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**Ph.D. Thesis Summary**

# Molecular basis of deficit of $F_1F_o$-ATP synthase and its impact on energy metabolism of cell

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## ABBREVIATIONS

**AA:** Amino Acid
**ADP:** Adenosine diphosphate
**ANT:** Adenine Nucleotide translocator
**ATP:** Adenosine triphosphate
**ATPAF1-2:** ATP synthase mitochondrial F1 complex assembly factor 1-2
**bp:** base pair
**BN-PAGE:** Blue Native Polyacrylamide Gel Electrophoresis
**Cfrm:** confirm
**COX:** Cytochrome c oxidase
**COX10:** Cytochrome c oxidase, assembly factor
**CS:** Citrate synthase
**CV:** Complex V
**DAPIT:** Diabetes Associated Protein in Insulin sensitive Tissues
**DNA:** Deoxyribonucleic Acid
**[Fe-S]:** Iron-Sulfur Cluster
**FMC1:** Formation Of Mitochondrial Complex V Assembly Factor 1 Homolog
**GFP:** Green Fluorescent Protein
**HEK293:** Human Embryonic Kidney 293 cells
**HSP70:** Heat Shock Protein 70
**kDa:** kilodalton
**LHON:** Leber Hereditary Optic Neuropathy
**LS**: Leigh Syndrome
**LP:** Like Pathogenic
**MILS:** Maternally Inherited Leigh Syndrome
**mRNA:** messenger Ribonucleic Acid
**mt:** mitochondrial
***MT-ATP6:*** Mitochondrial gene for subunit a, encoded by mtDNA
***MT-ATP8*:** Mitochondrial gene for subunit A6L, encoded by mtDNA
**mtDNA:** mitochondrial Deoxyribonucleic Acid
**mRNA:** messenger Ribonucleic Acid
**mt-mRNA:** mitochondrial messenger Ribonucleic Acid
**mt-rRNA:** mitochondrial ribosomal Ribonucleic Acid
**mt-tRNA:** mitochondrial transfer Ribonucleic Acid
***MTTK:*** mitochondrial gen, tRNA for lysine
**NARP:** Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa syndrome
**nDNA:** nuclear deoxyribonucleic acid
**OXPHOS:** oxidative phosphorylation system
**P:** Patient
**Pi:** inorganic phosphate
**PiC:** Inorganic Phosphate Carrier
**PTP:** permeability transition pore
**PUS1:** pseudouridylate synthase 1
**RC**: Respiratory Chain
**RNA:** Ribonucleic Acid
**rRNA:** ribosomal Ribonucleic Acid
**ROS:** Reactive Oxygen Species
**SA:** Sideroblastic anemia
**SDH:** Succinate Dehydrogenase
**SDS-PAGE:** Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
**TCA:** Tricarboxylic Acid Cycle
**TMEM242:** Transmembrane Protein 242
**TMEM70:** Transmembrane Protein 70
**tRNA:** transfer Ribonucleic Acid
**VUS:** Variant of Uncertain Significance
**WB:** Western Blot

**ABSTRAKT**

Hlavní funkcí mitochondrií je produkce energie prostřednictvím procesu oxidační fosforylace. ATP syntáza je makromolekulární rotační stroj lokalizovaný ve vnitřní mitochondriální membráně katalyzující syntézu adenosintrifosfátu (ATP) z adenosindifosfátu (ADP) a anorganického fosfátu (Pi).

Mitochondrialní poruchy ATP syntázy představují heterogenní skupinu onemocnění charakterizovanou různou závažností klinického fenotypu s nástupem od narození nebo v pozdějších fázích života. Mutace v mitochondriální nebo jaderné DNA mohou vést k poruše ATP syntázy, a to buď izolovaně, nebo v kombinaci s defekty dalších komplexů systému oxidační fosforylace.

Cílem práce bylo charakterizovat protein TMEM70, asemblační faktor ATP syntázy, a studovat vliv nových variant vedoucích k deficitu ATP syntázy v pacientských vzorcích.

TMEM70 je 21 kDa velký protein vnitřní mitochondriální membrány s orientací obou konců do mitochondriální matrix, který tvoří v membráně vyšší oligomerní struktury. Naše výsledky ukázaly, že absence proteinu TMEM70 vede ke vzniku izolovaného deficitu komplexu V, s přítomností adaptačního/kompenzačního efektu komplexů dýchacího řetězce. Ve svalových mitochondriích byly pozorovány různé stupně deficitu ATP syntázy způsobené extrémně vzácnými heteroplazmatickými variantami v genu *MT-ATP6*. Zatímco varianta m.8851T>C (p.Trp109Arg) vede jen k mírnému snížení množství holoenzymu ATP syntázy, varianta m.8719G>A (p.Gly65*) má za následek výrazné snížení hladiny komplexu V s přítomnosti několika asemblačních intermediátů. Podobně těžký dopad na hladinu ATP syntázy byl pozorován i u nových variant v genech *MTTK* a *PUS1*, což bylo doprovázeno deficitem dalších komplexů systému oxidační fosforylace.

**Klíčová slova: mitochondrie, systém oxidační fosforylace, ATP syntáza, mitochondriální poruchy, mitochondriální DNA, *MT-ATP6*, *MTTK, TMEM70***

**ABSTRACT**

Mitochondria's primary function is to produce energy through the process of oxidative phosphorylation. ATP synthase is a macromolecular rotary machine located in the inner mitochondrial membrane that catalyzes the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi).

The mitochondrial disorders due to ATP synthase deficiency represent a heterogeneous group of diseases characterized by variable severity of the phenotype with onset at birth or later in life till adulthood. Mutations in both, mitochondrial or nucelar DNA encoded genes, may result in ATP synthase impairment, either isolated or combined with deficits of other complexes of oxidative phosphorylation.

The aims of the thesis were to characterize TMEM70 protein, an ATP synthase assembly factor, and to analyze the impact of novel disease variants leading to ATP synthase deficiency in patients´ derived samples.

TMEM70 is a 21 kDa hairpin structure protein localized in the inner mitochondrial membrane, with both termini oriented into the matrix, which forms higher oligomer structures. Our results demonstrated that the absence of TMEM70 protein leads to an isolated deficiency of complex V followed in some stage by adaptive/compensatory effect of respiratory chain complexes. Different severities of ATP synthase deficiency were observed in muscle mitochondria due to extremely rare heteroplasmic variants of *MT-ATP6*. While m.8851T>C (p.Trp109Arg) variant lead only to mild reduction of ATP synthase, m.8719G>A (p.Gly65*) variant resulted in diminished levels of ATP synthase holoenzyme and the presence of assembly intermediates. Similarly, a profound impact on ATP synthase was observed in the case of novel variants in *MTTK* and *PUS1* genes, which was accompanied by deficiency of other oxidative phosphorylation system complexes.

**Keywords: mitochondria, oxidative phosphorylation system, ATP synthase, mitochondrial diseases, mitochondrial DNA, *MT-ATP6*, *MTTK*, *TMEM70***

# 1. INTRODUCTION

## 1.1. Mitochondria and Oxidative phosphorylation system

Mitochondria are semiautonomic organelles with their own deoxyribonucleic acid (mtDNA). They play a central role in energy generation in almost all eukaryotic cells. They produce more than 95% of the cellular adenosinetriphosphate (ATP) from metabolic fuels through the oxidative phosphorylation system (OXPHOS) *(1, 2)*. In addition, mitochondria are involved in the metabolisms of amino acids (AA), lipids and nucleotides, biosynthesis of heme, in the maturation of cellular iron-sulfur clusters [Fe-S]. They are also the site of the tricarboxylic acid cycle (TCA) and key steps of gluconeogenesis and the urea cycle. Mitochondria serve as a calcium storage and play a key role in apoptosis and the production of reactive oxygen species (ROS). Furthermore, mitochondria control proliferation, motility, and redox status of the cell *(3-6)*.

## 1.2. Complex V

Human mitochondrial ATP synthase ($F_1F_o$-ATP synthase, complex V, CV) is a unique macromolecular rotary machine of ~650 kDa located in the inner mitochondrial membrane. It consists of 18 different protein subunits organized in membrane-extrinsic $F_1$ catalytic and membrane-embedded $F_o$ domains, which are connected via peripheral and central stalk *(7-11)*. ATP synthase, adenine nucleotide translocator (ANT), and inorganic phosphate carrier PiC form a structure of ATP synthasome, the mitochondrial ADP-phosphorylating apparatus *(12, 13)*.

Complex V catalyzes the synthesis of ATP from ADP and inorganic phosphate (Pi) in the $F_1$ domain during the final step of oxidative phosphorylation. While the main function of ATP synthase is to synthesize ATP, it can also run in reverse to hydrolyze ATP under certain physiological and pathological conditions. ATP synthase forms dimers and higher oligomers *(14-17)* and thus contributes to the formation of the inner mitochondrial membrane. ATP synthase is also implicated in the formation of the permeability transition pore (PTP), which triggers cell death *(18)*.

The formation of ATP synthase is a not fully understood modular process that requires specific assembly factors and depends on the coordinated expression of the nuclear and mitochondrial genomes. The biogenesis of human ATP synthase is a process featuring the independent formation of several subcomplexes, aided by several assembly factors ATPAF1, ATPAF2, TMEM70, FMC1 and TMEM242 *(19-22)*.

One of the human ATP synthase assembly factor TMEM70 was determined by integrative genomics *(23)*. Its homologs were found in higher eukaryotes but not in yeasts and fungi.

Expression level of TMEM70 is very low as typically for ancillary factors *(22, 24)*. *TMEM70* gene is located on chromosome 8 and encodes 260 AA. The protein precursor (29 kDa) contains mitochondrial targeting N-terminal sequence. Processed TMEM70 is 21 kDa transmembrane protein localized in the inner mitochondrial membrane *(24)*. Mature TMEM70 protein consists of 179 AA *(24)*, contains conserved DUF1301 domain and two transmembrane regions with both termini facing the mitochondrial matrix *(25, 26)*. TMEM70 forms dimers and higher oligomers and stable interaction with ATP synthase subunits were not detected *(25)*. But, recently thanks to new methodological approaches, several studies have demonstrated the presence of TMEM70 and the subunit c interaction *(27-29)*. The absence/defect of TMEM70 protein results in reduced content/activity of ATP synthase but not absolutely. Therefore, TMEM70 has been characterized as an accessory assembly factor for ATP synthase but is not essential for its biogenesis. The role of the TMEM70 protein has been intensively studied in recent years. TMEM70 is localized restricted to cristae where it assembles in large oligomers that interact specifically with subunit c of ATP synthase and provide a scaffold for the assembly of the c-ring before its incorporation into functional complex *(21, 27, 29)*. The study from 2020 proposed that TMEM70 has a special role in the stability of membrane-bound subassemblies or in the membrane recruitment of subunits into the forming complex I *(28)*.

### 1.3. Mitochondrial disorders

Mitochondrial diseases are a clinically heterogeneous group of disorders with impaired mitochondrial function and aberrant energy metabolism. Nowadays, more than 400 genes, either in nDNA or mtDNA, mutations are known to cause mitochondrial disease *(30)*. Clinical symptoms arise in childhood or later in life, affect one organ in isolation or more *(31)*. Mitochondrial disease prevalence in adults is 1: 8 000 and 1: 5 000 in childhood *(32, 33)*

Mitochondrial diseases of nuclear origin are caused by mutations in structural genes of OXPHOS complexes as well as in their specific assembly factors *(34, 35)*. Moreover, nuclear origin defects have been demonstrated in upstream pathways generating substrates for OXPHOS (pyruvate dehydrogenase complex, Krebs cycle, fatty acid beta-oxidation or substrate import) or in genes encoding factors involved in mtDNA expression. The mitochondrial proteosynthesis requires proteins involved in mtDNA maintenance, transcription, RNA processing/maturation, translation and posttranslational modification. Furthermore, all of these proteins need to be correctly targeted and imported into the mitochondria. Thus, any defects in mitochondrial protein import or the structure, caused by aberrant cristae formation or abnormal membrane lipid composition, as well as factors affecting mitochondrial fission/fusion or quality control negatively impact the OXPHOS complexes *(35, 36)*.

Mutations in the maternally transmitted mtDNA occur in structural as well as mt-tRNA, and mt-rRNA genes. More than 600 different pathogenetic variants in mtDNA are involved in human diseases. An overview of all variants in mtDNA is published in the MITOMAP compendium https://www.mitomap.org *(37)*. Clinical and biochemical phenotypes of the patients with mtDNA variants depend on the level of heteroplasmy. Heteroplasmic mutations often have a variable threshold, the level to which the cell can tolerate defective mtDNA molecules *(38, 39)*. When the mutation load exceeds this threshold, metabolic dysfunction and associated clinical symptoms occur.

The mitochondrial disorders impacting OXPHOS complexes in derived cells and tissues can be isolated (defect in single complexes) or combined (impact on multiple complexes). The combined defects of OXPHOS complexes are usually caused by aberrant molecular mechanisms: mtDNA (replication, transcription, RNA processing, and modification, translation, large mtDNA deletions), cofactor biosynthesis (coenzyme Q, [Fe-S] clusters, heme/cytochromes, riboflavin), maintenance of mitochondrial homeostasis (mitochondrial protein import, lipid metabolism, division/fusion, mitophagy/quality control) or supercomplex formation *(40)*.

### *1.3.1. Defects of ATP synthase*

Mitochondrial disorders of ATP synthase are caused by mutations in mtDNA or nDNA. ATP synthase disorders are divided into primary defects caused by a mutation in genes encoding structural subunits or assembly factors and secondary defects caused by mutations in genes related to mitochondrial replication, transcription, processing, modification, or translation.

#### *1.3.1.1. Primary defects of ATP synthase due to mutation in mtDNA*

Two structural subunits of ATP synthase, subunit a, encoded by *MT-ATP6* gene, and subunit A6L encoded by *MT-ATP8* gene, are encoded by mtDNA. Primary disorders of ATP synthase by mitochondrial origin can be caused by mutations in both of them. However, the mutations in the *MT-ATP6* gene are much more frequent. Subunit a and subunit A6L are synthesized from a bi-cistronic mRNA unit. The genes show an overlap of 46 nucleotides. Mutations in this unit can therefore affect either subunit a or A6L, or both.

Currently, over 70 different variants in *MT-ATP6*, 10 various mutation in *MT-ATP8* and 8 in the overlapping region have been reported in MITOMAP compendium *(37)*. The mtDNA defects of ATP synthase typical clinically manifest as neuropathy, ataxia, retinitis pigmentosa (NARP), Leigh syndrome (LS), maternally inherited Leigh's syndrome (MILS), Leber hereditary optic neuropathy (LHON) *(41)* or hypertrophic cardiomyopathy *(42)*. The wide

variability in clinical outcomes likely resides in the levels of heteroplasmy and different distributions of mtDNA mutations in cells and tissues. In addition to; ATP deficiency, defects in ATP synthase may have a number of secondary effects, such as increased production of ROS and changes in upstream metabolic processes, which together unpredictably influence the disease process.

Mutations in mtDNA genes encoding ATP synthase subunits can result in impaired Fo proton channel function, complex stability or protein-protein interactions can be damaged *(43)*. Defects in *MT-ATP6* often affected ATP production *(41, 44-48)*. Steady-state ATP synthase levels were reduced and/or incomplete forms of ATP synthase were present in patient cells/tissues *(49, 50)*. However, the absence of these subcomplexes has also been reported *(51, 52)*.

#### *1.3.1.2. Primary defects of ATP synthase due to mutation in nDNA*

Primary ATP synthase defects caused by nuclear mutations belong to the most severe metabolic diseases, usually manifesting as early-onset mitochondrial encephalo-cardiomyopathies. Up to now, mutations in eight nuclear genes related to primary nuclear defects of ATP synthase were found. Six of them, *ATP5F1A (53-56)*, *ATP5F1E (56, 57)*, *ATP5F1D (58)*, *ATP5MK (59)*, *ATP5PO (56)*, and *ATP5MC3 (56, 60)* encode structural subunits α, ε, δ, DAPIT, OSCP, and c, while the other two *ATPAF2(61)* and *TMEM70 (22)* genes encode specific ancillary factors of biosynthesis of ATP synthase. However, differences can be found in the frequency of mutations. While the first seven types of nuclear ATP synthase defects are extremely rare, various mutations in the *TMEM70* gene are very frequent.

In a group of patients with isolated ATP synthase deficiency, severe neonatal lactic acidosis, and encephalocardiomyopathy, the presence of a mutation in the *TMEM70* gene was identified as the cause of the disease *(22)*. The homozygous splicing mutation c.317-2A>G was found in the second intron preventing the synthesis of TMEM70 protein *(22, 62)*. The patient´s fibroblasts presented decreased ADP-stimulated respiration, decreased ATP synthase activities and significantly reduced amount of ATP synthase. All defects in fibroblasts were corrected after complementation with wild-type TMEM70.

Clinically, TMEM70 protein defect was manifested in patients mainly as hypertrophic cardiomyopathy, 3-methylglutaconic aciduria, lactate acidosis, dysmorphism, hypotonia, ataxia, and psychomotor retardation. Less common symptoms include oligohydramnios, prematurity, low birth weight, intrauterine growth restriction, persistent pulmonary hypertension, Wolff-Parkinson-White syndrome, and hyperuricemia. Since 2008, 27 different mutations in the *TMEM70* gene leading to the clinical phenotype have been described *(63)*.

The study performed on fibroblasts prom a patient carrying the prevalent homozygous splicing mutation c.317-2A>G showed an increased level of complex III and complex IV. Surprisingly, the increase in these enzyme complexes was detected only at the protein level, while not at mRNA level. Thus, the compensatory increase in complex III and complex IV proteins appears to be due to specific adaptive changes in mitochondrial biogenesis that occur during posttranslational phase *(64)*.

Mitochondrial ultrastructure is often impaired in *TMEM70* patient´s cells and tissues, because ATP synthase plays important role in the formation of mitochondrial cristae (see chapter 1.2.6.2). Defective morphological features have been observed in the skeletal muscle mitochondria of patients with two different compound heterozygous mutations c.317-2A>G and c.118_119insGT (p.Ser40Cysfs*11) *(65)*; c.317-2A>G and c.783A>G (p.*261Trpext*17) *(66)* and patients´ fibroblast and myoblasts *(26, 66-68)*. Several swollen and irregularly shaped mitochondria with disruption of the central part and complete loss of cristae were reported. Furthermore, some mitochondria were small with simplified cristae. Reduction of ATP synthase levels results in the absence of cristae and formation of an ”onion-like” mitochondrial ultrastructure characterized by concentric rings of the inner mitochondrial membrane *(66)*. A spectrum of mild to more severe pathological changes have been observed in patients with the prevalent mutation, including giant mitochondria, subsarcolemmal accumulation of mitochondria, globular inclusions, concentric cristae formation, cristae fragmentation, and crystalloid inclusions *(67)*. The ultrastructural morphology of affected mitochondria is restored by complementation *(26)*.

Increased fragmentation of the mitochondrial network, suggesting altered fission/fusion dynamics was observed also in fibroblast patient with compound heterozygous mutation c.317-2A>G; c.349_352del (p.Ile117Alafs*36) and homozygous splicing c.317-2A>G *(66)*.

## 2. AIMS OF STUDY

Mitochondrial diseases represent one of the most common groups of inherited metabolic disorders affecting children and adults *(31, 35)*. Due to the dual genetic control of mitochondria, defects in mitochondrial processes can be caused by mutations in the mtDNA or nDNA. Inheritance of mitochondrial disorders is either maternal (mtDNA), sporadic (de-novo), autosomal recessive, autosomal dominant, or X-linked. Genetic defects affecting mitochondrial functions have so far been identified in more than 400 genes *(30, 35, 69)*.

The aim of this study was to characterize the protein TMEM70, an ATP synthase assembly factor and specifically to characterize the impact of identified novel disease variants leading to primary or secondary ATP synthase deficiency in patients´ cell lines or tissues samples.

**The specific aims:**

A) **To characterize TMEM70 protein, the assembly factor of human mitochondrial $F_1F_o$-ATP synthase**
B) **To study the impact of prevalent splicing homozygous mutation c.317-2A>G in *TMEM70* gene on OXPHOS complexes and mitochondrial ultrastructure**
C) **To study the impact of various *MT-ATP6* variants on OXPHOS complexes and energy metabolism**
D) **To study the molecular and biochemical aspects of selected mitochondrial diseases with complex V deficiency**

## 3. MATERIALS AND METHODS

**Ad published results, see individual articles/manuscripts**

## 4. RESULTS AND DISCUSSION

### 4.1. Results and discussion related to the specific aim A) To characterize TMEM70 protein, the assembly factor of human mitochondrial $F_1F_o$-ATP synthase

Hejzlarová, K., Tesařová, M., Vrbacká-Čížková, A., Vrbacký, M., Hartmannová, H., Kaplanová, V., Nosková, L., **Kratochvílová, H**., Buzková, J., Havlíčková, V., Zeman, J., Kmoch, S., Houštěk, J. (2011). **Expression and processing of the TMEM70 protein.** Biochimica et biophysica acta; 1807: 144-149. IF = 4.843, Quartile Score = Q1 (2011).

The *TMEM70* gene encoding a mitochondrial membrane protein was first identified by integrative genomics in 2006 *(23)*. In 2008, the TMEM70 protein was determined as an assembly factor of mammalian ATP synthase *(22)*. In this study, we investigated the import, localization, sublocalization, and processing of this factor in human cells using GFP- and FLAG-tagged forms of TMEM70 protein.

TMEM70 was found to be synthesized as a 29 kDa precursor protein processed to a 21 kDa mature form. Mitochondrial localization was demonstrated by immunocytochemical colocalization of TMEM70-FLAG, mitochondrial dye (MitoTracker Red), and ATP synthase subunit β. Furthermore, TMEM70 was found only in isolated mitochondria from the cells expressing the TMEM70-FLAG protein, while it was absent in the cytosolic fraction. Moreover, we fractionated the isolated mitochondria by sonication and treated the mitochondrial membranes with $Na_2CO_3$. TMEM70-FLAG was found only in the sediment, indicating its localization in the mitochondrial membrane. The mass spectroscopy analysis confirmed the mitochondrial location of TMEM70 and further indicated low cellular content of the TMEM70 protein as well as ATP synthase assembly factors ATPAF1 and ATPAF2 *(8)*. Although the TMEM70 protein has been described as an ATP synthase assembly factor, in this study we did not identify a direct interaction of TMEM70 with ATP synthase, but we did identify the presence of a dimeric form of the TMEM70 protein. In this study, we only tested the possible interaction of TMEM70 protein with the subunit α/β and possible subassembly complexes in which these subunits are present ($F_1$, $F_1$-c). As later shown, the TMEM70 protein interacts with the subunit c and helps in the assembly of the c8 ring, which is present in the inner mitochondrial membrane *(21, 27-29)*.

**Kratochvílová, H.** *, Hejzlarová, K. *, Vrbacký, M., Mráček, T., Karbanová, V., Tesařová, M., Gombitová, A., Cmarko, D., Wittig, I., Zeman, J., Houštěk, J. * Equal contribution, (2014). **Mitochondrial membrane assembly of TMEM70 protein.** Mitochondrion 15: 1-9. IF = 3.249, Quartile Score = Q2 (2014).

In this study, we focused on description of TMEM70 protein structure, to elucidate its function. Therefore, we characterized the membrane topology of TMEM70 protein in the inner mitochondrial membrane by several independent approaches using GFP, FLAG and MYC-FLAG as the C-terminal tags of the protein.

TMEM70 protein structure was predicted to form a hairpin-like membrane assembly of the protein. Based on the accessibility to membrane impermeable protease (trypsin) and to the dye (Trypan blue) at differently permeabilized mitochondria we demonstrated that TMEM70 has a hairpin structure with the N- and C-termini oriented towards the mitochondrial matrix. Such conformation is also consistent with previous computational predictions *(26)*. Two-dimensional electrophoresis and co-imunnoprecipitation analysis of different tagged forms confirmed our previous findings that TMEM70 protein forms dimers and higher oligomer structures *(24)*. Nevertheless, no evidence of a specific association with the ATP synthase complex or the $F_1$ subcomplexes was found. We have analyzed HEK293 cells expressing either TMEM70-GFP or TMEM70-FLAG alone or coexpressing both constructs simultaneously. Mitochondria were solubilized with Triton X-100 immunoprecipitated with anti-FLAG antibody. These experiments demonstrated that TMEM70 protein forms oligomers.

A key question for understanding the biological function of the TMEM70 protein, however, is to reveal physiological interactions with the ATP synthase components, assembly intermediates or other specific assembly factors. Neither immunoprecipitation nor electron microscopy studies with immunogold labeling uncovers a direct interaction of TMEM70 protein with ATP synthase or its intermediates. And although we tested the interactions with the subunit c by immunocapture of an antibody against ATP synthase, no signal of TMEM70 protein was observed. However, as later shown, TMEM70 interacts with the subunit c and helps its, along with another assembly factor TMEM242, to organize the subunits c in the inner mitochondrial membrane by assembling into larger oligomeric structures *(21, 27)*. Our work has thus provided the first steps towards unraveling the function of this protein.

## 4.2. Results and discussion related to the specific aim B) To study the impact of prevalent splicing homozygous mutation c.317-2A>G in *TMEM70* gene on OXPHOS complexes and mitochondrial ultrastructure

**Štufková, H.,** Hůlková, H., Stránecký, V., Smíšková, Z., Pavrovská, S., Wenchich, L., Hansíková, H., Honzík, T., Mráček, T., Houštěk, J., Zeman, J., Tesařová, M. **The impact of prevalent homozygous splicing c.317-2A>G *TMEM70* mutation on OXPHOS complexes, mitochondrial reticulum, and ultrastructure in patient tissues, fibroblasts, and myoblasts** (manuscript prepared for submission).

This study represents a detailed analysis of the impact of the prevalent homozygous splicing mutation c.317-2A>G in the *TMEM70* gene on the amount, stability, and activity of OXPHOS complexes and mitochondrial ultrastructure in predominantly affected tissues (brain, muscle, heart, and liver), in myoblasts, and fibroblasts.

TMEM70 was found to be involved in the assembly of the c8-ring in the inner mitochondrial membrane *(27)*. In other studies *(21, 28)*, the authors suggested that TMEM70 is not only involved in the assembly of complex V but also has a role in the stabilization and assembly of complex I.

In a set of patient tissues (muscle, heart, liver, brain), steady-state level of individual OXPHOS complexes in mitochondria were determined by BN-PAGE/WB. In all analyzed tissues, a significant deficiency of complex V was detected. The decreased levels of complex I were observed in all patient muscle samples. While, no specific pattern was observed in the levels of other OXPHOS complexes. SDS-PAGE/WB analysis revealed significantly reduced level of all ATP synthase subunits analysed, including subunit c.

In the case of fibroblasts, a compensatory effect was observed in all cases, but this phenomenon was not apparent in all available tissues. In cardiomyocytes, an increase in mitochondrial mass and increased OXPHOS activity was observed based on SDH, COX staining. Increased levels and activities of OXPHOS complexes, associated with compensatory effect, have been previously reported in several mitochondrial defects *(57, 70)*. The adaptive response is probably enabled by post-transcriptional events *(64)* in addition a compensatory effect was also observed in the *TMEM70* knockout mouse model *(29, 71)*.

The mitochondrial ultrastructure was analyzed in heart, fibroblasts and myoblasts. Elongated mitochondria were predominant in control samples, whereas structural changes, including unusually aberrant and sparse cristae, were common in patient samples. Moreover, mitochondria with concentric cristae were also found. Dimerization of complex V plays a pivotal role in cristae formation and thus in determining the morphology of the inner mitochondrial membrane *(17)*. The role of ATP synthase dimers in mitochondria cristae formation was first described in yeast models, where a downregulation of ATP synthase

(Lefebvre-Legendre et al. 2005) or altered formation of ATP synthase dimers due to deficiency of the subunits e or g (Paumard et al. 2002) led to the absence of cristae and formation of concentric (“onion-like”) membrane structures. Both in tissues from TMEM70 patients and in cultured cells, strongly altered mitochondrial cristae and "onion-like" mitochondria were present. Our data confirmed previously published findings *(27, 65-68, 72)*. Similar changes in mitochondrial ultrastructure have also been demonstrated in TMEM70 deficient models *(27, 71)*. However, deficiency of the mitophilin protein has also led to aberrant mitochondrial morphology and the formation of concentric cristae (“onion-like“ morphology) in HeLa cells *(73)*, thus this morphological feature is not exclusive to TMEM70 defects.

It was shown, that in the absence of TMEM70 protein, minimal ammounts of ATP synthase are assembled and therefore the existence of an additional assembly factor was assumed. The hypothesis of a second protein required for the c8-ring assembly was recently confirmed by the Walker’s group *(21)*. Carroll and coauthors showed that assembly of the c8-ring requires not only TMEM70 but also a second protein TMEM242. TMEM242 is directly involved in the assembly of ATP synthase and both TMEM242 and TMEM70 interact specifically with subunit c *(21)*. TMEM70 and TMEM242 have similar overlapping functions, but in addition, TMEM242 may influence the incorporation of subunits a, A6L, DAPIT, and 6.8PL *(21)*. Finally, in our previous study, we obtained gene expression data in a set of 10 TMEM70 patient fibroblasts *(74)*. In this group, we analysed the mRNA expression level of TMEM242. However, data does not show any specific trend in the expression levels of TMEM242 in the patients.

Furthermore, TMEM70 and TMEM242 interact with the subunit c as well as with the mitochondrial assembly intermediates of complex I *(21, 28)*. Nevertheless, this functional connection needs to be further studied as reduction of complex I is not a consistent feature *(29, 64)* including this study. Across various types of patient tissues, a decreased of complex I levels were rather individual and seem to be tissue specific. Moreover, no assembly intermediates were observed.

### 4.3. Results and discussion related to the specific aim C) To study the impact of various *MT-ATP6* variants on OXPHOS complexes and energy metabolism

Honzík, T., Tesařová, M., Vinšová, K., Hansíková, H., Magner, M., **Kratochvílová, H.**, Zámečník, J., Zeman, J., and Ješina, P. (2013). **Different laboratory and muscle biopsy findings in a family with an m.8851T>C mutation in the mitochondrial *MT-ATP6* gene**. Mol Genet Metab 108, 102-105. IF = 2.827, Quartile Score = Q2 (2013).

In this study, we reported a very rare maternally inherited missense m.8851T>C (p.Trp109Arg) mutation in the mitochondrial *MT-ATP6* gene. In 2013 a second family with this mtDNA variant was reported so far. Several additional patients have been reported *(37, 75-77)*.

In our study, clinical symptoms such as a failure to thrive, microcephaly, psychomotor retardation and hypotonia were present in a 3-year-old girl (proband) with a high mtDNA mutational load (87–97%). LS findings on magnetic resonance imaging were documented. Her 36-year-old mother (68% blood heteroplasmy) developed peripheral neuropathy and muscle weakness at the age of 22 years.

We uncover pathological features in the proband´s muscle sample. Enzyme histochemical analysis revealed focal subsarcolemmal accumulation of SDH reaction products in up to 5% of muscle fibers. Typical COX-negative fibers were not observed. Electron microscopy revealed a focal increase in subsarcolemmal mitochondria without altered morphology. The energy-generating capacity of OXPHOS in the muscle sample, indicated diminished OXPHOS activity due to disrupted complex V. Analysis of RC enzyme activities in a muscle homogenate and isolated mitochondria showed normal or even increased activities of complexes I, II, III, I+III, II+III, and IV and CS compared to age-matched controls. Muscle mitochondrial proteins were separated by BN-PAGE. There was a significant decrease in steady-state levels of complex V and the accumulation of its subcomplexes.

In skin fibroblasts, BN-PAGE followed by WB and immunodetection revealed a slight decrease (80% of controls) amount of complex V. However, no assembly subcomplexes were observed. Decreased complex V holoenzyme and the presence of assembly subcomplexes were common phenotypic features of the *MT-ATP6* variant in patient tissues or fibroblasts *(48, 76, 78-81)*. However, studies have been reported where ATP synthase holoenzyme was not reduced, despite high heteroplasty load *(51, 82)*. The inconsistency of complex V assembly in individuals with the same genotype and similar levels of heteroplasmy points to biochemical variability caused by pathogenic variants in *MT-ATP6*. Our result suggests that the treshold effect varies according to the type of material analysed.

Our findings extend the clinical and laboratory phenotype associated with the m.8851T>C (p.Trp109Arg) mutation in the *MT-ATP6* gene. We classified this variant as pathogenic in

2013. However, in 2018 this classification was changed to like pathogenetic (LP). Recently, this variant was indicated as "Cfrm" in MITOMAP database *(37)*, indicating that at least two or more independent laboratories have published report on the pathogenicity of this particular variant. However, this variant has also been reported as "Variant of Uncertain Significance" (VUS), due to the high variability of clinical symptoms in patients with the same variant and a similar level of heteroplasmy *(75, 76)*. Recently, one individual was described who lived to the 9th decade despite carrying 98% m.8851T>C levels, a mutant load. Although this variant has been previously associated with severe pediatric phenotypes *(52, 83)*. However, this may be due to overall unique aspect of each patient and the different levels of heteroplasmy.

**Štufková, H**., Kelifová, S., Kousal, B., Lokvencová, K., Hansíková, H., Zámečník, J., Vaněčková, M., Zeman, J., Lišková, P., Honzík, T., Tesařová, M. **Clinical and laboratory findings in a rare maternally inherited variant m.8719G>A in *MT-ATP6* gene** (manuscript prepared for submission).

In the second study related to the third specific aim, we study the effect of the m.8719G>A variant in *MT-ATP6* (p.Gly65*) on the OXPHOS complexes, in particular on the complex V assembly. This extremely rare variant was found in a 57-year-old patient with cataract, pigmentary retinopathy, hearing loss, and leukoencephalopathy.

Tested tissues (blood, buccal swab, skin fibroblasts, skeletal muscle) showed a variable heteroplasmy load from 9% in fibroblasts to 70% in muscle. Normal or elevated activities of respiratory chain complexes were observed in muscle mitochondria. Analysis of the mitochondrial energy-generating system indicated a complex V deficiency.

Separation of muscle mitochondria by BN-PAGE followed by WB and immunodetection revealed reduced amounts of complex V (approx. 20% of controls) with accumulated subcomplexes, V* ($F_1$-part with c8-ring) and free $F_1$-part (Figure 12A). In addition, steady-state levels of complex IV holoenzyme were also diminished in patient mitochondria (approx. 70% of control. A similar profile was found in a patient carrying 2 bp microdeletion m.9205_9206delTA in mtDNA *(84)*.

The maternally inherited missense variant m.8719G>A (p.Gly65*) has so far been reported only once in a patient (without clinical and laboratory findings) from a cohort of more than 10,000 patients with suspected mitochondrial disease *(85)*. Our findings thus provide for the first time the clinical and biochemical phenotypes associated with this ultra rare pathogenic mtDNA variant.

### 4.4. Results and discussion related to the specific aim D) <u>To study the molecular and biochemical aspects of selected mitochondrial diseases with complex V deficiency</u>

Tesařová, M., Vondráčková, A., **Štufková, H**., Vepřeková, L., Stránecký, V., Beránková, K., Hansíková, H., Magner, M., Gáloová, N., Honzík, T., Vodičková, E., Starý, J., Zeman, J. (2019). **Sideroblastic anemia associated with multisystem mitochondrial disorders**. Pediatr Blood Cancer 66, e27591. IF = 2.355, Quartile Score = Q2 (2019).

Sideroblastic anemia (SA) is a heterogeneous group of inherited or acquired diseases with disrupted iron utilization in erythroblast, ineffective erythropoiesis, and variable systemic iron overload *(86)*. Inherited SA can be caused by mutations in various genes associated with heme biosynthesis, [Fe-S] biosynthesis/transport with the thiamine-transport cluster, structural subunits of the OXPHOS complexes or mutations in mtDNA *(87-94)*.

In a cohort of 421 patients with multisystem mitochondrial diseases, SA was found in 8 individuals. Two patients developed SA with ring sideroblasts at the ages of three and six years, respectively. Both had mitochondrial myopathy, lactic acidosis, and SA due to homozygous 6-kb deletion of all of exon 4, a partial deletion of exon 5, and a 9-bp insertion derived from intron 3 (c.896+2551_1061delinsATTTTACCA) resulting in a putative truncation of the PUS1 protein (p.Gly148ValfsX41). Large deletions represent a novel cause of presumed PUS1 loss-of-function phenotype.

The PUS1 protein is essential for proper postransriptional modification of tRNA mitochndrial proteosynthesis *(95)*. PUS1 converts uridine into pseudouridine. Pseudouridine may have a functional role in tRNAs and may assist in the peptidyl transfer reaction of rRNAs. Mutations in the *PUS1* gene have been associated with mitochondrial myopathy and SA *(87, 96)*.

In our study, SA was also found in a child with heterozygous composed mutations c.610A>G (p.Asn204Asp) and c.674C>T (p.Pro225Leu) in the *COX10* gene that encodes the COX10 protein, an assembly factor for complex IV. The insertion of heme A is a two-step process performed by COX10 *(97)*. Mutations in the assembly factors *COX10* have been reported to cause variable severe phenotypes including lactic acidosis, Leigh syndrome, hypertrophic cardiomyopathy and hypotonia *(98)*.

In our study the activity of respiratory chain complexes III and IV in the muscles of *PUS1* patients was significantly reduced. In addition, the activity of I + III was also reduced in one of them. The patient with mutation in the *COX10* gene showed low activities of complexes I + III and IV in muscles. Reduced activities of complexes I + III and IV were also found in all three analyzed patients with mtDNA deletion. All patients, except one with mtDNA deletion, had very low COX/CS ratios.

Protein analyses of the OXPHOS complexes revealed reduced levels of complex IV in muscle and liver mitochondria in the patient with *COX10* variant. Steady-state levels of complexes I, IV and V were markedly reduced in liver mitochondria of *PUS1* patients. The combined deficiency of OXPHOS complexes was more profound in the muscle mitochondria of these two patients, where reduced levels of complexes I, III, IV, and V were observed. In addition, we also found an increased number of intermediates of complex V assembly in both muscle and liver tissue of *PUS1* patients.

Our results confirm that defects in complex V are primarily caused by defects in structural subunits or ATP synthase assembly factors, but also in genes providing other mitochondrial functions. In this case, we observe a combined defect in multiple OXPHOS complexes in muscle and liver tissue in *PUS1* patients. While the defect in complex IV assembly factor primarily disrupts complex IV biogenesis. Moreover, the presence of ATP synthase assembly intermediates is also evident in liver and muscle. The combined defect was also observed in other *PUS1* patients *(96)*.

**Štufková, H.,** Kolářová, H., Lokvencová, K., Honzík, T., Zeman, J., Hansíková, H., and Tesařová, M. (2022). **A Novel *MTTK* Gene Variant m.8315A>C as a Cause of MERRF Syndrome**. Genes (Basel) 13 (7) IF = 4.141, Quartile Score = Q2 (2021).

ATP synthase disorders can be caused by primary defects in structural subunits or assembly factors, but also by secondary defects in genes important for other mitochondrial functions. These include genes for mt-tRNA encoded by mtDNA. Which are essential for mitochondrial proteosynthesis of all subunits of mitochondrially encoded OXPHOS complexes. Damage to mt-tRNAs commonlly leads to combined defects in the OXPHOS complexes *(99)*.

In this study, we report a novel pathogenic variant in mtDNA in mt-tRNA gene for lysin. Since first description in 1990, the number of pathogenic variants in mt-tRNA genes has steadily increased *(37, 99)*. Mt-tRNA-Lys is encoded by the *MTTK* gene, which is the second most common site of pathogenic variants in mtDNA. To date, 28 pathogenic variants have been described *(37)*.

Our proband, a 39-year-old woman with myoclonus, epilepsy, muscle weakness, and hearing impairment carried the heteroplasmic m.8315A>C variant in the *MTTK* gene with a mutation load ranging from 71% to >96% in the tested tissues.

In the muscle sample, analysis of the capacity of the mitochondrial energy-generating system showed impaired OXPHOS activity with reduced ADP stimulation. The activities of respiratory chain enzymes in isolated muscle mitochondria showed significantly reduced activities of complexes I + III and complex IV as well as the COX/CS ratio. Interestingly, the

activities of RC complexes II and complexes II + III were slightly increased, suggesting a compensatory effect.

Analysis of steady-state muscle mitochondrial protein levels using BN-PAGE followed by WB and immunodetection revealed mildly reduced amounts of complex I and complex V with accumulated sub-complexes, most likely V* (ATP synthase with several subunits c) and $F_1$ domain. In addition, the steady-state level of complex IV holoenzyme was significantly reduced. A similar effect of mutations in the *MTTK* gene on OXPHOS complexes in muscle mitochondria was observed in our previous study *(100)*. Two patients with pathogenic *MTTK* variants (m.8363G>A and m.8344A>G) showed the same pattern of combined OXPHOS complexes deficiency (diminished CIV level, with a profound decrease in CI and CV, and accumulated CV sub-complexes). Interestingly, even though the individual variants are localized in different parts of the mt-tRNA the effect on OXPHOS remains the similar *(100)*.

The variant m.8315A>C, is localized in the linker between the D-loop arm and the anticodon stem, which has not been previously predicted as a mutation hotspot. Based on *MTTK* sequence alignment, there is only a single purine nucleotide, either adenine or guanine, found in this position among 40 species *(101)*. Thus, the substitution of adenine for cytosine at position m.8315 may disrupt interactions that stabilize the secondary structure of mt-tRNA-Lys.

## 5. CONCLUSIONS

The overall aim of the presented thesis was to study the biochemical and molecular genetic aspects of primary and secondary ATP synthase deficiency and its impact on mitochondrial energy metabolism. Furthermore, the study of the structural characteristics of the TMEM70 protein, whose defects are the most common cause of primary nuclear mitochondrial ATP synthase disorders.

The core of the thesis is based on the five published articles by the author and coauthors, extended by two manuscripts prepared for submission. Four specific aims of the thesis have been proposed and each has been addressed in its corresponding section.

The first specific aim focused on the characterizationof the protein TMEM70, an assembly factor of human mitochondrial ATP synthase. It was found that the 21 kDa TMEM70 protein localized in the inner mitochondrial membrane forms a higher oligomeric structure. TMEM70 has a hairpin structure with the N- and C-termini oriented into the mitochondrial matrix. Our results contributed to the later discovery of the function of TMEM70 protein in the assembly of ATP synthase.

Secondly, we were able to characterize the impact of the prevalent splicing homozygous mutation c.317-2A>G in the *TMEM70* gene on OXPHOS complexes and mitochondrial ultrastructure in board spectrum of tissues and cells. Our results demonstrate that prevalent *TMEM70* mutation leads to a certain degree of adaptive/compensatory effect, which is especially evident in fibroblasts, and affects mitochondrial reticulum and ultrastructure. The absence of TMEM70 protein always leads to an isolated deficiency of complex V, but only to the inconsistent formation of a combined defect in OXPHOS complexes.

The third specific aim was to study the effect of various *MT-ATP6* variants on OXPHOS complexes and energy metabolism. Our results extend the clinical, biochemical, and laboratory phenotype associated with two rare variants m.8851T>C (p.Trp109Arg) and m.8719G>A (p.Gly65*) in the *MT-ATP6* gene. Our findings may contribute to further diagnosis of maternally inherited ATP synthase disorders.

In the last section, we focus on the molecular and biochemical aspects of selected mitochondrial diseases with combinated OXPHOS defects including complex V deficiency. We observed combined defects of OXPHOS complexes in tissues of patients with *PUS1* and *MTTK*. The novel results extend the knowledge of secondary ATP synthase defects in combination with defects in other OXPHOS complexes.

In conclusion, the proposed aims of the Ph.D. Thesis were accomplished.

## 6. SUMMARY

- TMEM70 is a 21 kDa mitochondrial protein localised in the inner mitochondrial membrane, has hairpin structure with the N- and C-termini oriented into the mitochondrial matrix, and forms higher oligomer structures.
- The prevalent mutation of the *TMEM70* gene leads to a some stage of adaptive/compensatory effect, which is especially evident in fibroblasts; the absence of TMEM70 protein always leads to an isolated deficiency of complex V, but only to an inconsistent combined defects of OXPHOS complexes.
- The findings extend the clinical and biochemical description of the phenotype associated with two rare maternally inherited variants in *MT-ATP6* gene: m.8851T>C (p.Trp109Arg) and m.8719G>A (p.Gly65*).
- Pathogenic mutations in *PUS1* and *MTTK* genes lead to a specific pattern of combined OXPHOS complexes deficit in tissues´mitochondria.

## 7. LIST OF ORIGINAL ARTICLES

**Publications *in extenso*, that constitutes the basis of the PhD thesis:**

Hejzlarová, K., Tesařová, M., Vrbacká-Čížková, A., Vrbacký, M., Hartmannová, H., Kaplanová, V., Nosková, L., **Kratochvílová, H**., Buzková, J., Havlíčková, V., Zeman, J., Kmoch, S., Houštěk, J. (2011). **Expression and processing of the TMEM70 protein**. Biochimica et biophysica acta 1807, 144-149. IF = 4.843, Quartile Score = Q1 (2011).

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**Other publications *in extenso*:**

Vondráčková, A., Veselá, K., **Kratochvílová, H**., Kučerová Vidrová, V., Vinšová, K., Stránecký, V., Honzík, T., Hansíková, H., Zeman, J., and Tesařová, M. (2014). **Large copy number variations in combination with point mutations in the TYMP and SCO2 genes found in two patients with mitochondrial disorders**. Eur J Hum Genet 22, 431-434. IF = 4.349, Quartile Score = Q1 (2014).

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