and subsequent HRM analyses, a total of 15 to 50 ng of gDNA was amplified (NanoDrop ND-1000 UV-Vis Spectrophotometer, Nano-Drop Technologies, Inc.). The HRM analysis was performed using a LightScanner instrument (Idaho Technology Inc.) according to the instructions in the LightScanner's manual. The melting profiles of 60 patient samples were analysed blindly, along with 14 reference control samples. If a new sequence variant was found by HRM analysis, all the remaining exons of the suspected gene were then sequenced by Sanger methodology.

Variants of both *COX4I2* (rs6088855) and *COX10* (rs113058506) were clearly distinguishable from the wild type when the DNA was mixed at a 1:1 ratio. The use of High Sensitivity Master Mix (Idaho Technology) allowed superior resolution of all genotypes for exon 5 of the *COX10* gene and exon 9 of the *COX15* gene. All other common variants were readily identified by HRM during the first experiment with only the use of LCGreen® Plus Melting Dye (Idaho Technology).

4.6 Restriction analysis

All the identified missense mutations were verified by PCR-RFLP analysis. The frequency of rare sequence variants was ascertained by PCR-RFLP and/or HRM analysis in the Czech population by using set of 100 – 250 Czech healthy control samples or a set of 80 control gDNA samples of Roma origin.

4.7 *In silico* analysis

The web servers SIFT (http://sift.jcvi.org/) [213], SNAP (https://rostlab.org/services/snap/) [214], PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) [215], MutPred (http://mutpred.mutdb.org/) [216], PMut (http://mmb2.pcb.ub.es:8080/PMut/) [217], PANTHER (http://www.pantherdb.org/tools/csnpScoreForm.jsp) [218] and SNPs&GO (http://snps-and-go.biocomp.unibo.it/snps-and-go/) [219] were used to evaluate the possible pathogenicity of all identified missense substitutions with unknown genetic effects.

4.8 Biochemical and follow-up electrophoretic analysis

4.8.1 Thymidine levels in plasma

Thymidine and deoxyuridine levels were analysed by reversed-phase high-performance liquid chromatography with UV detection at the Institute of Inherited

Metabolic Disorders of First Faculty of Medicine Charles University in Prague and General University Hospital in Prague [220,221].

4.8.2 Thymidine phosphorylase activity

Thymidine phosphorylase activity was measured spectrophotometrically in isolated lymphocytes according to Spinazzola et al. [222]. Briefly, lymphocytes were isolated in a Ficoll gradient. Lymphocytes were homogenised in lysis buffer, sonicated and centrifuged. In the supernatant, the protein concentration was determined according to Lowry [223]. A 150 mg aliquot of supernatant protein was added to the reaction mixture and incubated at 37°C for 30 min. The reaction was inhibited, and the amount of thymine was determined spectrophotometrically at 300 nm.

4.8.3 SDS-PAGE electrophoresis and immunoblot analysis

Ten micrograms of mitochondrial protein was separated by tricine SDS-PAGE carried out on 12% polyacrymide, 0.1% SDS and 5.5 M urea gels. Mitochondrial fractions were dissociated in 50 mM Tris/HCl (pH 6.8), 12% glycerol, 4% SDS, 2% 2-mercaptoethanol and 0.01% Bromophenol Blue for 30 min at 37 °C, as described earlier [224]. Proteins were electroblotted from the gels onto Immobilon^{TM}-P PVDF membranes (Millipore, Carrigtwohill, Ireland) using semi-dry transfer. The membranes were decorated with rabbit polyclonal antiserum raised against human SCO2 (1:1000), with mouse monoclonal antibodies raised against cytochrome c oxidase subunits COX1 (Abcam-Mitosciences, Eugene (OR), USA; 1 μg/ml), COX2 (Abcam-Mitosciences; 1 μg/ml) and porin (Abcam-Mitosciences; 1 μg/ml) under the same conditions, as described previously [224].

4.8.4 Total copper content in tissues

The total copper content in the dry matter of liver, brain and muscle tissues was assessed by FAA (Perkin Elmer 3300 AAS, Perkin-Elmer Corp., USA) or ICP-MS (Elan DRC-e Perkin Elmer SCIEX, PerkinElmer Inc., USA) at The National Reference Laboratory for Genetic Toxicology in The Centre of Toxicology and Health Safety (The National Institute of Public Health, Prague, Czech Republic).

4.9 DNA array and copy number variation analysis

The nuclear DNA of patients (P1, P5, P8, P12, P17, P29, P43, P49) was analysed using a Genome-Wide Human SNP 6.0 microarray chip (Affymetrix), allowing the detection of deletions larger than 700 bp at The Centre for Applied

Genomics (Toronto, Canada, http://www.tcag.ca/facilities/statisticalAnalysis.html). Additionally, patient 61 with detected combined deficiency of complexes I-III, III and IV in muscle was included in the examined group of patients based on a disease phenotype resembling the clinical manifestation observed in patient 5. Altogether, the DNA array was applied for analysis of 16/61 patients.

The presence of only one copy of the *SCO2* and *TYMP* genes in both affected patients was verified by a real-time PCR copy-number variation assay (Hs00093549_cn, Hs00001601_cn, Hs00137275_cn, Hs00574610_cn; Applied Biosystems). The frequency of deletions covering *SCO2* and *TYMP* genes was assessed in a set of 50 Czech healthy control samples. As for the copy number of *PUS1* gene, TaqMan probes were also employed (Hs01998936_cn, Hs01410383_cn; Applied Biosystems) according to manufacturer`s instruction.

4.10 Next-generation sequencing

Targeted sequencing of the mitochondrial exome, containing 1233 genes, was performed for 25/57 patients. This collection of candidate genes was originally derived from MitoCarta, which contained 1013 genes that were assumed to be functional or structural components of the mitochondria. Our updated list of candidate genes was enlarged based on our internal laboratory selection to add newly characterised mitochondria-related genes and to allow for various inter-molecular interactions provided by the selected genes. We analysed 1.2 μg of gDNA from the patients and/or both of their parents (blood, skeletal muscle biopsy or cultured fibroblasts) or their unaffected siblings.

Next-generation sequencing was performed on a SOLiD^{TM} 4 System (Life Technologies, Czech Republic) using an optimised sequence capture protocol derived from the standard NimbleGen SeqCap EZ Library SR User`s Guide version 3.0, which is available from Roche NimbleGen, Inc. (http://www.nimblegen.com). Sonicated patient DNA (Covaris) was purified using Agencourt AMPure XP Reagent beads (Beckman Coulter) and treated with the Fast-End Repair Enzyme Mix (Thermo Scientific). Purified patient samples were ligated to specific oligonucleotide adaptors using the Rapid DNA Ligation Kit (Thermo Science), bar-coded with specific oligonucleotides and amplified by PCR. Patient samples were purified with the QIAquick PCR Purification Kit (Qiagen) and their concentrations were measured with the Qubit^{TM} Quantification Platform using the dsDNA BR Assay Kit (Life

Technologies). The quality of the amplified patient library was checked using the DNA 1000 Kit (Agilent) and an Agilent 2100 Bioanalyzer. The sets of barcoded patient samples were mixed equally to become 1 ng patient library samples that were then hybridised to the Mitoexome SeqCap EZ Library using Human COT-1 DNA (Invitrogen) and the SeqCap EZ Hybridisation and Wash Kit (NimbleGen, Roche). After the hybridisation procedure, the mixed patients DNA samples were washed using the SeqCap EZ Hybridisation and Wash Kit and bound to M-270 Streptavidin Dynabeads (Life Technologies). Captured patient DNA samples were ligated to identical universal oligonucleotides and amplified by PCR. The amplified samples were purified using the QIAquick PCR Purification Kit. Sample concentration was measured with the Qubit^{TM} Quantification Platform using the dsDNA HS Assay Kit (Life Technologies) and sample quality was checked using the HS DNA Kit (Agilent) and an Agilent 2100 Bioanalyzer. Emulsion PCR, associated with the enrichment of templated beads, was performed according to the available protocol (SureSelect Target Enrichment System for the Applied Biosystems SOLiD System, Agilent), and the mixed patient samples were sequenced. Statistical analysis of sequencing data was followed by a comparison of the identified sequence variants in the patients, family members and reference controls with available databases of genetic variants, tissue specific expression databases and *in silico* tools. To confirm the NGS data, Sanger sequencing was used. If available, parental DNA was tested for the identified mutations.

The complete and optimised protocol for next-generation sequencing was provided by the Institute of Inherited Metabolic Disorders in the First Faculty of Medicine at Charles University in Prague and General University Hospital in Prague; this institute also performed the sequencing procedure and statistical analysis of the raw NGS data. Output sequence reads were aligned to the reference genome (hg19) using NovoalignCS version 1.08 (Novocraft, Malaysia) with default parameters. Sequence variants in the analysed samples were identified using the SAMtools package version 1.08. The high confidence variants list was annotated using the ANNOVAR annotation tool (hg19). For further analysis, we prioritised sequence variants present in affected individuals that were not found in unaffected relatives and that had a frequency lower than 0.05 in the dbSNP, 1000 Genomes, Exome Variant Server and internal exome database. Candidate variants were visualised using the Integrative Genomics Viewer (IGV) version 1.5.65.

5 Results and discussion

5.1 High-resolution melting analysis used for mutation screening of genes encoding COX structural subunits and selected COX assembly factors

5.1.1 Sanger sequencing of patient mtDNA prior to HRM analysis

Sanger sequencing of the mtDNA revealed only known common polymorphisms listed in Mitomap (http://www.mitomap.org/MITOMAP) or mtDB - Human Mitochondrial Genome Database (http://www.mtdb.igp.uu.se/index.html) in 59/60 patients. However, in patient P25, a homoplasmic variant m.15866A>G (p.N374D) in the *MT-CYB* gene was detected that has not been yet reported. It was found to be homoplasmic in patient skeletal muscle, blood and cultivated fibroblasts and was not present among the 200 healthy controls, which was confirmed by BbsI-RFLP analysis. Since we lost the contact with the patient family, the presence of m.15866A>G (p.N374D) in the *MT-CYB* gene could not be tested in maternal relatives. The protein alignment showed that the Asp 374 of cytochrome b is not evolutionary conserved. Additionally, the western blot assembly profile and the activity of complex III performed on patient cultured fibroblasts were within the physiologic range. Considering the highly polymorphic nature of mitochondrial genome and the strict application of postulated pathogenicity criteria [121,122], we assessed the m.15866G variant as polymorphic. These data suggested a nuclear genetic origin of the COX deficiency in the patient. However, a subtle modification in the rate of complex III biogenesis cannot be excluded, as documented by other publications [20,91]. With the exception of the new homoplasmic m.15866G variant, the patient P25 carried only common SNPs in her mtDNA.

5.1.2 Optimisation of HRM analysis

[225]. Irrespective of the position of the base-pair variant within the PCR product, HRM analysis is capable of detecting homozygous and/or heterozygous sequence variations in amplified PCR products by monitoring differences in their thermal stability and evaluating the shape and/or shift in their melting curves [226-229]. The sensitivity and specificity of HRM analysis are better than those of many conventional methods used to detect mutations [225]. Although HRM analysis makes it possible to screen the entire amplicon region, sequencing is still needed to determine the precise sequence variation that is present in an amplicon. The accuracy of HRM analysis is dependent on the salt concentration, GC content, length and the primary

sequence of the duplex. Additionally, it could be affected by the presence of many melting domains [230-232]. Currently, several strategies are used to achieve better resolution by HRM, for example, small amplicons, unlabelled probes, snapback primers, internal temperature calibrators and mixing patient samples with the reference control genotype [233-238].

Because the exact occurrence and distribution of common SNPs was not known in the examined Czech population, the HRM mutation screening was performed using probe-free HRM; this approach is especially suitable for large-scale genetic studies [239-242]. A total of 70 amplicons covering 65 coding regions were analysed; their length ranged from 191 bp to 565 bp. PCR primer sequences and specific PCR conditions are available online at the *Journal of Human Genetics* (http://www.nature.com/jhg/journal/v57/n7/suppinfo/jhg201249s1.html, Supplementary Table 1) [243]. However, the applied PCR design is described below for clarity (Table 4). Moreover, internal calibrators and DNA mixing were applied to improve the resolution of individual genotypes for four amplicons of the COX genes that were used. However, the majority of examined amplicons did not require these adjustments.

Table 4 Primers and PCR conditions used for HRM analysis.

Gene and exon	Forward primer (5´→3´) Reverse primer (5´→3´)	Length of amplicon	Annealing temperature	Cycles	PCR mixture[d]
COX10[a] **1**	AGACACCACGCTCTCCTTTC GAGAAGAATTTCCCCCAAGG	240bp	61,8°C	35	1x PlM, 4% DMSO, 800 nM primers
2	TTGCTTCTGGGGAGGTGTAG TCTCAACAGAGAAAAAGGCAGA	271bp	55,5°C	30	1x PlM, 1 mM $MgCl_2$, 4% DMSO, 800 nM primers
3	AAAAGCTGGTCTGATTGAAGATG TGAAGAGAGGAAAAATACTAAGACAGG	544bp	58,4°C	30	1x PlCM, 4% DMSO, 800 nM primers
4	TGGTAACAGTGTGTCTGCTCTGT ACAGCCATCTAGGAAAAAGTGA	218bp	66,8°C	35	1x PlCM, 2% DMSO, 800 nM primers
5	GTGCCAGGGTATTGATTTATG CACACTTTGGTTAGAGGTGGA	369bp	61,8°C	35	1x HSM, 8% DMSO, 640 nM primers
6	CAGGTTCTCTGCTCTTTTTCC CTCCTTGACCGAGTGTGCT	306bp	58,4°C	30	1x PlM, 4% DMSO, 400 nM primers
7a[b]	TCTGGTGATGACTGCCTTTG GTCCACGTAGAAGCGGAAGC	328bp	61,8°C	35	1x PlCM, 0,5 mM MgCl2, 8% DMSO, 800 nM primers
7b[b]	TTCCCATCAATGCGTACATC TTCCAGAATTACCACAACATGC	243bp	61,8°C	40	1x PlCM, 8% DMSO, 800 nM primers
COX15[a] **1**	GTTGTGGAAGAGGTGGCTGT TATCTTTATCCCGGCCCTTT	191bp	61,8°C	30	1x PlCM, 4% DMSO, 800 nM primers
2	CCAGTTGGAAGGCTGATG GAACTCAGGAGACGGAGG	518bp	66,8°C	30	1x PlCM, 4% DMSO, 800 nM primers

Table 4 Primers and PCR conditions used for HRM analysis (continued)

Gene and exon	Forward primer (5´→3´) Reverse primer (5´→3´)	Length of amplicon	Annealing temperature	Cycles	PCR mixture
3	CCTGATGGCAGCTGTTTCT TGCCCTCTTCCTCATCAAC	482bp	61,8°C	30	1x PlCM, 4% DMSO, 800 nM primers
4	GGATGTTTCCTCCTCCTCCT TGG GAGCATTTCTGGTTTCT	271bp	55,5°C	30	1x PlCM, 1mM $MgCl_2$, 4% DMSO, 240 nM primers
5	CCAAGATCCCGCCACT CCATCCACCATCCCTTCT	520bp	61,8°C	30	1x PlCM, 4% DMSO, 1200 nM primers
6	ATGGGGTAGAAGGGAAAACA TGAAGATGGGGGAATGAGA	429bp	61,8°C	30	1x PlCM, 4% DMSO, 1200 nM primers
7	TTG GGGTGGGAGCAGGT GGTAGGGGGACAGGGGTG	565bp	66,8°C	30	1x PlCM, 4% DMSO, 400 nM primers
8	GAAGAGGATGGTGGAAGAG TTTGTAGAGATGGGGTTTTG	473bp	61,8°C	30	1x PlCM, 4,6% DMSO, 400 nM primers
9a[b]	TGTGGAGGTTTGTGTGTG GTCACAGTCCCAGGAGG	258bp	61,8°C	30	1x PlCM, 800 nM primers
9b[b]	GCCCAGCTAGTTCCTCTTT TCTCGATGGGGTCATTCT	365bp	61,8°C	35	1x HSM, 0,32 mM MgCl2, 400 nM primers
COX4I1 **1**	AGACTCCAGTCGCGCTTC CTGCGGACGTGCAGACTT	331bp	61,8°C	35	1x PlCM, 10% DMSO, 1000 nM primers
2	GCTCTGGGGCAAAAAGAAG AACTCCAGCACAGGGCTTTA	234bp	64,6°C	30	1x PlCM, 1 mM $MgCl_2$, 800 nM primers
3	CTGTGACCCCCTGAGATGAT GAGGCTCTGTCACACACACG	364bp	64,6°C	35	1x PlM, 800 nM primers
COX4I1 **4**	TGGTTGAATGTTGCAGAGGA AGCCTCAAGGTATGGAGGTC	298bp	61,8°C	30	1x PlCM, 4% DMSO, 800 nM primers
5	GAGGGATTGGCCTAGAAACA_CCCTTG GGAGAAACCTATTG	398bp	58,4°C	30	1x PlM, 4% DMSO, 800 nM primers
COX4I2 **1**	CTGCCGAAGCAGGACGTT AACCCTCTAAGACAGGGACCA	267bp	58,4°C	35	1x PlM, 8% DMSO, 800 nM primers
2	TGATGTGGGGGCAGAACT TGGGAAGTGTGGTAGGAACA	242bp	64,6°C	35	1x PlM, 2% DMSO, 800 nM primers
3	CCCGGCCACCTTCTTTATTA GGCCATTCTTTCCAAAGTCA	313bp	58,4°C	35	1x PlM, 2% DMSO, 800 nM primers
4	GAAGCCGGGATCACTTAGAG GTGACCACAAGGGCATGG	249bp	64,6°C	35	1x PlM, 2% DMSO, 800 nM primers
5	CCTGGCTGGTGTAGGAAGAC TGCCTAATTTTAGGTGCCAAGT	356bp	61,8°C	35	1x PlM, 4% DMSO, 800 nM primers
COX5A **1**	GTCACCTGACCAGAGACAAGG AGGTCACCGCAAGGACAC	390bp	61,8°C	30	1x PlCM, 10% DMSO, 1000 nM primers
2	TTCAATATTTTTGCTGCCACA GCAAGTTGCATGAAGTAACCA	354bp	61,8°C	30	1x PlM, 2% DMSO, 800 nM primers
3	GGAGACCCAGACAGATAAGATCA TTCTGATACCTCAGCAATAGCC	298bp	55,5°C	30	1x PlM, 2% DMSO, 800 nM primers
4	TCTGTCCTACCTGCCTCTGC GCTCACGGCCATTACCTCTA	354bp	61,8°C	30	1x PlM, 2% DMSO, 800 nM primers
5	TCGCTTGTGGGTTGACAGTA CAGCAAAACCATGAAACCAA	397bp	61,8°C	30	1x PlM, 2% DMSO, 800 nM primers

Table 4 Primers and PCR conditions used for HRM analysis (continued)

Gene and exon	Forward primer (5´→3´) Reverse primer (5´→3´)	Length of amplicon	Annealing temperature	Cycles	PCR mixture
COX5B **1**	ACTACGCGGTGCAGAAAGAG CCACTGGGACCTCGAGAAG	399bp	58,4°C	30	1x PlCM, 8% DMSO, 1200 nM primers
2	GCACCATTTTCCTTGATCATT CTCCCAGAGAGGAGACCACT	237bp	61,8°C	30	1x PlM, 800 nM primers
3	AACAGTCCCCTGAGCTTCTG CAACATGCACTCACACACTGA	291bp	66,8°C	30	1x PlCM, 2% DMSO, 800 nM primers
4	TCAACCATAGTCTTACTTGTGTATCA GGCAAGCTAGCATTAACAGACA	397bp	61,8°C	30	1x PlCM, 2% DMSO, 800 nM primers
COX6A1 **1**	GGCGCCCAATAGTAACTTCC AGGTCACAGTCCCTCCCTGT	267bp	64,6°C	35	1x PlCM, 0,5 mM $MgCl_2$, 6% DMSO, 800 nM primers
2	CGGGAGGGAAAGTGAGACC CACCCATGCCTTCAGAGAA	297bp	64,6°C	30	1x PlM, 4% DMSO, 800 nM primers
3a[b]	CACCCCGTTATAAGCAGTTCA TAACGGTCCAAACCAGTGCT	222bp	58,4°C	30	1x PlCM, 4% DMSO, 800 nM primers
3b[b]	CCAACTGGCTACGAAGATGA CAGCCTAGACCTTCACTGTGG	354bp	58,4°C	30	1x PlM, 4% DMSO, 600 nM primers
COX6A2 **1**	TGCCTCCTTGCCAAAATAAG AGCAGACGCCAGGTACGAG	400bp	61,8°C	35	1x PlM, 6% DMSO, 800 nM primers
2	TGCCTCCTTGCCAAAATAAG CGTGGCTATTGTGGAACAGA	724bp	61,8°C	30	1x PlM, 8% DMSO, 800 nM primers
2[c]	CTACCCTGCCCACCTGTTC CGTGGCTATTGTGGAACAGA	411bp	50,5°C	30	1x PlM, 0,5 mM $MgCl_2$, 14% DMSO, 600 nM primers
3	CTCTCTCCACAGCCCTACCC AGGAGCGCTTACCAAGCTG	228bp	66,8°C	35	1x PlCM, 6% DMSO, 800 nM primers
COX6B1 **1**	GGCCAGAAGTGAGGATGAAC CCTCAGCCCGCTAGACTG	288bp	64,6°C	30	1x PlM, 2% DMSO, 800 nM primers
2	CTGGGTAGTCTGGCTTGCTC GGGTCCCCTAGGAAGAGG	249bp	64,6°C	30	1x PlM, 640 nM primers
3	CAGATTGAGACCCTACCTCAAAA CACACTCCCCTCTGCTAAGA	245bp	61,8°C	30	1x PlM, 4% DMSO, 800 nM primers
4	TAGAGGTTGGCACACAGCAG GGTCAGGGCACTGATTCC	378bp	64,6°C	30	1x PlM, 2% DMSO, 800 nM primers
COX6C **1**	ATGAACTTCGGCTGTCACCT CGACTAAATCCGAGGCAGAG	248bp	64,6°C	30	1x PlM, 2% DMSO, 800 nM primers
2	AGCTCCAATCAATGCTTCCA AAAGATTTTTCAACCAAAAACACA	397bp	58,4°C	30	1x PlM, 4% DMSO, 800 nM primers
3	AAAACATGTGTTCTACCTTGTCTTTA GGGACAGTCACCTGTATTTGC	298bp	61,8°C	30	1x PlCM, 2% DMSO, 800 nM primers
4	CTCAGTTGATCCTCAAAGATGG GCTTCATAAACAGTTAAATCCCAAA	284bp	61,8°C	30	1x PlM, 2% DMSO, 800 nM primers
COX7A1 **1a**[b]	TAAATACCGTTTTACTCCCAAA CTCGGATTCGTCCACCAC	398bp	55,5°C	35	1x PlCM, 6% DMSO, 600 nM primers
1b[b]	TATTCCCTGGTACCGCTTTG GCACTTGGAGAGTCGCGTAT	377bp	55,5°C	35	1x PlCM, 10% DMSO, 800 nM primers
3	GCTAGGGATGGGGCTGTC CTGATGAGAAAGGGGTGCTG	297bp	64,6°C	35	1x PlCM, 8% DMSO, 800 nM primers
4	CTCTAAGGAGCAGCCAGCAC AGTCCTGCCCAGAAACCAG	215bp	61,8°C	35	1x PlM, 8% DMSO, 800nM primers

Table 4 Primers and PCR conditions used for HRM analysis (continued)

Gene and exon	Forward primer (5´→3´) Reverse primer (5´→3´)	Length of amplicon	Annealing temperature	Cycles	PCR mixture
5	GATGTCCAGGGAGGGGATTA TCCACAGGGCAGAGATCC	243bp	61,8°C	35	1x PIM, 6% DMSO, 800nM primers
COX7A2 **1a**[b]	GTTTTACGCCTTCTCGCTCA GCTTGCGCTCCTAACCATAG	257bp	61,8°C	35	1x PICM, 800 nM primers
1b[b]	CCGTACTGCCGCTCTAGTTT CCAGGTGAGGGTTTTCTGTC	285bp	61,8°C	35	1x PICM, 4% DMSO, 800 nM primers
2	CGACTGAAAATAGTTGGTTTTGAA CTATGGTACATGTCCTTGACTTTTT	300bp	58,4°C	30	1x PIM, 2% DMSO, 800 nM primers
3	TCTCAAATTAACGGTGAAAGAAGA TGGCATAGCAAAAGCAATAAA	399bp	58,4°C	30	1x PIM, 2% DMSO, 800 nM primers
4	CAAACTTACAACTTTTGAACTGGA CAAACGGCAAGTTGAGACAG	486bp	58,4°C	30	1x PIM, 2% DMSO, 800 nM primers
COX7B **1**	AAGGGATTGCAATTACTATAGGTTT CGTAAAAGGAAAGCACACGA	291bp	58,4°C	30	1x PIM, 2% DMSO, 800 nM primers
2	TTCCTTGGCTTTCCTGATTG GACACTTTGAATGCATAGACTGAGA	292bp	58,4°C	30	1x PICM, 4% DMSO, 800 nM primers
3	TCCCAGGTGAGTTTCTGTGT AACATAAAGGCTAAAGTGATCAAGC	384bp	61,8°C	30	1x PICM, 800 nM primers
COX7C **1**	CCGCAATGGTCTGAACTACAA AGCCTGGTTTCTGGCTATCA	394bp	58,4°C	30	1x PICM, 4% DMSO, 600 nM primers
2	GCCATGTAGTGTTTTGTGATGAA GTGATGGGGAAGAGGCTACT	400bp	61,8°C	30	1x PIM, 2% DMSO, 800 nM primers
3	TCATGAAACTACATGATTTCTGTTAAA CACCATTAAATAAGCTAAATCACAGA	285bp	61,8°C	30	1x PICM, 2 mM $MgCl_2$, 2% DMSO, 600 nM primers
COX8A **1**	GCGTCATTTCCGAGAGACTT TCCAGACATGCCCAAACC	387bp	61,8°C	30	1x PIM, 6% DMSO, 800 nM primers
2	CTTTCTGCTGCCTGGGAACT TCCACACCTCCACCCAGT	476bp	61,8°C	30	1x PICM, 1 mM $MgCl_2$, 8% DMSO, 400 nM primers

Abbreviations: PIM = Plain PP Master Mix (Top-Bio); PICM = Plain Combi PP Master Mix (Top-Bio); HSM = High-Sensitivity Master Mix (Idaho Technology); DMSO , dimethyl sulfoxide (Sigma-Aldrich)

[a] HRM analysis used the accessory sequence for forward (5´-cag gaa aca gct atg ac-3´) and reverse (5´-aat acg act cac tat ag-3´) primers for the amplicons 2, 3, 5 and 6.

[b] The analysed amplicon was separateted into two overlapping fragments.

[c] Primers designed for nested PCR.

[d] PCR conditions were identical for all amplicons: 95°C 2 min; cycling: 95°C 30 sec, annealing temperature 30 sec, 72°C 1 min; 72°C 7 min.

5.1.3 HRM analysis in patients with COX deficiency

In all, the HRM technique directly detected 52 known homozygous and/or heterozygous sequence variants located in *COX*-related genes (Table 5). Thirty-three amplicons had a wild-type profile and did not have any heterozygous sequence variants. The 175 homozygous and/or heterozygous genetic variants, which when combined resulted in 152 distinct genotypes, were correctly detected.

Table 5 Known sequence variations identified by HRM analysis

Gene	Location of SNP
COX10	rs28680987, rs8076787, rs8077302, rs139962310, rs2159132, rs16949103, rs151110639, rs78809149, rs2230354, rs151441, rs111541535, rs113058506
COX15	rs3215694, rs11190255, rs2231687
COX4I1	rs2233447, rs11557187
COX4I2	rs6088855, rs61759491, rs149245323, rs1190725
COX5A	rs6495131, rs113828200, rs150174803
COX5B	rs117644956
COX6A1	rs116850387, rs117492062, rs8903, rs77136374
COX6A2	rs12240
COX6B1	rs10420252, rs7991, rs612220012
COX6C	rs1130474, rs11555138, rs139479312, rs1130569, rs4626565, rs138800666
COX7A1	rs75342, rs74398621, rs68159832, rs2285599, rs2285598, rs2008683, rs80050273
COX7A2	rs117825852, rs9360898, rs240418, rs57613317, rs75996601
COX8A	rs61759492

HRM analysis has expanded the spectrum of known SNPs in *COX*-related genes. Seven sequence variants leading to change in affected proteins were identified in a total of eight patients (P4, P8, P12, P17, P25, P29, P33, P39) located in four of the nuclear-encoded subunits *COX4I2*, *COX5A*, *COX6A2*, *COX7A1*, in an assembly factor *COX10* (Figure 5, Table 6) and in mtDNA. All the newly identified nucleotide sequence data are available in the EMBL database under the WEBIN ID accession numbers HE647854 – HE647864. In total, nine new sequence variants were documented, two of which were located in exons of structural subunits *COX7A1* (c.91_93delAAG, p.K31del) and *COX6A2* (c.34T>G, p.L12V) and had not been previously described. The remaining seven variants were located in introns of *COX4I2, COX6A1, COX7A1, COX7A2* and *COX10* (Table 7). Three heterozygous missense variations in *COX4I2* (c.253C>T, p.Arg85Trp), *COX5A* (c.212G>A, p.Arg71His) and *COX7A1* (c.91_93delAAG, p. K31del) were found in both the group of 60 patients and a set of 100 healthy control samples, which was confirmed by PCR-RFLP analysis. Using

HRM, the variant c.1291C>T (p.Arg431Trp, rs113058506) in *COX10* was found to be a heterozygous variant in two patients (2/60) and in two control samples (2/250). No homozygotes for c.1291T of *COX10* were detected. Additionally, the protein alignment showed that the affected codons are evolutionary conserved. Even though all the remaining exons and adjacent intronic regions of the suspected genes were sequenced in P8 (*COX4I2*), P17 (*COX5A*), P29 (*COX10*) and P12 (*COX7A1*, *COX10*), only common SNPs were identified. No mutations in the promoter regions and/or alternative cDNA splicing products were detected in *COX4I2*, *COX5A*, *COX7A1* and *COX10* of the patients P8, P12, P17 and P29. Besides, the deletions overlapping *COX4I2*, *COX5A* and *COX7A1* genes were proved to be absent in all four investigated patient gDNAs by microarray analysis.

Table 6 Non-synonymous variants identified in 60 investigated patients

Patient	Site of variation	Type of variation	Prediction of pathogenicity - conclusion
P25	***MT-CYB,*** m.15866A>G (p.N374D)	homoplasmic	**ambigous**
P8[1]	***COX4I2*** c.253C>T (p.R85W) rs149245323	heterozygous	**pathological**
P17	***COX5A*** c.212G>A (p.R71H) rs150174803	heterozygous	**pathological**
P4, P33	***COX6A2*** c.34T>G (p.L12V)	heterozygous	**neutral**
P8[1]**, P39**	***COX10*** c.1096G>T (p.V366L) rs111541535	heterozygous	**neutral**
P12[2]**, P29**	***COX10*** c.1291C>T (p.R431W) rs113058506	heterozygous	**probably damaging**

[1] In patient P8, two diverse heterozygous missense substitutions were identified in *COX4I2* (p.R85W) and *COX10* (p.V366L).

[2] Patient P12 harboured two distinct heterozygous variants of *COX7A1* (p.K31del) and *COX10* (p.R431W).

Six rare heterozygous base-pair variations were found in five nuclear-encoded genes that affect codons of *COX4I2* (p.R85W), *COX5A* (p.R71H), *COX6A2* (p.L12V), *COX7A1* (p.K31del) and *COX10* (p.V366L, p.R431W) and were classified by predictive bioinformatics tools to help differentiate neutral variants from those that affect protein function (Table 6). Importantly, proper interpretation of the predictive outcomes that were extracted from the web must take into account the differences in criteria and in the sequence and structural data that was used as the standard for the

functional comparison of the analysed mutant protein [244-246]. Keeping this in mind, three missense variants were evaluated as disease-related and two variants as neutral. The effect of m.15866A>G and p.K31del (*COX7A1*) were not possible to reliably assessed via currently available on-line tools (Table 6).

Table 7 New intronic sequence variants identified by HRM analysis

Gene	Site of variation	Comments
COX10	c.929-8_-7insCCC	heterozygous in 11 patient and 1 control samples
COX4I2	c.*63C>A	heterozygous in 1 patient sample
COX6A1	c.-45G>A	heterozygous in 1 patient sample
	c.*21C>T	homozygous in 1 patient sample and heterozygous in 2 patient and 2 control samples
	c.*147C>T	heterozygous in 1 patient sample
COX7A1	c.102+16G>C	heterozygous in 1 patient sample
COX7A2	c.-6T>A	heterozygous in 2 patient and 2 control samples

To the best of my knowledge, this was the first time that p.R85W (*COX4I2)*, p.R71H *(COX5A)*, p.L12V (*COX6A2)*, p.K31del (*COX7A1)* and p.V366L along with p.R431W (*COX10)* variants were detected in COX-deficient patients. With regard to findings of the NHLBI-ESP (https://esp.gs.washington.edu/drupal) and the 1000 Genomes (http://www.1000genomes.org/home) research projects, the homozygous c.253T (*COX4I2*), c.212A (*COX5A*) and c.1291T (*COX10*) variants are extremely rare and could be pathogenic. Because the defects caused by mutations in nuclear-encoded COX-related genes are autosomal recessive, patients P8, P12, P17 and P29 should be considered heterozygous carriers of pathogenic mutations (Table 6). Thus, it is evident that every human individual is a complex variable mosaic of potential pathogenic variants, which is in accordance with the results from exome sequencing and whole genome microarray analyses [107,247-249]. Nevertheless, an additional study of the four non-synonymous variations, p.R85W *(COX4I2)*, p.R71H *(COX5A)*, p.K31del *(COX7A1)* and p.R431W *(COX10)* should be performed to evaluate their pathogenicity, significance and severity. For this purpose, cells with stable down-regulated expression of individual subunits may be utilized [192].

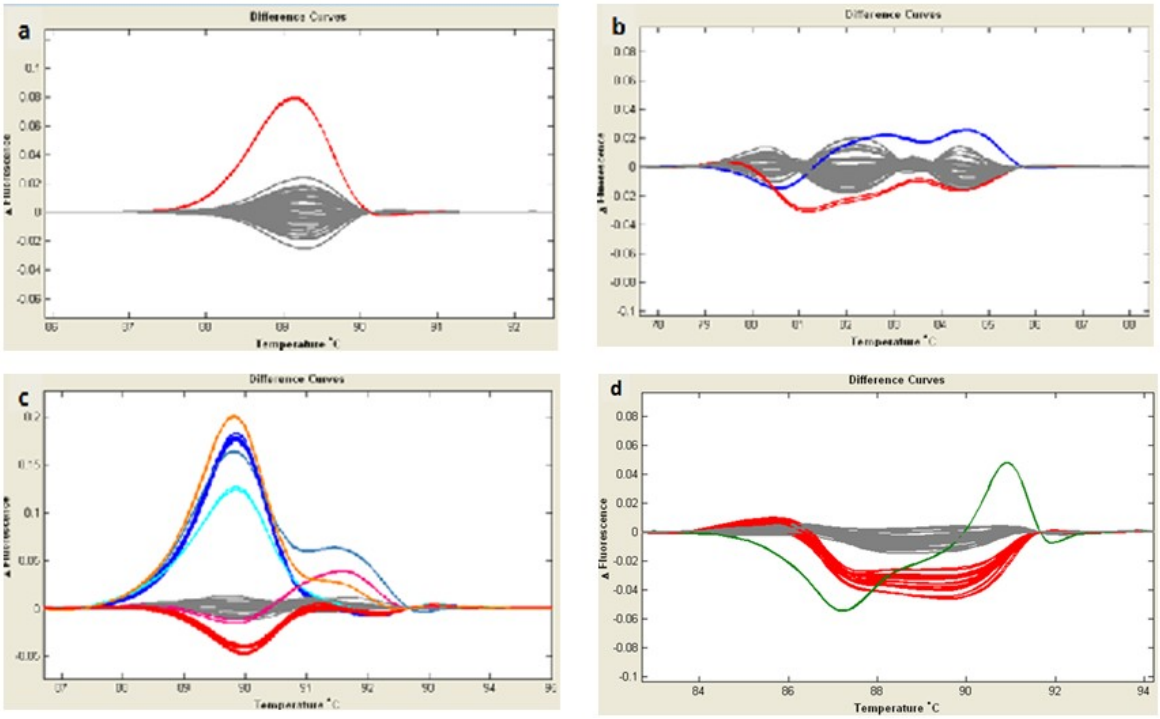

Figure 5 Melting curve plots for the amplicons covering three exons of *COX4I2*, *COX5A* and *COX7A1* and *COX10*.

Melting curves detected for exon 4 of *COX4I2*. The grey line depicts the wild type, and the red line depicts the heterozygous variant c.253C>T (p.R85W) (**a**). Melting curves detected for exon 2 of *COX5A*. The grey line depicts the wild type, the red line depicts the heterozygous variant c.101-63G>A, and the blue line depicts the heterozygous variant c.212G>A (p.R71H) (**b**). Melting curves detected for exon 2 of *COX7A1*. Seven distinct sequence genotypes were detected in these amplicons, which demonstrates the efficiency of the HRM assay. The orange line depicts rs68159832 (G), rs2285599 (G), and rs2285598 (G/C). The dark-blue line depicts rs68159832 (G), rs2285599 (G), and rs2285598 (G). The middle blue line depicts rs68159832 (G), rs2285599 (T/G), and rs2285598 (G/C). The light-blue line depicts heterozygous c.91_93delAAG (p.K31del), rs68159832 (G), rs2285599 (G), and rs2285598 (G). The grey line depicts rs68159832 (delG/G), rs2285599 (T), and rs2285598 (G). The rose line depicts rs68159832 (delG), rs2285599 (T), rs2285598 (G), and c.102+16G>C. The red line depicts rs68159832 (delG), rs2285599 (T), and rs2285598 (G) (**c**). Melting curves detected for exon 7 of *COX10* (fragment a). The grey line depicts the wild type, the red line depicts the heterozygous intronic variant c.929-8_-7insCCC/-, and the green line depicts the heterozygous exonic variant c.1096G>T (p.V366L) (**d**).

In summary, HRM and predictive methodologies are suitable low-cost screening tools. The reliability of pathogenicity prediction methods has been verified by several comparative studies as approximately 81-92% [250]. Despite the abovementioned drawbacks of the current predictive tools, they are an invaluable resource for genetic testing, especially because of their ability to determine when a rare sequence variant may be the cause of a Mendelian disorder. However, the application of only one predictive algorithm could be misleading [251]. HRM technology has been shown and confirmed as a simple and sensitive method, which is in accordance with previously published studies. Thus, application of HRM technology contributed to update of the contemporary spectrum of known genetic sequence variations present in the Czech population. These variants will be important for future targeted mutation screening in Czech COX-deficient children.

5.2 Copy-number variations in 16 patients with COX deficiency

Using a Genome-Wide Human SNP 6.0 microarray chip (Affymetrix), CNVs that affected the gene dosage of whole genes or part of annotated genetic regions and that led to loss or gain were identified in 16 patients. The genetic bases for mitochondrial disorder were found in four unrelated paediatric patients; their clinical manifestation and disease phenotype are summarised in Table 8. In these patients, at least one large and causal deletion was identified.

Table 8 Phenotype and genetic findings of the patients

Patient	Gene and type of mutation	Phenotype	Biomarkers of the disease
P49	***TYMP*** c.261G>T (p.E87D); Chr.22: del49275958_49451008	**Age of onset:** 4-5 years **Clinical presentation:** failure to thrive, cachexia, attacks of abdominal pain and alternation of diarrhoea and constipation due to gastrointestinal dysmotility, extreme nausea and intermittent vomiting, jejunal diverticulosis (X-ray investigation), hypotonic syndrome, general hyporeflexia and muscle hypotrophy, leukodystrophy, polyneuropathy, nowadays (16 years) wheelchair-bound	**respiratory chain enzyme activities:** normal in patient fibroblasts, muscle tissue not available on analysis **others:** mild hyperlactaciduria, increased urinary excretion of Krebs cycle intermediates (fumarate, aconitate), increased levels of uracil, thymine, deoxyuridine and thymidine
P1	***SCO2*** c.667G>A (p.D223N); Chr.22: del49275958_49362964	**Age of onset:** 7.5 months (20 months) **Clinical presentation:** microcephaly, myoclonic movements on extremities, severe hypotonic syndrome, failure to thrive, bilateral ptosis, divergent strabismus, ataxia, regression of psychomotor development, delayed myelination (brain MRI)	**respiratory chain enzyme activities:** isolated deficiency of complex IV in brain ; combined deficiency of complexes I and IV in muscle and liver **others:** elevated lactate in CSF, mild hyperlactaciduria, increased urinary excretion of Krebs cycle intermediates (fumarate, aconitate, 2-oxoglutarate)
P5	***PUS1*** c.[896+2551_1061 delinsATTTTACCA], p.Gly148ValfsX41; Chr.12: del130985486_130991463	**Age of onset (death):** 3 years (5 years 10 months) **Clinical presentation:** muscle hypotony, sideroblastic anemia, hypotrophy, cardiomyopathy, encephalopathy, Pearson syndrome	**respiratory chain enzyme activities:** isolated deficiency of complex IV in liver and fibroblasts, combined deficiency of complexes III and IV in muscle **others:** mild increase in glutamine and glutamate, greatly elevated lactate in CSF, lactacidemia, mild increase in lactate/pyruvate ratio
P61[1]		**Age of onset (death):** 6 years (24 years 8 months) **Clinical presentation:** microcephaly, hypotrophy, normocytic anemia, delayed psychomotor development, myopathy, dystrophy, cachexia, osteoporesis, scoliosis, delayed puberty, short stature, strabismus, arachnodactyly, IgG monoclonal gammopathy	**respiratory chain enzyme activities:** combined deficiency of complexes I, I-III and IV in muscle **others:** elevated alanine and lactate in blood

Table 8 Phenotype and genetic findings of the patients (continued)

[1] Patient 61 was included in the examined group of patients because his disease phenotype resembled the clinical manifestation of patient 5.

5.2.1 Patients with large deletions on chromosome 22q13.33

In patient 49, a maternally inherited 175-kb deletion on Chr.22: g.[(49275958_49451008)del(NCBI Build 36.1)] that spanned 12 genes (*LMF2, NCAPH2, SCO2, TYMP, ODF3B, KLHDC7B, c22orf41, CPT1B, CHKB, LOC100144603, MAPKIP2, ARSA*) was identified, in addition to a paternally inherited point mutation c.261G>T (p.Glu87Asp) in the *TYMP* gene (Table 9). Although the nucleotide substitution c.261G>T has not been described previously, the resulting amino acid replacement p.Glu87Asp has already been characterised [252]. The 175-kb deletion spans 12 genes and corresponds to known CNV-variation_5192, which occurs with almost 7% frequency in control samples [253]. In this patient, the MNGIE diagnosis was biochemically confirmed by diminished thymidine phosphorylase activity in patient lymphocytes. No pathogenic mutation was found in *SCO2* gene.

Table 9 Hemizygous genes on 22q13.33 found in our patients

Gene (OMIM)	Phenotype of the disease (OMIM)	Type of inheritance	Hemizygous in patient
***LMF2* (-)**	-	-	P1, P49
***NCAPH2* (611230)**	-	-	P1, P49
***SCO2* (604272)**	fatal infantile cardioencephalomyopathy due to cytochromec oxidase deficiency (604377)	AR	P1, P49
***TYMP* (131222)**	mitochondrial DNA depletion syndrome 1 (603041)	AR	P1, P49
***ODF3B* (-)**	-	-	P1, P49
***KLHDC7B* (-)**	-	-	P1, P49
***c22orf41* (-)**	-	-	P1, P49
***CPT1B* (601987)**	-	AR in mice	P1, P49
***CHKB* (612395)**	muscular dystrophy, congenital megaconial type (602541)	AR	P1 (partialy), P49
***LOC10144603* (-)**	-	-	P49
***MAPK8IP2* (607755)**	-	-	P49
***ARSA* (607574)**	metachromatic leukodystrophy (250100)	AR	P49

In patient 1, a paternally inherited 87-kb deletion on Chr.22: g.[(49275958_49362964)del(NCBI Build 36.1)] spanning 8 genes (*LMF2, NCAPH2, SCO2, TYMP, ODF3B, KLHDC7B, c22orf41, CPT1B*) was identified by microarrays and confirmed by a real-time PCR copy-number variation assay (Table 9). This deletion corresponds to a known CNV-variation_4139 that occurs with a frequency of approximately 0.4% in the control samples [253]. In combination with this deletion, a novel point mutation, c.667G>A (p.Asp223Asn), was found in the *SCO2* gene. The alignment of SCO2 proteins from multiple species showed that the affected Asp223 is completely conserved. The novel missense *SCO2* mutation was not present in 100 Czech healthy control samples. No pathogenic mutation was found in *TYMP* gene. In muscle and heart mitochondria, a markedly decreased quantity of SCO2 protein was found by immunoblot analysis (Figure 7).

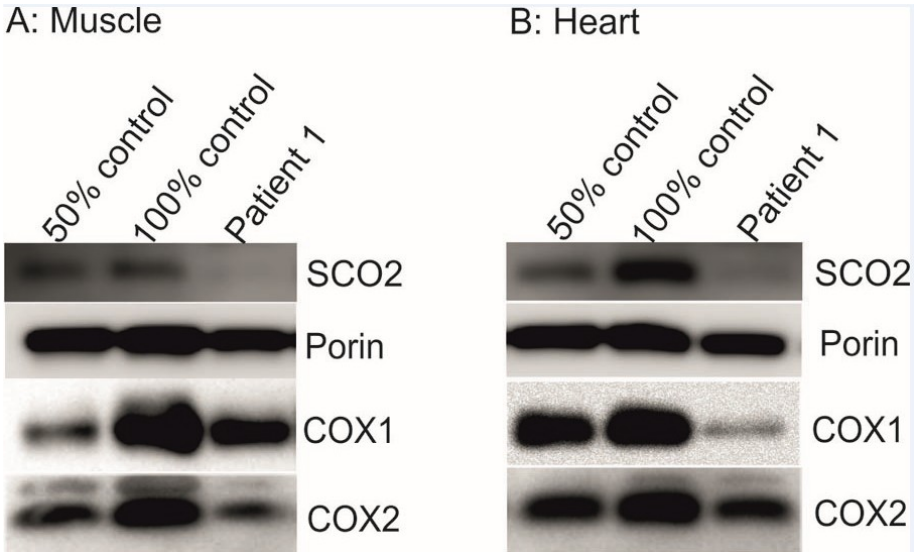

Figure 7 Immunoblot analysis of the muscle and heart mitochondria from the patient with causal mutations in the SCO2 gene

The analysis and image were kindly provided by Mgr. Hana Kratochvílová.

Lower levels of copper content (Table 10) were detected in autoptic liver (10% of control value), brain, heart and muscle (60-70% of control value) and further suggested COX deficiency due to *SCO2* dysfunction in this patient [254]. In addition, the pathogenicity of this mutation was predicted by *in silico* analysis. With regard to knowledge of the human SCO2 protein structure, the newly identified hemizygous mutation p.D223N is likely to affect the coordination of copper (I) by the two cysteines of the CXXXC conserved motif and by the conserved His224 [255]. Furthermore, markedly decreased levels of SCO2 protein and border low copper content found in

tissues of P1 are in accordance with previously reported findings in *SCO2* patients [224,254].

Table 10 Total copper content in autoptic tissues of P1 with SCO2 deficiency (expressed in µg in 1g of dry tissue)

Type of patient tissue	Copper content (µg/g)	
	Patient	Controls
Muscle	1.12	1.74 ± 0.53 (n=9)
Heart	2.2	2.98 ± 0.87 (n=8)
Brain (frontal cortex)	2.26	3.06 ± 1.62 (n=6)
Liver	4.49	49.03 ± 24.01 (n=10)

According to the Database of Genomic Variants (DGV), only three documented copy number variations of approximately 87-175 kb on chromosome 22q13.33 and overlapping the *TYMP* and *SCO2* genes, including CNV-variation_4139 and CNV-variation_5192 found in presented patients, have been identified so far with total frequency 2.2% in control samples [253].

To evaluate the occurrence of CNVs spanning the *SCO2* gene in the Czech population, a real-time PCR copy-number variation assay was carried out in 50 control samples. However, no CNV encompassing *SCO2* was found in the 50 tested Czech control samples. Regarding the deletions found in P49 and P1, only 2 out of 12 affected genes, apart from *SCO2* and *TYMP,* are associated to human disease (Table 9). Although mutations in both *ARSA* and *CHKB* result in autosomal recessive disorders their clinical features, neonatal manifestation of cardiomyopathy with increased creatine kinase activity found in *CHKB* related muscular dystrophy [256] or mental decline specific for juvenile form of metachromatic leukodystrophy, are distinct from symptoms observed in our patients.

5.2.2 Patients with large deletions on chromosome 12q24.33

In patients P5 and P61, a 6 kb homozygous deletion affecting exon 4 of the *PUS1* gene on Chr.12: g.[(130985486_130991463)del(NCBI Build 36.1)] was identified. Analysis of the *PUS1*-cDNA from these patients showed a deletion of the whole of exon 4 and a part of exon 5, along with a 9-bp insertion derived from intron 3. As a result, both patients were homozygous for c.[896+2551_1061delinsATTTTACCA], which lead to a truncation of the PUS1 protein

(p.Gly148ValfsX41). These patients were of Roma origin. The identified pathological deletion was not present in the 80 control DNA samples of Roma origin or in 200 samples of the general Czech population. Presently, the DGV identifies only one wider deletion (CNV-variation_8742) affecting exon 4 of *PUS1*, whose prevalence was assessed to be 0.1% [253]. Thus, the short, homozygous disease-causing deletion identified in our two patients seems to be unique.

5.2.3 Side genetic effects of CNVs and their clinical consequences in our patients

According to recent studies, CNVs are significant genetic factors that can influence the phenotype of every individual [179]. CNV variants can expose sequence differences that have an unclear effect on genes of unknown function, change epigenetic factors or alter the genomic neighbourhood [257-259]. These facts, together with the nuclear and mitochondrial genetic backgrounds, could further contribute to the complex clinical phenotype of mitochondrial disorders [93,257]. The existence of large deletions in mtDNA is well known, whereas deletions affecting nuclear genes are not commonly described in patients with mitochondrial disorders. A homozygous deletion spanning 3 genes, including *NDUFAF2*, was found in a patient with fatal multisystem disorder [260]. Leary et al. reported a de novo 15-30 kb heterozygous deletion of a *SCO2* allele that occurred in combination with a point mutation in a patient with neonatal hypertrophic cardiomyopathy; the patient presented a phenotype similar to that observed of other patients that were compound heterozygotes for a E140K mutation of *SCO2* [261]. Additionally, homozygous or heterozygous deletions of several exons of other OXPHOS-related disease genes were identified by the application of targeted array CGH in several patients with mitochondrial diseases [262,263].

Whether such factors influence the clinical presentation of our four patients is unknown. Due to the rarity of the causal mutations identified in patients P5 and P61, this question cannot be answered because the mutations have not yet been reported in patients suffering from *PUS1* deficiency. There is no satisfactory explanation for the phenotype-genotype variability observed in patients suffering from mitochondrial diseases; however, the clinical phenotypes of affected patients should be compared to note whether the same causal mutations are reported frequently and/or at least occur in the same genes. To date, we have diagnosed the late non-neonatal onset of *SCO2* deficiency in 8 patients [264], but ataxia was not noted in these or other *SCO2* patients

reported. All observed symptoms are typical of *SCO2* deficiency. Based on a review of published cases, hypertrophic cardiomyopathy, a key feature of *SCO2* deficient patients with neonatal onset, is present in approximately 50% of *SCO2* patients with non-neonatal presentation. Considering the biochemical and genetic findings, MNGIE was diagnosed in P49. The manifested symptoms of P49 were similar to those described previously [265]. Although no pathogenic mutation was found in *SCO2* gene of P49, mildly decreased COX activity in the lymphocytes could be a consequence of an accumulation of mtDNA point mutations due to diminished thymidine phosphorylase activity, as reported previously in MNGIE patients [266].

To conclude, it can be hypothesised that improvement in our knowledge of mechanisms based on the presence of variable gene dosage, epigenetic modifications, repetitive sequences, non-coding RNAs, cis- and trans-splicing, diverse penetrance of diseases, the complex effects of the hundreds of loss-of-function variants present in every individual, and general agreement on the classification of particular genetic variants will most likely clarify the phenotypic diversity in humans [267-270]. Notably, consensual guidelines on the classification of all risk and/or pathological genetic variants identified in a patient have not been established yet, which thwarts publicaly accepted meritorious intentions to match a complex patient genotype with the clinically differentiable and annotated disease-phenotype [271-273]. This might explain, why some of variants assessed earlier as deleterious in small cohort studies are nowadays ascertained to be quite common polymorphism and vice versa [274].

5.3 Targeted sequencing of mitochondrial exome in a group of 25 children with COX deficiency

Samples of 25 patients were subjected to targeted sequencing of mitochondrial exome based on a selection of mitochondria-related candidate genes thought to be employed in the maintenance of cellular energy homeostasis. The application of targeted NGS highlighted the cause of mitochondrial disorders in 5/25 patients (20%) who harboured genetic defects in previously characterised genes (Table 11). All the patients harboured at least one mutation that had not yet been characterised. Moreover, causative mutations located in four genes, *AARS2*, *TSFM*, *AIFM1* and *MGME1*, have been documented in very few families across the world. The manifested disease phenotypes of our patients resemble those of recently reported index patients, which is especially important to paediatricians. All the affected genes are listed in Supplementary table 1 (see the Attachment section) and have been recently identified as causes of combined COX deficiency. This fact emphasizes the need for such a systematically updated genetic depository.

The in-depth analysis of NGS data contributed to the identification of probable candidate genes (*ACOX2*, *UQCRH*, *QARS*, *SUCLG2*, *ACBD3*) in 5/25 patients (20%), but the pathogenicity of the suspected sequence variants remains to be confirmed experimentally. In spite of great effort, no candidate gene has been identified in 15/25 patients (60%). The proportion of definite molecular diagnoses yielded by NGS in these preliminary data is slightly lower than documented by other authors [275-277]. This discrepancy could result from a less-strict selection process for the examined group of Czech paediatric patients, though they all presented biochemically confirmed isolated or combined COX deficiency. Unexpected genetic specificity of the investigated group of patients could influence the presented findings, which, when combined with incomplete selection of candidate genes, could lead to coincidental omission of some genes causing primary and/or secondary mitochondrial dysfunction. Moreover, phenotypic overlap typical of patients with mitochondrial disorders could disguise the genuine genetic cause of a complex patient`s clinical presentation [107].

The targeted sequencing size was 2.03 Mb, which spanned exons of 1233 mitochondria-related genes that were covered ≥ 1x (96%), ≥ 10x (83%), ≥ 15x (79%) and/or ≥ 20x (75%). The number of identified exome sequence variants compared to the reference sample was calculated as approximately 1306 per individual patient sample,

of which rare variants occurred in <5% of the overall Czech population and totalled 365 events that resulted in the modification of 269 proteins.

Table 11 Patients with an identified genetic cause of their mitochondrial disease based on next-generation sequencing data

Patient (gender)	Gene (OMIM)	Pathogenic variants	Phenotype of the patients
P16 (female)	***AARS2* (612035)**	**c.[1774C>T];[2188G>A], p.R592W;p.V730M**	**Age of onset (death):** at birth (9 weeks)**Clinical presentation:** hypertrophic cardiomyopathy, anemia, dystrophy, delayed psychomotor development, encephalopathy, failure to thrive **Biochemical findings:** lactic acidosis, hyperglycemia, combined deficiency of COX and complex I in heart and musle **Support of pathogenicity:** clinical phenotype similar to described patients, segregation in family
P21 + one affected sibling (males)	***TSFM* (604723)**	**c.[446G>A];[856C>T], p.Cys149Tyr;Gln286Stop**	**Age of onset (death):** at birth (6 weeks, 8weeks resp.) **Clinical presentation:** hypotonia, severe encephalopathy, hypertrophic cardiomyopathy **Biochemical findings:** lactic acidosis, combined OXPHOS deficiency in muscle **Support of pathogenicity:** decreased mitochondrial proteosynthesis in fibroblasts
46 (female)	***TK2* (188250)**	**c.[209T>C];[416C>T], p.Phe70Ser;Ala139Val**	**Age of onset (death):** 15 months (2 years) **Clinical presentation:** myopathy, hypotonia, hypetrophic cardiomyopathy, leukodystrophy **Biochemical findings:** combined OXPHOS deficiency in muscle **Support of pathogenicity:** segregation in family
P48 (male)	***AIFM1* (300169)**	**c.[1391T>G];[0], p.Leu464Trp**	**Age of onset (death):** at birth (18 months) **Clinical presentation:** hypotonia, encephalopathy, myoclonic epilepsy, brain atrophy **Biochemical findings:** tissue specific deficiency of COX and complex I **Support of pathogenicity:** clinical phenotype similar to described patients, segregation in family
P60 (female)	***MGME1* (615076)**	**c.[794C>T];[971G>A], p.Thr256Ile;Arg324Gln**	**Age of onset (death):** at birth (4 years) **Clinical presentation:** epilepsy, hypotonia, encephalopathy, hyporeflexia **Biochemical findings:** lactic acidosis, tissue specific deficiency of COX and complex I **Support of pathogenicity:** dynamics of mtDNA repopulation in fibroblasts (ongoing)

Newly identified mutations are highlighted in red.

High-throughput NGS has enabled the sequencing of whole DNA or RNA samples in a cost-efficient manner, which may improve the diagnosis and counselling of affected families. Thus, NGS has permitted the discovery of new mitochondrial disease-causing or disease-associated variants [277]. However, NGS facilities are also facing new challenges, particularly in the processing, analysing and interpreting of data. Future improvements in advanced sequencing will most likely address increasing read length and coverage, particularly in genetic regions that include plentiful repeat sequences, to developing more efficient *in silico* predictive algorithms that enable reliable

classification of rare sequence variants and/or variants with dominant effects and enhancing the enrichment procedure [278-281].

Advances in the knowledge of genes involved in mitochondrial homeostasis have been made. For instance, new pathological mutations located in *FLAD1*, *ELAC2*, *SFXN4* and *FBXL4* were reported at the conference "Mitochondrial Disease: Translating biology into new treatments" (Hinxton, UK, 2013). The spatial distribution and function of *LYRM7*, *OPA3*, *MRM2*, *MRM3*, *METTL20* and *COQ4* were also discussed at the conference. This report suggests that the list of genes in MitoCarta must be continually updated. As the existence of new and uncharacterised assembly factors with roles in the biogenesis of individual respiratory complexes are highly probable, whole exome and/or genome sequencing would be the optimal approach to find the molecular bases of COX deficiency in patients. Molecular defects in genes resulting in secondary deterioration of mitochondrial function and/or ultrastructure also require attention [282]. Additionally, future refinement of advanced whole exome or genome techniques will provide information concerning the genetic bases of mitochondrial defects, as well as the regulation of tissue-specific energy demands [15,258,259,283].

5.4 Summary of the clinically relevant findings in the investigated group of paediatric patients

Genetic defects causing isolated or combined COX deficiency were revealed and confirmed in 9/61 (14.75%) patients using DNA array (4/15, 26.7%) and NGS technology (5/25, 20%). The clinical impact of suspected deficiency-causing sequence variants remains to be assessed in 5/25 patients (20%). Consequently, our findings allowed us to provide genetic counselling in nine affected families that had rare pathogenic mutations leading to mitochondrial disease.

6 Conclusions and impact of the PhD thesis

♣ A high-resolution melting assay was designed and validated for the examination of 15 nuclear-encoded genes of cytochrome c oxidase that may be possible causes of COX deficiency. Nine new exonic and intronic variants of COX-related genes were documented, which updated the contemporary spectrum of known genetic sequence variations present in the Czech population. The variants that were likely to be damaging will be important for a future targeted-mutation screen in Czech COX-deficient children.

♣ HRM and predictive methodologies have been shown to be suitable low-cost screening tools for the identification of pathological sequence variants. *In silico* tools appeared to be helpful in the classification of new missense variants. This utility encouraged us to implement the predictive modalities in the routine genetic analysis that is provided by our laboratory. Further improvements are essential to improve the reliability of the predictive tests and studies should evaluate the variant types and their effects on protein structure, given knowledge of an affected COX-related protein function.

♣ SNP DNA array analysis was an attainable laboratory analysis that contributed to the elucidation of causal genetic defects in 4/16 patients. Two patients harboured large heterozygous deletions covering both *SCO2* and *TYMP* genes, in addition to point mutations in the second allele. Two unrelated patients carried 6-kb homozygous deletions affecting splicing of the *PUS1* gene. To evaluate the occurrence of CNVs spanning the *SCO2* gene and exon 4 of *PUS1* gene in the Czech population, a real-time PCR copy-number variation assay was carried out in 50 control samples; such CNVs were not found in any of the samples.

♣ For the purpose of targeted mitochondrial exome testing, we updated the recently published MitoCarta, which covers the genetic regions that are considered to cause mitochondrial disorders. Despite continuous characterisation of further genes implicated in mitochondrial dysfunction, we found our updated list to be a basic but invaluable tool usable for mutation screening of 1233 candidate genes using only a very small amount of patient sample. The preliminary results of next-generation sequencing data analysis found pathological mutations in 5/25 patient DNA samples investigated. Our findings enabled us to identify several highly suspected candidate genetic variants, *ACOX2*, *UQCRH*, *QARS*, *SUCLG2* and *ACBD3*, in 5/25 patients (20%), but their

pathogenicity remains to be confirmed. Overall, our results allowed us to provide genetic counselling for nine affected families (9/61).

7 Attachment - Supplementary table 1

Supplementary table 1 Mutations leading to isolated or combined COX deficiency

Gene	Location	Function of a gene product	Disease phenotype [References]
MTCO1	mtDNA	catalytic subunit of COX, ensuring oxygen binding	therapy resistant epilepsy, multisystem disorder, myopathy and cortical lesions, EXIT/myoglobinuria, MM and rhabdomyolysis, DEAF, SNHL, LHON, SIDA, motor neuron disease, prostate cancer, MELAS-like syndrome, mild EXIT and MR
MTCO2	mtDNA	catalytic subunit of COX, ensuring cytochrome c binding	encephalopathy, myopathy, encephalomyopathy, cortical lesions, lactic acidosis, MM, EXIT/rhabdomyolysis, SNHL, multisystem diorder, developmental delay, ataxia, seizures, hypotonia, DEAF, SNHL, PEG glaucoma, Alpers- Huttennlocher - like syndrome, rhabdomyolysis, LHON, PD risk factor
MTCO3	mtDNA	catalytic subunit of COX, ensuring proton pumping	Leigh syndrome, encephalopathy, encephalomyopathy, MELAS/PEM/NAION, myopathy and myoglobinuria, rhabdomyolysis, LHON, AD, MM and lactic acidosis, sporadic bilateral optic neuropathy, EXIT and APS2 - possible link
MT-TF	mtDNA (622G>A)	mitochondrial translation	late-onset mild myopathy and peripheral neuropathy [284]
MT-TF	mtDNA (616T>C, T>G)	mitochondrial translation	maternally inherited epilepsy [285]
MTTS1	mtDNA (1095T>C)	mitochondrial translation	SNHL [286]
MTTL1	mtDNA (3243A>G)	mitochondrial translation	MELAS [287], Leigh syndrome with PDHC deficiency [288], Barth`s-like syndrome [289]
MTTL1	mtDNA (3260A>G)	mitochondrial translation	MMC [290]
MT-TI	mtDNA (4277T>C)	mitochondrial translation	hypertrophic cardiomyopathy [291]
MT-TI	mtDNA (4308G>A)	mitochondrial translation	CPEO [292]
MT-TI	mtDNA (4290T>C)	mitochondrial translation	progressive necrotising encephalopathy [293]
MT-TI	mtDNA (4300A>G)	mitochondrial translation	maternally inherited hypetrophic cardiomyopathy [294]
MTTW	mtDNA (5514A>G)	mitochondrial translation	neonatal encephalomyopathy [295]
MTTW	mtDNA (5545C>T)	mitochondrial translation	hypetrophic cardiomyopathy with severe multisystem disorder [296]
MT-TN	mtDNA (5728A>G)	mitochondrial translation	multiorgan failure [297]
MTTS1	mtDNA (7453G>A)	mitochondrial translation	fatal neonatal lactic acidosis [298]
MTTS1	mtDNA (7497G>A, 7512T>C, 7472insC)	mitochondrial translation	**m.7512T>C, 7472insC:** myoclonus epilepsy, deafness, ataxia, cognitive impairment [299,300] and diabetes mellitus [301]; **m.7497G>A:** myopathy, SNHL, psychomotor retardation, exercise intolerance [299,300]

Supplementary table 1 (continued)

Gene	Location	Function of a gene product	Disease phenotype
MT-TK	mtDNA (8328G>A)	mitochondrial translation	encephalopathy, EXIT with myopathy and ptosis
MT-TK	mtDNA (8344A>G)	mitochondrial translation	MERRF [302]
MT-TK	mtDNA (8363G>A)	mitochondrial translation	encephalopathy, SNHL, hypertrophic cardiomyopathy [303]
MTATP6	mtDNA (8993T>G)	structural subunit of F1F(o)-ATP synthase	Leigh syndrome [304]
MTATP6	mtDNA (9205delTA)	structural subunit of F1F(o)-ATP synthase	failure to thrive, spastic quadruparesis and microcephalia [163]
MTTS2	mtDNA (12264C>T)	mitochondrial translation	neurodevelopmental delay, myopathy, epilepsy, deafness [305]; severe multisystem disease with cataracts [306]
MTTE	mtDNA (14674T>C, T>G)	mitochondrial translation	reversible infantile myopathy with COX deficiency [204,205]
MTTE	mtDNA (14709T>C)	mitochondrial translation	hydrops fetalis [307]
MT-TT	mtDNA (15923A>G)	mitochondrial translation	newborn cardiopulmonary arrest [308]
MT-TV	mtDNA (1643A>G)	mitochondrial translation	late infantile encephalomyopathy [295]
COX4I2	20q11.21	structural subunit of COX	exocrine pancreatic insufficiency, dyserythropoietic anemia, and calvarial hyperostosis (OMIM 612714)
COX6B1	19q13.1	structural subunit of COX	neuropathy, seizures, ataxia, musle weakness, unsteady gait, visual disturbances (OMIM 220110)
COX7B	Xq21.1	structural subunit of COX	aplasia cutis congenita, reticulolinear, with microcephaly, facial dysmorphism and other congenital anomalies (OMIM 300887)
LRPPRC	2p21	stabilization of COXI and COXIII mRNAs	Leigh syndrome, French-Canadian type (OMIM 220111)
TACO1	17q23.3	translational activator of COXI subunit	Leigh syndrome (OMIM 256000)
COX10 (OMIM 602125)	17p12	biosynthesis of heme a prosthetic group located in COXI subunit	encephalopathy, progressive mitochondrial, with proximal renal tubulopathy
COX15	10q24		Leigh syndrome (OMIM 256000), cardioencephalomyopathy, fatal infantile (OMIM 615119)
SURF1	9q34.2	assisstance in association of COXII subunit with COX assembly intermediate S2	Leigh syndrome (OMIM 256000), Charcot-Marie-Tooth disease [309]
SCO1 (OMIM 603644)	17p13.1	mitochondrial chaperones delivering copper into COXI and COXII subunits, playing role in copper homeostasis	hepatic failure, early onset, and neurologic disorder
SCO2	22q13.33		cardioencephalomyopathy, fatal infantile (OMIM 604377), Myopia 6 (OMIM 608908), SMA-like phenotype [310]
COA5	2q11.2	COX assembly factor	lethal neonatal cardiomyopathy (OMIM 220110)

Supplementary table 1 (continued)

Gene	Location	Function of a gene product	Disease phenotype
COX14	12q13.12	COX assembly factor playing role in COXI assembly	fatal neonatal lactic acidosis wiht dysmorphic features (OMIM 220110)
COX20	1q44	COX assembly factor	mitochondrial complex IV dficiency with ataxia and muscle hypotonia (OMIM 220110)
NDUFA4	7p21.3	reassessed as a new additonal subunit of COX	congenital lactic acidosis, Leigh syndrome [11]
CEP89 (OMIM 615470)	19q13.11	mitochondrial integrity, support of complex IV activity, proper cognitive and neuronal function	delayed development, myopathy, cataracts, severe deafness, facial dysmorphism [311]
NDUFAF5	20p12.1	complex I assembly factor	mitochondrial complex I deficiency with Leigh syndrome (OMIM 252010)
NDUFV1	11q13	structural subunit of complex I	mitochondrial comlex I deficiency (OMIM 252010) [312]
BCS1L	2q33	complex III assembly factor	encephalopathy [313]
FLAD1 (OMIM 610595)	1q21.3	catalysis of the adenylation of flavin mononucleotide FMN into the redox cofactor FAD	muscular hypotonia with lipid storage myopathy and combined respiratory deficiency (Gutierrez-Rios P, et al.; 2013 - in Mitochondrial Disease: Translating biology into new treatment held in a Wellcome Trust Scientific Conference, Hinxton, UK)
FBXL4	6q16.1-q16.3	phosphorylation-dependent ubiquitination, maintenance of mtDNA, networking of mitochondria, distribution of nucleoids	mitochondrial encephalomyopathy with mtDNA depletion and lactic acidosis [314]
FASTKD2	2q33.3	role in regulation of mitochondrial apoptosis	infantile mitochondrial encephalomyopathy (OMIM 220110)
OPA1	3q28-q29	mitochondrial network stabilization, cytochrome c distribution driven by cristae remodeling	optic atrophy 1 (OMIM 165500), optic atrophy plus syndrome (OMIM 125250)
ETHE1	19q13.31	sulfide catabolism	ethylmalonic encephalopythy (602473)
TRMU	22q13	engaged in modification, structural stabilization and function of mitochondrial tRNAs (Glu, Lys, Gln)	liver failure, transient infantile (OMIM 613070)
YARS2	12p11.21	mitochondrial enzyme catalyzing attachment of tyrosine to the corresponding tRNA	myopathy with lactic acidosis and sideroblastic anemia, type 2 (OMIM 613 561)
TK2	16q22-q23.1	mitochondrial DNA synthesis	myopathic form of mitochondrial DNA depletion syndrome 2 (OMIM 609560)
GFM1	3q25	mitochondrial translational elongation factor	combined oxidative phosphorylation deficiency 1 (OMIM 609060)
MRPS16	10q22.1	mitochondrial ribosomal protein	combined oxidative phosphorylation deficiency 2 (OMIM 610498)
TSFM	12q14.1	EFTs enzyme acting during a mitochodrial translation process	combined oxidative phosphorylation deficiency 3 (OMIM 610505)
TUFM	16p11.2	mitochondrial translational elongation factor	combined oxidative phosphorylation deficiency 4 (OMIM 610678)

Supplementary table 1 (continued)

Gene	Location	Function of a gene product	Disease phenotype
MRPS22	3q23	mitochondrial ribosomal protein	combined oxidative phosphorylation deficiency 5 (OMIM 611719)
AIFM1	Xq26.1	flavoprotein essential for apoptotic processess acting on a nucleus and mitochonria	combined oxidative phosphorylation deficiency 6 (OMIM 300816)
C12orf65	12q24.31	translation of mtDNA-encoded proteins	combined oxidative phosphorylation deficiency 7 (OMIM 613559), autosomal recessive spastic paraplegia 55 (OMIM 615035)
AARS2	6p21.1	mitochondrial alanyl-tRNA synthetase acting in mitochondrial mRNA translation	combined oxidative phosphorylation deficiency 8 (OMIM 614096)
MRPL3	3q22.1	mitochondrial ribosomal protein	combined oxidative phosphorylation deficiency 9 (OMIM 614582)
MTO1	6q13	catalysis of mitochondrial tRNA modification	combined oxidative phosphorylation deficiency 10 (OMIM 614702)
RMND1	6q25.1	involved in mitochondrial translation process by putative coordination of assembly or maintenance of mitochondrial ribosome (Janer A, 2012)	combined oxidative phosphorylation deficiency 11 (OMIM 614922)
EARS2	16p12.2	mitochondrial glutamyl-tRNA synthetase 2 acting in mitochondrial translation	combined oxidative phosphorylation deficiency 12 (OMIM 614924)
PNPT1	2p15	enzyme involved in RNA import into the mitochondria	combined oxidative phosphorylation deficiency 13 (OMIM 614932)
FARS2	6p25.1	mitochondrial phenylalanyl-tRNA synthetase 2	combined oxidative phosphorylation deficiency 14 (OMIM 614946)
MTFMT	15q22.31	methionyl-tRNA formyltransferaseacting in initiation of mitochondrial translation	combined oxidative phosphorylation deficiency 15 (OMIM 614947)
MRPL44	2q36.1	mitochondrial ribosomal protein	combined oxidative phosphorylation deficiency 16 (OMIM 615395)
MRPL12	17q25	mitochondrial ribosomal protein	growth retardation and neurological deterioration [315]
LYRM4 (OMIM 613311)	6p25.1	factor of Fe-S cluster biogenesis	combined OXPHOS deficiency with lactic acidosis, hepatopathy, stridor and failure to thrive [316]
SCN1A	2q24.3	structural subunit of neuronal sodium channel	Dravet syndrome (OMIM 607208) [317]
Deletion of *C2orf34*, *PREPL* and *SLC3A1*	2p21	c2orf34 (CAMKMT) - formation of trimethyllysine in calmodulin; PREPL - serine peptidase with suggested facilitation of activation or degradation of neuropeptides and peptide hormones ; SLC3A1 - a component of the renal amino acid transporter	atypical 2p21 deletion syndrome (OMIM 606407) [318]

Supplementary table 1 (continued)

Gene	Location	Function of a gene product	Disease phenotype
IBA57	1q42.13	part of the iron-sulfur cluster assembly machinery in mitochondria	multiple mitochondrial dysfunction syndrome 3 (OMIM 615330)
NFU1	2p13.3	involved in the biogenesis of iron-sulfur clusters	multiple mitochondrial dysfunction syndrome 1 (OMIM 605711) [319]
TYMP	22q13.33	enzyme essential for the nucleotide salvage pathway	mitochondrial DNA depletion syndrome 1 (MNGIE type, OMIM 603041) [320]
POLG1 (OMIM 174763)	15q25	catalytic subunit of mtDNA polymerase	Pearson syndrome, mtDNA depletion syndrome [321], Alpers syndrome [322]
DGUOK	2p13.1	enzyme involved in phosphorylation of deoxyribonucleosides	mitochondrial DNA depletion syndrome 3, hepatocebral type (OMIM 251880)
SUCLA2	13q14.2	subunit of the succinyl-CoA synthetase	mitochondrial DNA depeltion syndrome 5 (OMIM 612073) [323]
MPV17	2p23.3	mtDNA maintenance and stability	mitochondrial DNA depletion syndrome 6, hepatocerebral type (OMIM 256810) [324,325]
TWINKLE	10q24.31	DNA helicase acting in mtDNA replication	mitochondrial DNA depletion syndrome 7, hepatocerebral type (OMIM 271245)
RRM2B (OMIM 604712)	8q22.3	subunit of a p53-controlled ribonucleotide reductase catalyzing the biosynthesis of deoxyribonucleotides	mitochondrial DNA depletion syndrome 8 [326]
SUCLG1	2p11.2	catalysis of the conversion of succinyl CoA and ADP or GDP to succinate and ATP or GTP	mtDNA depletion syndrome 9 (encephalomyopathic type with methylmalonic aciduria) (OMIM 245400)
AGK	7q34	mitochondrial membrane protein involved in lipid and glycerolipid metabolism	cardiomyopathic type of mtDNA depletion syndrome 10 = Sengers syndrome (OMIM 212350)
MGME1	20p11.23	mitochondrial membrane structure and shape, levels of ROS and mitochondrial redox state, mtDNA maintenance	mitochondrial DNA depletion syndrome 11 (OMIM 615084) [54]
ANT1	4q35.1	homodimer in the mitochondrial inner membrane transporting ADP and ATP across the membrane	mitochondrial depletion syndrome 12, cardiomyopathic type (OMIM 615418)

EXIT = exercise intolerance; DEAF = deafness; SNHL = sensorineural hearing loss; SIDA = sideroblastic anemia; LHON = Leber hereditary optic neuropathy; MELAS = mitochondrial encephalomyopathy; PEM = progressive encephalopathy; NAION = nonarteritic anterior ischemic optic neuropathy; MM = mitochondrial myopathy; AD = Alzeimer's disease; APS2 = autoimmune polyendocrinopathy type II; MR = mental retardation; PEG = pseudoexfoliation glaucoma; PD = Parkinson's disease; MMC = maternal myopathy and cardiomyopathy; CPEO = chronic progressive external opthalmoplegia, MNGIE = mitochondrial DNA depletin syndrome 1 [117]

8 References

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9 LIST OF ORIGINAL ARTICLES

Publications *in extenso* that are based on this PhD thesis

Vondrackova A, Vesela K, Hansikova H, Docekalova DZ, Rozsypalova E, Zeman J, Tesarova M: *High-resolution melting analysis of 15 genes in 60 patients with cytochrome-c oxidase deficiency.* J Hum Genet., 2012, 57(7):442-8. **IF = 2.365**

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Publications *in extenso* that are not based on this PhD thesis

Vondrácková A, Tesarová M, Magner M, Docekalová D, Chrastina P, Procházkova D, Zeman J, Honzík T: *Clinical, biochemical and molecular characteristics in 11 Czech children with tyrosinemia type I.* Cas Lek Cesk., 2010, 149(9):411-6. Czech.

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