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PhD thesis summary

Genetické příčiny deficitu cytochrom c oxidázy u dětí

Genetic causes of cytochrome c oxidase deficiency in children

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LIST OF CONTENTS

ABBREVIATIONS

ATP	adenosine triphosphate
cDNA	complementary DNA
Chr.	chromosome
COX	cytochrome c oxidase
CNV(s)	copy number variation(s)
DNA	deoxyribonucleic acid
gDNA	genomic DNA
HRM	high-resolution helting analysis
IGV	intergrative genomics viewer
mtDNA	mitochondrial DNA
NGS	next generation sequencing
OXPHOS	oxidative phosphorylation
P	patient
PCR	polymerase chain reaction
SNP(s)	single nucleotide polymorphism(s)
rCRS	revised Cambridge reference sequence
RFLP	restriction fragment length polymorphism
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis

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. 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

1 INTRODUCTION

1.1 Mitochondrial disorders

Mitochondrial diseases represent a heterogeneous group of disorders that are caused by defects in mitochondrial (mtDNA) and nuclear genes encoding the components of the OXPHOS system, as well as other nuclear genes involved in mitochondrial metabolism and the maintenance of energy homeostasis within a cell [1]. Thus, the proper function of bioenergetics in the mitochondrial compartment is modulated by many factors [2,3,4]. Mitochondrial diseases are one of the most common inherited metabolic deficiencies, with an incidence rate of 1:5000 [5]. Although the onset of mitochondrial disease can start at any age, the first clinical symptoms can occur within the first days of life. Diagnostic tests for mitochondrial disorders in children usually require invasive methods such as muscle, liver and skin biopsies, as they are based on biochemical analyses rather than direct molecular methods [6].

1.2 Cytochrome c oxidase deficiency

COX deficiency is clinically heterogeneous group of disorders that predominantly affect tissues with high-energy demand, raising from isolated myopathy to severe multi-system disease, with onset from infancy to adulthood, which are caused by mutations located in mtDNA and/or corresponding nuclear genes. 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* [7,8]. Deleterious mutations causing primary or secondary COX deficiency can be located in 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. Despite advances in the identification of an increasing number of mutations and genes involved in 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 [9]. 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 [10], although pathological mutations have only been identified in a small fraction of them despite advances in applied research methodologies [11]. 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 [12].

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 MATERIAL AND METHODS

3.1 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.

3.2 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.

3.3 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.

3.4 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.

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 [13]. 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.

3.5 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).

3.6 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.

3.7 *In silico* analysis

The web servers SIFT (http://sift.jcvi.org/) [14], SNAP (https://rostlab.org/services/snap/) [15], PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) [16], MutPred (http://mutpred.mutdb.org/) [17], PMut (http://mmb2.pcb.ub.es:8080/PMut/) [18], PANTHER (http://www.pantherdb.org/tools/csnpScoreForm.jsp) [19] and SNPs&GO (http://snps-and-go.biocomp.unibo.it/snps-and-go/) [20] were used to evaluate the possible pathogenicity of all identified missense substitutions with unknown genetic effects.

3.8 Biochemical and follow-up electrophoretic analysis

3.8.1 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 [21,22].

3.8.2 Thymidine phosphorylase activity

Thymidine phosphorylase activity was measured spectrophotometrically in isolated lymphocytes according to Spinazzola et al. [23]. 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 [24]. 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.

3.8.3 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 [25]. 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 [25].

3.8.4 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).

3.9 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.

3.10 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). 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.

4 RESULTS AND DISCUSSION

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

Prior to high-resolution melting analysis, the presence of mutations was excluded in 60 samples of patient mtDNA using Sanger sequencing. In P25, a new variant of the *MT-CYB* gene (m.15866A>G; p.N374D) was found. The variant was recorded as polymorphic because the assessments of both the western blot assembly profile and the activity of complex III of patient cultured fibroblasts were within the physiologic range. However, a subtle modification in the rate of complex III biogenesis cannot be excluded, as documented by other publications [4,26].

The sensitivity and specificity of HRM analysis are better than those of many conventional methods used to detect mutations [27]. 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 [28,29,30,31]. The HRM methodology was designed and optimised for two COX assembly factors, 13 COX structural subunits and their isoforms. 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 [32,33,34,35]. A total of 70 amplicons covering 65 coding regions were analysed; their length ranged from 191 bp to 565 bp. The 175 homozygous and/or heterozygous genetic variants, which when combined resulted in 152 distinct genotypes, were correctly detected by HRM. 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 [36,37,38,39,40,41]. In this study, internal calibrators and DNA mixing were applied to improve the resolution of individual genotypes for four amplicons of the COX genes. However, the majority of examined amplicons did not require these adjustments.

HRM analysis has expanded the spectrum of known SNPs in *COX*-related genes. In total, nine new sequence variants were documented, of which two 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. This finding was confirmed by PCR-RFLP analysis in

a set of 100 healthy control samples. The remaining seven variants were located in introns of *COX4I2, COX6A1, COX7A1, COX7A2* and *COX10*. From seven patient samples (P4, P8, P12, P17, P29, P33, P39), six rare heterozygous sequence variants located in four nuclear-encoded subunits, *COX4I2* (p.R85W), *COX5A* (p.R71H), *COX6A2* (p.L12V), *COX7A1* (p.K31del), and in an assembly factor, *COX10* (p.V366L, p.R431W), were identified and led to a change in the resultant proteins. *In silico* analysis was applied to differentiate neutral variants from those that affect protein function [42,43,44]. *COX4I2* (p.R85W), *COX5A* (p.R71H) and *COX10* (p.R431W) were classified by predictive bioinformatic tools as extremely rare and pathogenic variants. No mutations in the promoter regions and/or alternative cDNA splice products were detected in *COX4I2*, *COX5A*, *COX7A1* and *COX10* from the patients P8, P12, P17 and P29. Deletions overlapping the *COX4I2*, *COX5A* and *COX7A1* genes were absent in all four patient gDNA samples investigated by microarray analysis.

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% [45]. 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 utilised [46]. The nucleotide sequence data are available in the EMBL database under the WEBIN ID accession numbers HE647854 – HE647864. The newly identified genetic sequence variations present in the Czech population will be important for future targeted mutation screening of COX-deficient Czech children.

I contributed to this study by designing and optimising the HRM methodology, applying the *in silico* prioritisation method, performing genetic methods including the analysis of promoters, cDNA and PCR-RFLP, and writing the final manuscript.

4.2 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.

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. Although the nucleotide substitution c.261G>T has not been described previously, the resulting amino acid replacement p.Glu87Asp has already been characterised [47]. The 175-kb deletion spans 12 genes and corresponds to known CNV-variation_5192, which occurs with almost 7% frequency in control samples [48]. 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.

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. This deletion corresponds to a known CNV-variation_4139 that occurs with a frequency of approximately 0.4% in the control samples [48]. In combination with this deletion, a novel point mutation, c.667G>A (p.Asp223Asn), was found in the *SCO2* gene. Lower levels of copper content 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 [49].

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). 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% [48].

To evaluate the occurrence of CNVs spanning either the *SCO2* gene or exon 4 of the *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 this analysis.

According to recent studies, CNVs are significant genetic factors that can influence the phenotype of every individual [50]. CNV variants can expose sequence differences that

have an unclear effect on genes of unknown function, change epigenetic factors or alter the genomic neighbourhood [51,52,53]. These facts, together with the nuclear and mitochondrial genetic backgrounds, could further contribute to the complex clinical phenotype of mitochondrial disorders [51,54]. 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 [55], 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 [56]. 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 [57].

I contributed to this study by performing the *in silico* prioritisation method, analysing the SNP DNA array data and selecting candidate genes appropriate for further laboratory investigation, performing genetic analyses such as PCR-RFLP and the copy-number variation assay, and writing the final manuscript.

4.3 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. 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 have been recently identified as causes of combined COX deficiency. This fact emphasizes the need for such a systematically updated genetic depository, as we did.

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.

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 [58,59,60]. 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 [61].

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 [62]. 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 [11,52,53,63].

I contributed to this study by assisting in expanding the MitoCarta list of candidate mitochondria-related genes for next-generation sequencing, prioritising deleterious variants

and performing genetic methods such as verification analysis of causative mutations by Sanger sequencing and PCR-RFLP.

5 CONCLUSIONS

♣ 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).

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7 LIST OF ORIGINAL ARTICLES

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