Final report of the NKFIK_FK_132812 research project "Investigation of mitochondrial dynamics pathways in our own patients"

During the study period, our research group focused on two areas: understanding the intracellular role of MSTO1 and DNM2 proteins and investigating mitochondrial dysfunction in the context of various pathologies.

I. Investigating the intracellular role of MSTO1 and DNM2 proteins

I.1. Analyzing the intracellular role DNM2 proteins

To examine the role of DNM2, we focused on three main areas: (1) an in vitro silencing model of the DNM2 gene using three different siRNAs in HeLa cells; (2) examining gene expression changes in patients with the DNM2 R369W and R465W pathogenic variants compared to healthy controls; and (3) studying the R372W mutation in the DYN-1 gene (the nematode ortholog of the human R369W variant) in a *C. elegans* model. In each phase, RNA sequencing was performed, and gene expression changes were analyzed. Differential gene expression (DEG) and pathway analyses from previous and current sequencing data were standardized and validated. Interestingly, all experimental setups revealed an enrichment of genes and pathways related to cytoskeletal function, which may help explain how DNM2 mutations affect mitochondrial dynamics and the reduction in mitochondrial fusion observed in patient fibroblasts.

I.1.a. In vitro silencing of the DNM2 gene

In the first year of the project, we optimized and adapted the silencing methods in our laboratory. To study the effects of silencing the DNM2 gene, HeLa cells were transfected with DNM2 siRNAs. Scrambled siRNA was used as the control. We used HeLa cells, which provide a straightforward model for silencing. siRNAs were used at a concentration of 100 nM (specifically for DNM2 s223432, s4212, s4214, Life Technologies). As a transfection reagent, Lipofectamine RNAiMAX (Life Technologies) was used. After transfection, DNM2 mRNA and protein expression were determined by real-time PCR and Western blotting. During the experiments, 6-7 parallel silencing series were performed. Samples with adequate silencing efficiency, as confirmed by RT-PCR and Western blotting, were selected for RNA sequencing. The gene expression level was approximately 10% after silencing compared to mRNA levels from control (scrambled siRNA) and non-transfected HeLa cells, confirming adequate silencing efficiency.

For each RNA sample, the amount and RNA Integrity Number (RIN) were determined using Qubit RNA (Thermo Fisher Scientific) and Bioanalyzer RNA 6000 Nano (Agilent)

assays. The RIN was 10 in all sequenced samples. Library preparation was performed with PerkinElmer reagents using NEXTflex® RNA-Seq Kits according to the manufacturer's instructions. RNA sequencing was conducted on the Illumina NextSeq platform. During bioinformatics analysis, adapter-trimmed, quality-filtered (Q > 30) reads were mapped and quantified using the BaseSpace RNA Express program. Differential expression analysis was performed using the R limma-voom method (with prior screening: minimum count = 3, minimum sample = 3, TMM normalization).

As a first step, a baseline gene expression profile was created by comparing the three parallel untransfected samples. Next, each sample treated with DNM2-specific siRNA was compared to both scrambled control and untreated samples. Only changes in gene expression that showed a significant difference in all tested samples were considered. Using this method, only the downregulation of the FBLIM1 (filamin-binding LIM protein 1) gene expression was identified as the common variance. To test the effect of the Lipofectamine RNAiMAX transfection reagent, we examined the significant changes between scrambled controls and untransfected samples. This analysis revealed that Lipofectamine RNAiMAX significantly altered the expression of 332 genes. Therefore, for further analysis, scrambled siRNA-transfected samples were used as controls, which are more biologically relevant and widely accepted in the literature. Consequently, the observed alterations were considered solely due to the effect of DNM2 siRNAs. For DNM2 samples, the log2(fold change) of DNM2 was below -3 in each transcript run. Following these analyses, a total of 203 gene changes were observed in DNM2-silenced samples. Of these, 12 genes showed very high significance. The FBLIM1, KRT13, KRT19, TMEM139, and TMEM45A genes are involved in cytoskeletal function and trafficking and may indirectly modify mitochondrial dynamics. In DNM2-depleted samples, the expression of three uncharacterized proteins and one antisense RNA gene also significantly decreased. TNNC1 plays a major role in the regulation of muscle function, so the reduced expression may be related to the formation of centronuclear nuclei. CYP4F3 gene expression also significantly decreased. CYP4F3 is a monooxygenase involved in cholesterol and steroid biosynthesis. Its relationship with DNM2 remains unclear. However, the role of steroid biosynthesis in mitochondria suggests a potential link between DNM2 and CYP4F3. Notably, DNM2-positive patients often exhibit endocrine abnormalities, which may be explained by steroid biogenesis dysfunction. GO and KEGG pathway analyses revealed an enrichment of genes involved in serotonin transport and uptake, G-protein signaling, muscle function, focal adhesion, hypertrophic cardiomyopathy, and atherosclerosis.
For validation after silencing in HeLa cells, whole proteome analysis was conducted at the Proteomics Laboratory of the Szeged Biological Research Institute. The yield of the analysis met quality standards. The protein products of the genes with high significance in previous studies were confirmed through validation of the gene expression analysis results.

The publication is currently being drafted and written.

I.1.b. Examining gene expression changes in patients with the DNM2 R369W and R465W pathogenic variants compared to healthy controls

In this work phase, transcriptome analysis of 2 patients and 6 age and sex matched control samples was performed from blood samples. Previously, the patients had been confirmed to have the pathogenic rare variants of DNM2 (R369W and R456W) and both cases had centronuclear myopathy. Both patients had previously been found to have mitochondrial dysfunction on myopathological examination and their fiboblast samples showed a disturbance of mitochondrial dynamics. In this project, RNA sequencing was performed in 2 DNM2 positive patients. RNA was isolated from blood. RNA sequencing was performed using NEXTFLEX™ Rapid Directional RNA-seq kit and the libraries were sequenced on Illumina NovaSeq X Plus platform. Bioinformatic analysis focused on mRNA changes. In the principal components analysis, patients and controls were separated. Transcriptome analysis revealed a more pronounced difference in the patient carrying the R369W variant (Patient1) compared to controls. When considering the common variances in both patients, we found significant changes in 44 genes compared to the mean of the controls. For 17 of these genes (39% of the overall genes), the differences were found in genes associated with cytoskeletal function - PTPRK, COL19A1, WASF3 and 14 genes belonging to the protocadherin gamma subfamily. GO and KEGG-based pathway analyses in patient samples revealed an enrichment of genes involved in mitochondrial function (e.g. ATP synthesis, OXPHOS system), nucleotide transport and neurodegeneration. To confirm our results, whole proteomic analysis of plasma samples from patients and controls was performed at the Proteomics Laboratory of the Szeged Biological Research Centre. Unfortunately, the mass spectrometry did not give us enough data for the analysis, so our experiment was not successful and we could not perform the protein validation.

Both in the in silico DNM2 depletion model and in patients, the changes in the expression of a large number of genes involved in cytoskeletal function raise the possibility that the changes in mitochondrial dynamics that were previously observed in patient derivated fibroblast samples could result from the impairment of organelle movement due to dysregulation of the cell scaffold by DNM2 mutations.

I.1.c. Studying the R372W mutation in the DYN-1 gene (the nematode ortholog of the human R369W variant) in a C. elegans model

In the first year of the project, we commissioned InVivo Biosystems to produce humanised C. elegans transgenic animals. Our first aim was to see if the human DNM2 gene could rescue the dyn-1(-) null mutant worm population; we then used CRISPR/CAS9 to introduce the R369W and R465W mutations into the humanised worm population carrying the DNM2 gene to test the effect of clinically relevant human mutations. However, this

was complicated by the fact that a large percentage of animals were found to be non-viable when the human DNM2 sequence was introduced in the first step of the experimental phase. It is thought that the human DNM2 gene may be toxic to C. elegans, and thus unable to take over the role of the nematode DYN1 orthologue. For these reasons, we turned to investigating the effect of relevant mutations by modifying the corresponding conserved positions of the C. elegans DYN-1 gene. In this context, CRISPR/CAS9 has allowed us to generate a modified strain carrying mutation R372W in the DYN-1 gene, corresponding to the human mutation R369W in the DNM2 gene. Based on our experiments, no marked phenotypic differences were observed between mutant and wild-type *C. elegans*. Staining of the mitochondrial network showed significantly reduced signal intensity in the mutant animals, suggesting significant mitochondrial dysfunction in dyn-1 mutant worms. Transcriptome analysis of mutant and wild-type worms was also performed. The analysis revealed a very marked downregulation shift. Comparison of the significant nematode genes and their human orthologs obtained from DEG analysis of the nematodes with transcriptomic data from the patients described in section I.1.b is currently ongoing.

We plan to publish the results of experiments I.1.b and I.1.c together in the future

I.2. Analyzing the intracellular role MSTO1 and MSTO2P genes

For MSTO1 experiments, although the expression value identified by real-time PCR and Western blot prior to RNASeq showed a significant decrease for all sequenced samples, this was not detectable in the log2 (fold change) values during RNASeq. Several methods were outlined to address this, such as transfection with higher siRNA concentrations, repeated transfection, etc. However, this was not carried out because a 'comment' was received on our 2017 publication (PMID: 28554942) questioned the association of the mutation we described with the clinical symptoms in patients. A French research team has found the same variant in several patients, which we described earlier. As an explanation, it has been suggested that the detected mutation is a relatively common variant of the MSTO2P lncRNA n.83G>A (rs11264409), which shows a very strong homology with the previously reported MSTO1 V8M (rs762798018) missense mutation. After the publication of our study, it was known that MSTO2P (NR_024117.2) is a pseudogene of MSTO1 (PMID: 28618927). Sequence homology revealed that MSTO1 and MSTO2P, both consist of 14 exon, share more than 97.3% nucleotide homology. The isoforms differ in only 143 nucleotides, of which 136 are intronic and 7 are exonic only. One nucleotide difference was found in exons 8, 10 and 11, while exon 9 has 4 nucleotides that distinguish MSTO1 from MSTO2P (https://blast.ncbi.nlm.nih.gov). To clarify the issue, we re-sequenced our patients both by whole exome analysis and Sanger sequencing. Using the original primer, the sequencing repeatedly detected a heterozygous variant of MSTO1, while newly

designed specific primers for intronic different sequences showed that the variation seen was a homozygous n.83G>A alteration of the MSTO2P gene while the MSTO1 homologue was wild-type. The new analysis suggests that mutations in MSTO1 in our patients are not responsible for the decrease in gene and protein expression seen previously in patients' fibroblast cells, and does not provide a clear explanation of how MSTO1 'rescue' could normalize mitochondrial dynamics in this case. The question arises as to how the decrease in MSTO1 expression seen can be explained. One possibility could be that the homozygous gene variant in MSTO2P can regulate MSTO1 expression in lncRNA. Alternatively, it has been shown that in tumors, MSTO2P acts as a long non-coding RNA (lncRNA) that can regulate gene expression by increasing EZH2 expression, thereby promoting cell proliferation and autophagy (PMID: 31081092; PMID: 35346226). To clarify this issue, we performed separate gene silencing and RNA sequencing of MSTO1 and MSTO2P. In this case, the design of gene-specific siRNAs was challenging, as all commercially available siRNA vectors are specific for a region of the gene that shares full homology with the pseudogene.

To differentiate the knockout of the MSTO1 and MSTO2P genes, we designed a specific siRNA vector. Silencing experiments were performed in HeLa cells with MSTO1, MSTO2P, and double transfections, using a control vector (scramble siRNA) and an untreated group as controls. After silencing, RNA and protein isolation, the RNA samples underwent sequencing, while protein samples were used for total proteome analysis. Principal component analysis (PCA) showed a clear separation of the sequenced groups. While MSTO1 exhibited a larger decrease in gene expression compared to MSTO2P during individual silencing, heatmap analysis revealed that the effects of the two genes could still be distinguished. KEGG and GO pathway analysis indicated slight differences between MSTO1 and MSTO2P, with both genes enriched in pathways related to cell division and organellar transport. Due to the insufficient silencing efficiency of MSTO2P (logfoldchange: -0.78), we plan to repeat the experiment using higher siRNA concentrations.

Currently, the analysis of these transcriptomic results is ongoing and we hope that our results will help us to understand the observed mitochondrial dynamical changes in patients and in cell biological models. We plan to publish the results in the near future.

II: Investigation of mitochondrial dysfunction in different disorders

II.1. The role of mitochondrial dysfunction in reproductive endocrinological disorders complicating infertility

In the previous phase of the project, we studied mitochondrial function in insulin resistance (IR), polycystic ovary syndrome (PCOS), and primary ovarian insufficiency (POI). We enrolled 115 women with PCOS and/or IR, and 45 age-matched healthy volunteers. The study cohort is registered on ClinicalTrials.gov (ID: NCT06167135 - POMODORI). Participants were selected based on a clinical picture and family history suggesting multisystemic involvement or significantly impaired carbohydrate metabolism and endocrine parameters. Mitochondrial dysfunction was assessed using plasma biomarkers (GDF-15, FGF-21, and total plasma peroxide) and DNA-based biomarkers for mtDNA deletions.

II.1.a. Investigating the significance of GDF-15 and mtDNA deletions in IR, PCOS and POI

In this study cohort, we analyzed growth differentiation factor-15 (GDF-15) plasma levels, investigating its role as a biomarker for IR and PCOS. Our results showed significantly higher GDF-15 levels in patients compared to controls, particularly in those with BMI > 25 kg/m^2, associated with insulin resistance. GDF-15 levels correlated directly with BMI, and more severe metabolic abnormalities. Elevated GDF-15 and mitochondrial DNA deletions may reflect carbohydrate metabolism disorders. GDF-15 levels were also linked to metformin dose, supporting higher doses for patients with severe metabolic issues. Our findings suggest mitochondrial dysfunction contributes more to disease in the IR-only subgroup than in IR-PCOS patients. Elevated GDF-15 may be related to the severity of insulin resistance and accelerated aging. (PMID: 39456699).

In the next phase, we examined in the same cohort how GDF-15 associates with endocrine changes in female reproduction and the presence of multisystemic symptoms. Thyroid function significantly correlated with GDF-15 levels, and the association between GDF-15 and vitamin D3 was stronger in the mtDNA deletion group, and also the multi-organ involvement was more frequent in them, while mitochondrial dysfunction was strikingly greater in the IR-only and IR-POI groups than in IR-PCOS patients. This study is now ready for submission to the Journal of Clinical Medicine.

II.1.b. The comparison of the main pharmacotherapies for insulin resistance based on transcriptome analysis

In this project phase, we investigated the gene expression response to three pharmacotherapies for IR - metformin, metformin-GLP-1 agonist combination, and inositol vitamin supplementation - compared to healthy controls by RNA sequencing. Bioinformatic analysis revealed significant differences in gene expression between patient groups and healthy controls. Firstly, an overall downregulation majority was observed, which corresponds with other research data in analogous articles. Several functional clusters were identified in order to gain further knowledge of the functions of the differentially expressed genes. The majority of the genes were found to belong to inflammatory pathways as well as to glucose-associated pathways. Additionally, there were more obvious functions, such as B-cell activity or classical insulin signaling genes. The signalling pathway analysis revealed that the GLUT, PI3K/Akt, and Wnt signalling pathways were the most significantly affected among the genes belonging to the aforementioned functions. Furthermore, the gene expression patterns of the GLP-1 agonist-treated group exhibited a greater resemblance to those of healthy controls, suggesting a higher efficacy of metabolic therapy. These findings offer valuable insights into the potential of personalised therapy targeting the GLUT and PI3K/Akt signalling pathways. We plan to validate the genes with the highest significance by real-time PCR shortly. A paper based on these data is being written and will be submitted for publication within the next six months.

II.1.c. The role of plasma peroxide levels and multiple deletions of mtDNA in the background of insulin resistance and PCOS

In our previous experiments, we found mitochondrial DNA deletions in 61.5% of IR-PCOS patients and 8.7% of age- and sex-matched controls, suggestive of mitochondrial dysfunction (PMID: 39456699). Thus, we hypothesised that these deletions were the consequence of increased oxidative stress therefore we measured plasma peroxide levels by the Biomedica OxyStat Assay. The plasma peroxide levels were found to be significantly higher in patients compared to controls, with the highest values observed in the PCOS group. However, the presence or absence of mtDNA deletions did not affect peroxide levels. Higher BMI was associated with slightly higher peroxide levels, although not significantly. Among patients receiving different insulin resistance therapies, those receiving metformin-GLP-1 agonist combination therapy had the highest peroxide levels, probably reflecting the severity of IR. Furthermore, patients with elevated peroxide levels exhibited a higher prevalence of mtDNA deletions. In the transcriptomic analysis, RNA sequencing clearly distinguished patients with and without mtDNA deletions from each other by PCA. Differential expression analysis (DEG analysis) revealed increased expression of mtDNA-encoded genes, as evidenced by the upregulation of oxidative phosphorylation-responsive genes, suggesting a compensatory

response. Furthermore, we identified the upregulation of mitochondria-activated apoptosis through MHC II class genes. In addition, following elevated oxidative stress, we identified genes involved in cell stress signalling. An rRNA gene cluster was also found to be upregulated, connected to the cell's response to elevated oxidative stress. The analysis also revealed several genes associated with apoptosis, autophagy, and mitophagy.It is important to note that the patient cohort under study is associated with IR, and thus, there are also genes connected to inflammatory processes, predominantly in the NF-κB and GPCR signalling pathways, and also to metabolic pathways connected to mitochondrial activity. In addition, significant changes in immune-related genes were identified. Moreover, in order to establish a foundation for further investigation, we sought to identify which genes are implicated in the ageing process. Our research revealed that the majority of these genes are components of the OXPHOS system, with a single gene belonging to the 6mA epigenetic regulation pathway, which is associated with cellular ageing. In order to complete the study, it is intended that approximately 20 additional healthy controls will be enrolled with a view to determine the plasma peroxide level cut-off with greater precision.A paper based on this data is being written and is planned for submission for publication within the next six months.

II.1.d. *Investigation of mitochondrial dysfunction and accelerated ageing in polycystic ovary syndrome with insulin resistance*

Based on our previous findings, we hypothesized that accelerated aging may play a role in the pathomechanisms of IR, PCOS, and POI. Recent research suggests that accelerated aging, where biological age as measured by biological clocks exceeds chronological age, is a feature of many pathologies. Aging is a natural phenomenon, but certain pathological conditions, such as diabetes, neurodegeneration, or chronic stress, can accelerate this process. During normal aging, cells experience oxidative stress, contributing to decreased cell renewal and the development of various diseases. Recent literature indicates that the biological age of individuals with type 2 diabetes, the most common metabolic disease, increases by an average of 12 years compared to chronological age. Similarly, in type 1 diabetes, the average increase in biological age is 16.61 years (PMID: 34773197). Based on these, in collaboration with Prof. Dr. Tibor Vellai, we investigated mitochondrial 6-methyladenine levels in patient and control samples as an alternative method to determine biological age. Our preliminary results reveal that the biological age of our patients differs significantly from their chronological age. This "delta-age" slope is significantly associated with BMI, and the effect of metformin treatment is notably smaller in metformin-treated subjects compared to non-treated individuals (Gal A, Vellai T, Varbíró Sz, Molnar MJ, personal communication).

II.2. Analysis of intracellular pathomechanisms of Leber's Hereditary Optic Neuropathy (LHON) at the RNA level

In collaboration with Semmelweis University's Department of Ophthalmology, we studied mitochondrial dysfunction in a family with the homoplasmic ND1_m.3460G>A variant in Leber's optic neuropathy, observing a phenotypic difference among 1 asymptomatic carrier, 1 affected female, and 1 affected male patient with late-onset symptoms and toxic exposure. We hypothesized that protective factors might influence disease presentation and aimed to show the phenotypic exacerbating effect of toxic exposure, which was investigated by whole transcriptome analysis. After DEG and pathway enrichment analysis mainly OXPHOS-related and inflammatory response were enrichment. A paper based on this data is being written and is planned for submission within the next six months.

II.3. *Investigation of GAA mutations known in the background of Pompe disease*

Pompe disease results from the accumulation of glycogen within lysosomes, attributable to a deficiency of the lysosomal acid-α-glucosidase (GAA) enzyme. The clinical presentation of this disease presents two distinct phenotypes, depending on the level of residual enzyme activity. In this study, we conducted an enzymatic and genetic analysis of Hungarian patients diagnosed with Pompe disease. We identified fifteen different pathogenic variants of the GAA gene, with the c.-32-13 T > G splice site alteration being the most prevalent. When comparing α-glucosidase enzyme activity between homozygous cases and compound heterozygous cases carrying the c.-32-13 T > G variant, the mean GAA activity in homozygous cases was found to be significantly lower. Looking ahead, we intended to perform targeted mitochondrial analyses involving these patients; however, this aspect could not be completed during the project timeline. The findings of this study have been published in a paper (PMID: 34072668) and have also been presented at two conferences.

II.4. *Analysis of the mitochondrial 13513G>A mutation: A case report of 5 Hungarian patients*

The m.13513 G>A (p.D355N) mutation in the MT-ND5 gene has been reported in the literature in the background of Leigh syndrome, MELAS, and MELAS / LHON overlap syndrome. In this study, 537 patients examined for the m.13513 G>A mutation by bidirectional sequencing. DNA isolation was performed in 390 cases from blood and in 147 cases from postmitotic tissue (muscle, urinary squamous cell). Among the studied

patients, the m.13513 G>A mutation was detected in 5 cases. The heteroplasmy ratio was in 50 - >95%. Clinical symptoms ranged from a broad spectrum of phenotypes such as Leigh syndrome, ataxia, strabismus, neuropathy, visual impairment, and renal failure. In four cases, the disease was associated with early onset Leigh syndrome, while in one patient, clinical symptoms manifested in adulthood with multisystemic involvement with severe visual and renal impairment and hypoacusis. The m.13513 G>A mtDNA alteration was present in about 1% of the total investigated cohort, while in the cases examined from postmitotic tissues this proportion was found to be 2.88%.

II.5. Investigation of adverse pregnancy outcomes in rare autosomal trisomy

In recent years, in addition to the project's main aims, the sensitivity and specificity of genome-wide non-invasive prenatal tests (NIPT) have been analysed chromosome-by-chromosome using meta-analysis (PMID: 39499701). We then focused on false positive NIPT results to determine their association with adverse pregnancy outcomes. Our meta-analysis summarising the false positive results has been finalised and is currently under internal review, so publication is expected in the near future.

During this project period, 3 PhD students and 6 TDK students have been supervised. The project resulted in 5 scientific publications (PMID: 37431815; PMID: 37431817; PMID: 34072668; PMID: 39456699; PMID: 39499701). Two publications are expected to be submitted soon. Further 4 papers are currently in the process of being written. The results of the project were presented at 8 international and 23 national conferences. During the project, a total of 14 TDK students joined the research team, of which 3 students were nominated in TDK competitions. In total, we have written 9 Master's theses and 2 Rector's dissertations based on the scientific findings.

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Aniko Gal, PhD
Principal investigator