Case report

Pathology of skeletal muscle fibres and small blood vessels in MERRF syndrome: an ultrastructural study

Paulina Felczak[1], Iwona Stępniak[2], Paweł Kowalski[3], Tomasz Stępień[1], Teresa Wierzba-Bobrowicz[1]

[1]Department of Neuropathology, Institute of Psychiatry and Neurology, Warsaw, Poland
[2]Department of Genetics, Institute of Psychiatry and Neurology, Warsaw, Poland
[3]Laboratory of Molecular Genetics, The Children's Memorial Health Institute, Warsaw, Poland

Our studies concerned skeletal muscle biopsy specimens from a patient with clinically suspected MERRF syndrome, confirmed by genetic tests showing the presence of point mutation in the m.8344A> G in the *tRNALys* gene. Ultrastructurally, extensive damage of mitochondria in skeletal muscle fibres was observed, including the presence of two types of mitochondrial inclusions. Mild damage of mitochondria was revealed in small blood vessels and the presence of calcium deposits in the vascular walls were observed. The differences in mitochondrial damage may be related to different origin and expenditure of biologically useful energy in these cells.

Key words: MERRF, A8344G mutation, abnormal mitochondria, ultrastructure, calcium precipitate.

Introduction

Mitochondria are intracellular organelles that produce energy through processes involving oxygen. They consist of a double-membrane system that envelops the mitochondrial matrix compartment. Mitochondria are key players in cellular ATP production via oxidative phosphorylation (OXPHOS). They are also responsible for fatty acid oxidation, heme biosynthesis, apoptosis induction, heat generation, and calcium homeostasis [1].

Mitochondria are found in all human cells except mature erythrocytes. The largest numbers of mitochondria occur in the most metabolically active cells, such as skeletal and cardiac muscles, liver, and brain [2, 3, 4, 5].

Mitochondria contain their own genetic material. They are under the dual genetic control of both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Because mitochondria were originally derived from bacteria, the DNA inside the mitochondria differs from the DNA inside the nucleus. Mitochondrial DNA is a circular molecule of 16.5 kb, encoding 13 polypeptides, 22 transfer RNAs (tRNA), and two ribosomal RNAs (rRNA) [6]. The 13 polypeptides are subunits of a series of large enzyme complexes necessary for ATP production via oxidative phosphorylation [7]. This process depends on five multi-subunit polypeptide complexes (I-V) located within the inner mitochondrial membrane. Mitochondrial DNA encodes for four of five enzyme complexes. It codes for seven subunits of complex I (NADH dehydrogenase), one of complex III (cytochrome c reductase), three of complex IV (cytochrome c oxidase), and two of complex V (ATP synthase). Complex II (succinate dehydrogenase) is encoded by nuclear DNA only [8].

Defects of mitochondrial metabolism cause a wide range of human diseases [9]. Mitochondrial disorders are caused by mutations in the mtDNA, or nDNA,

resulting in impaired respiratory chain activity or oxidative phosphorylation. Nuclear gene defects may be inherited in an autosomal recessive or autosomal dominant manner. Mitochondrial DNA defects are transmitted by maternal inheritance. Mitochondrial DNA deletions generally occur *de novo* and thus cause disease in one family member only, with no significant risk to other family members. Mitochondrial DNA point mutations and duplications may be transmitted down the maternal line [10]. Phenotypically, mitochondrial disorders present as single or multi-system diseases, with onset between birth and senescence [11]. Mitochondrial disorders are manifested in tissues with high-energy demand, being aggravated by fever, infection, stress, or certain drugs [12]. Systems or organs most frequently clinically affected by these disorders include the peripheral nervous system, the central nervous system (CNS), endocrine glands, heart, ears, eyes, gastrointestinal tract, liver, kidneys, bone marrow, skeletal muscles, and dermis [13]. It has recently been shown that arteries may also be affected, although rarely, and this pathology is known as mitochondrial arteriopathy [14].

The central nervous system may be affected alone or frequently together with skeletal muscles, for which the term "encephalomyopathy" has been coined [5, 15, 16].

The most common clinical presentations of mitochondrial encephalomyopathy are: mitochondrial encephalomyopathies, lactic acidosis, stroke-like episodes, (MELAS) Leigh disease, Kearns-Sayre syndrome (KSS), and Myoclonic Epilepsy with Ragged Red Fibres (MERRF) [8, 10].

MERRF syndrome is an acronym of myoclonic epilepsy associated with ragged-red fibres [17], and it was first described by Tsairis *et al*. in 1973 [18, 19]. Typical manifestations of the disease, such as: myoclonus, generalised epilepsy, cerebellar ataxia, and ragged-red fibres (RRF) on muscle biopsy are adopted as the MERRF diagnostic criteria [17]. Other multisystemic alterations can also be found, e.g. deafness, exercise intolerance, dementia, peripheral neuropathy, short stature, abnormal cardiac conduction, cardiomyopathies, ophthalmoparesis, pigmentary retinopathy, and lipoma [19, 20, 21].

MERRF syndrome is induced by pathogenic mutations in the mtDNA. It is known that the mt-tRNA Lys point mutation exchanging an A base for a G base at nucleotide position 8344 in the mt-tRNA gene *MTTK* (https://www.omim.org/entry/545000) is most commonly responsible for all MERRF conditions in 80% of patients. Other mutations in mitochondrial genes, including but not limited to: m.8356 T>C, m.8361 G>A, and m.8363 G>A, account for another 10% and m.611 G>A and m.15967 G>A for the other 5%. The remaining mutations, for example m.611 G>A and m.15967 G>A, account for less than 5% of cases, but up to 10% of MERRF patients still have no identifiable mutations in mtDNA [19, 21].

There is no single criterion of standard diagnostic test for mitochondrial disease. A comprehensive investigation, including neuroimaging, metabolite profiling, as well as histological, biochemical, and genetic analyses of muscle biopsy, is often necessary to make a definitive diagnosis [22]. In addition, the ultrastructural studies should also be carried out.

The aim of our study was to compare pathology of skeletal muscle fibres and small blood vessels in MERRF syndrome.

Material and methods

Material derived from the First Polish Brain Bank in the Institute Psychiatry and Neurology in Warsaw.

The ultrastructural investigations were performed on the muscle biopsy samples derived from biceps. For electron microscopic evaluation, small fragments of tissues were fixed in 2.5% glutaraldehyde solution in cacodylate buffer pH 7.4, and postfixed in 1% osmium tetroxide solution in the same buffer. After dehydration in a graded ethanol alcohol series and propylene oxide, specimens were embedded in Spurr resin. Semithin sections were stained with toluidine blue to choose appropriate areas. Ultrathin sections were contrasted with uranyl acetate and lead citrate. The sections were examined and photographed with transmission electron microscope (TEM) JEOL model 1400 in the Nencki Institute of Experimental Biology PAS in Warsaw.

The molecular experiment involved three stages:

1. Isolation of total DNA from peripheral blood using phenol–chloroform standard procedure.
2. PCR analysis. To amplify the fragment of 640 bp (base pair), two primers corresponding to mitochondrial DNA (mtDNA) positions nt 7960-7979 (forward) and nt 8640-8621 (reverse) were used. The conditions for PCR amplification were as follows: 35 cycles of denaturation (94°C for 1 min); annealing (63°C for 1 min); and extension (72°C for 1,5 min). PCR product was separated on a 1.5% agarose gel and purified using QIAquick PCR Purification Kit (Qiagen).
3. Direct sequencing analysis performed on ABI PRISM 3130 DNA Analyzer (Applied Biosystems®). The nucleotide sequences were compared with the Revised Cambridge Reference Sequence (GeneBank number: NC_012920).

Molecular data

Mutation A8344G tRNALys (*MTTK* gene) in the mitochondrial DNA was revealed in our patient. The analysed mitochondrial DNA was collected from

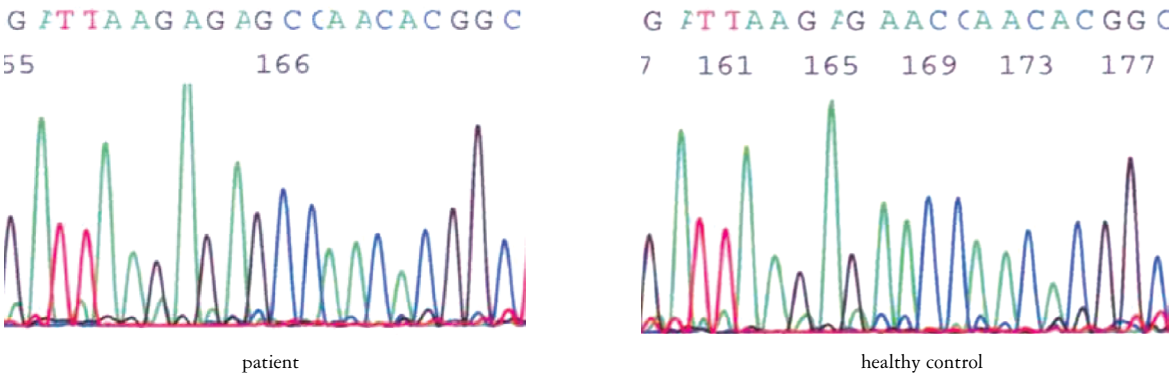

Fig. 1A, B. *MTTK* gene (forward mtDNA strand): m.8344A>G mutation in the patient versus healthy control

peripheral blood. It is a heteroplasmic form of mutation with a significant predominance of abnormal alleles (approximately 95%) in the *MTTK* gene sequence (the whole gene, comprising nucleotides in positions m.8295-8364, was investigated). The result indicates with high probability that A8344G mutation is responsible for the presence of MERRF syndrome in our patient. Genetic tests were carried out in the Department of Medical Genetics, Molecular Genetics Laboratory, The Children's Memorial Health Institute in Warsaw. Figure 1A, B presents the sequence electropherogram of m8344A>G mutation.

Case report

A 30-year-old female patient was admitted to our Department of Neurology because of suspected mitochondrial encephalopathy. The patient became ill when she was 22 years old. For several years, she suffered from severe migraine headache with vomiting. At that time, she was also suffering from severe dizziness and imbalances that affected her movement and her posture. The patient felt general weakness and drowsiness. The patient had been diagnosed with hypothyroidism, subclinical adrenal insufficiency, menstrual disorders, and hyperprolactinemia and therefore was under the care of an endocrinologist. She had also required treatment for severe hypertension. She was characterised by growth hormone deficiency (153 cm), poor weight gain (35 kg), and creatine kinase (CK) of about 132 U/l. The patient had been diagnosed with bilateral hearing loss (hearing aid in the right ear). Ophthalmological examination, performed due to progressive visual acuity and blindness of the left eye, revealed bilateral neuropathy of the optic nerve. Cerebral computed tomography (CT) revealed a lipoma in the corpus callosum. In magnetic resonance imaging (MRI) no abnormalities were found. The MRI focused on the evaluation of the pituitary gland, performed due to the short stature of the patient, and showed on T1-weighted image (T1W1) a pituitary microadenoma measuring 4x4x4 mm. The patient was referred to the Genetic Clinic where, on the clinical picture, mitochondrial cytopathy was suspected. Additionally, the patient had a positive family history with mitochondrial maternal transmission. Several family members presented walking and speech problems; in one of them severe myoclonic epilepsy was observed. Phenotype of proband and family history indicated MERRF syndrome. Clinical diagnosis was confirmed by a molecular test in the proband and then in seven family members. All have been covered by genetic counselling.

Results

Ultrastructural examinations were performed on skeletal muscle fibres and small blood vessels (capillaries and arterioles). Skeletal muscle pathology was compared to the pathology of small blood vessels. In skeletal muscle fibres we observed mitochondrial proliferation and pleomorphism, as well as structural fibre defects with changes of different phenotypes including fibre fragments or entire muscle fibres. There were mild changes in the number and morphology of mitochondria in the capillaries and arterioles. In small vessels, damage to the endothelium, pericytes, smooth muscle cells, and the presence of calcium deposits in the arteriolar walls were revealed.

1. Skeletal muscle

In the skeletal muscle fibres the mitochondria most frequently accumulated on the periphery, near the sarcolemma (Figs. 2, 3, 5) and in the deeper regions of the fibres. In the damaged mitochondria, crystalline mitochondrial inclusion of type I (Figs. 2-4) or type II (Fig. 5) were visible. Mitochondria often differed in size and morphology from mitochondria with the appearance characteristic of skeletal muscle fibres. Among them there were mitochondria

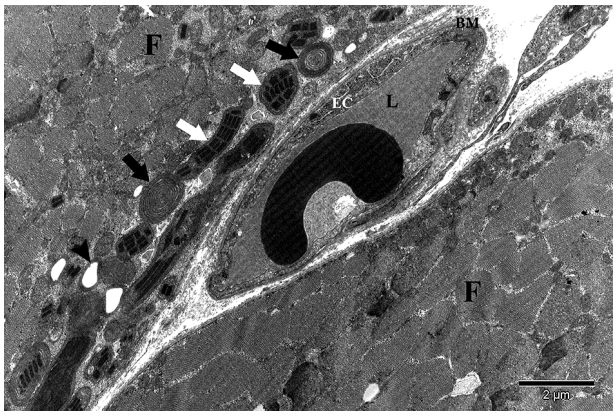

Fig. 2. A cluster of the damaged mitochondria with: crystalline inclusions of type I (white arrows), concentric systems of internal membranes (black arrows), and vacuoles (arrowhead) in the skeletal muscle fibre (F). A capillary between muscle fibres. L – lumen, EC – endothelial cell, BM – basement membrane. Original magnification 12,000×

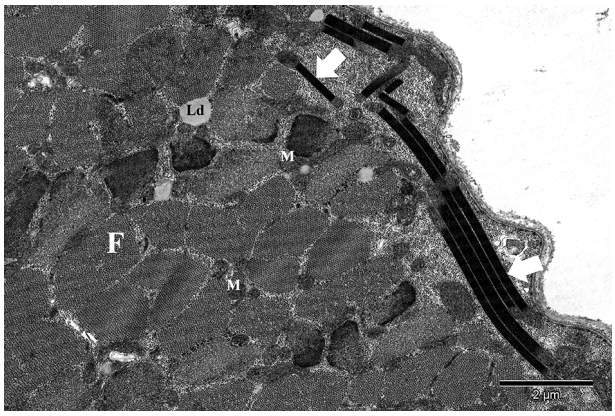

Fig. 5. A cluster of mitochondria with crystalline type II inclusions (arrows) in the skeletal muscle (F) fibre, M – mitochondria, Ld – lipid droplet. Original magnification 15,000×

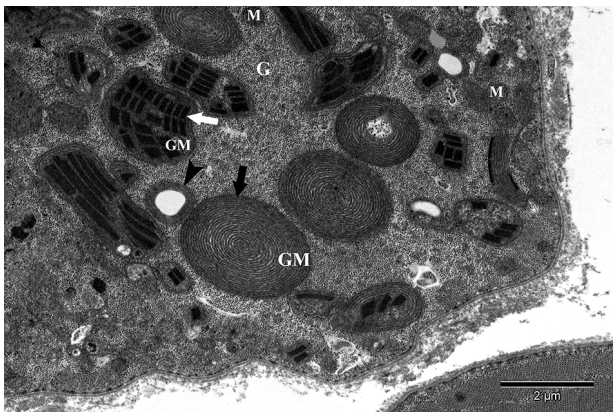

Fig. 3. Mitochondrial pleomorphism in the skeletal muscle fibre. Giant mitochondria (GM) with crystalline inclusions of type I (white arrow), with concentric membrane systems (black arrow) and mitochondria with vacuoles (arrowhead), M – mitochondria, G – glycogen clusters. Original magnification 15,000×

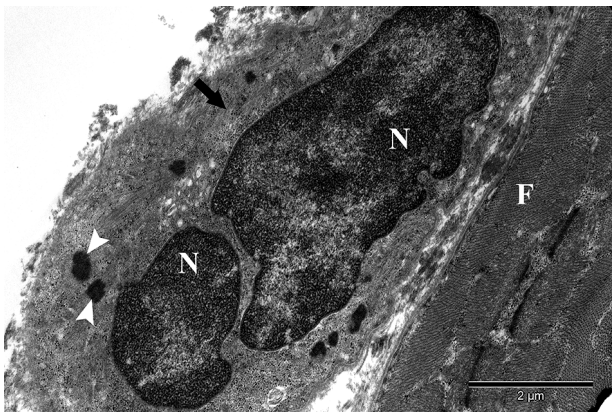

Fig. 6. A fragment of the skeletal muscle fibre with a fused myofilament structure (arrow), with cell nuclei (N), with rods-like structures (arrowheads), and muscle fibre (F). Original magnification 20,000×

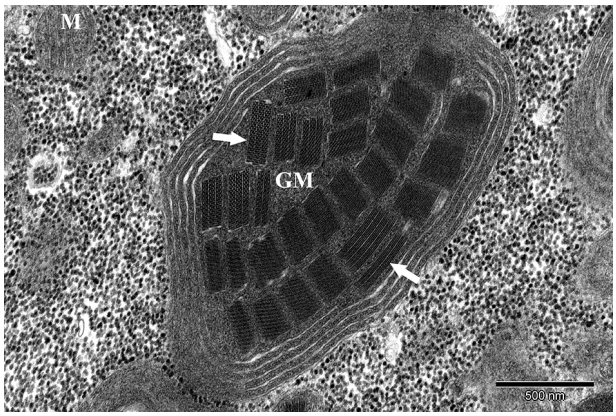

Fig. 4. Giant mitochondria (GM) with numerous crystalline inclusions of type I (arrows), M – mitochondria. Original magnification 60,000×

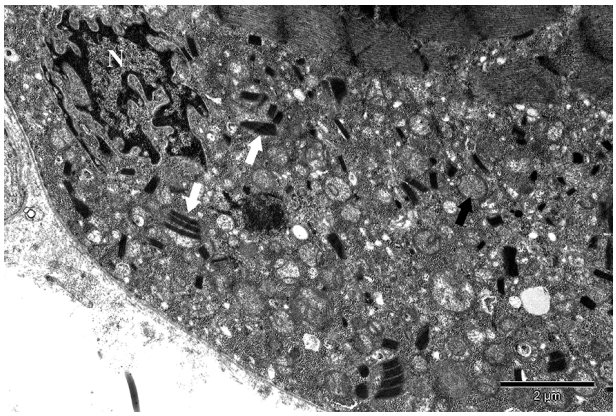

Fig. 7. Degenerated skeletal muscle fibre. Visible damaged mitochondria with cavities of cristae (black arrow) and with crystalline inclusions (white arrows), cell nucleus (N) divided into fragments. Original magnification 15,000×

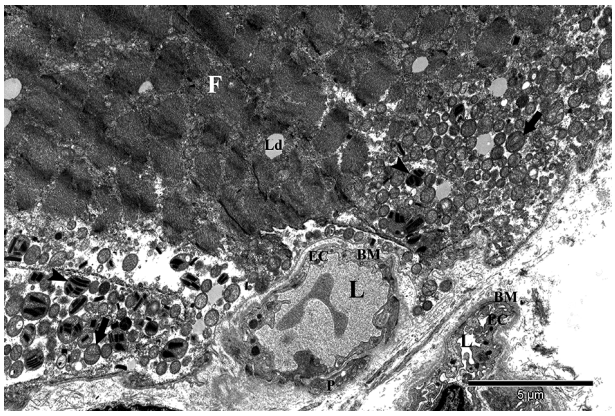

Fig. 8. Skeletal muscle fibre (F) and fragments of degenerate fibres filled with numerous defective mitochondria, with crystalline inclusions (arrowheads) and with cristae (black arrows). In the vicinity of the fibres visible capillaries: L – lumen, EC – endothelial cell, P – pericyte, BM – basement membrane, Ld – lipid droplet. Original magnification 8000×

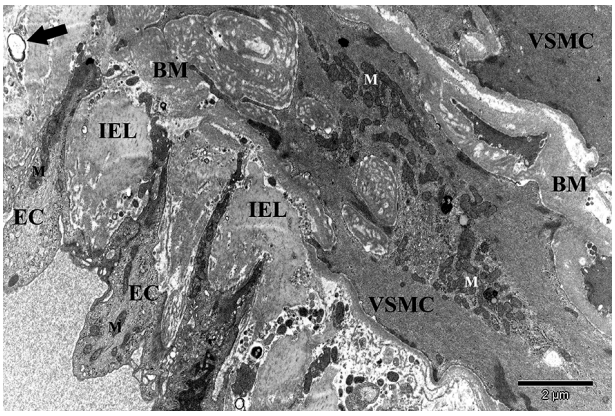

Fig. 11. Fragment of arteriole: mitochondria (M) in damaged vascular smooth muscle cells (VSMC) and proliferation endothelial cell (EC), damaged, mineralised, and thickened basement membranes (BM) and internal elastic lamina (IEL), precipitates calcium in the areas of damaged membranes (arrow). Original magnification 12,000×

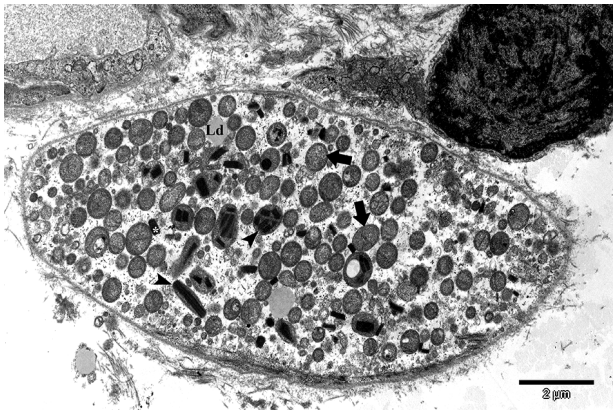

Fig. 9. Residual skeletal muscle fibre. In the electronically bright area surrounded by the membrane, numerous damaged mitochondria are visible without cristae (black arrows), with crystalline inclusions (arrowheads) and crystalline inclusions outside the mitochondria (white star), Ld – lipid droplet. Original magnification 12,000×

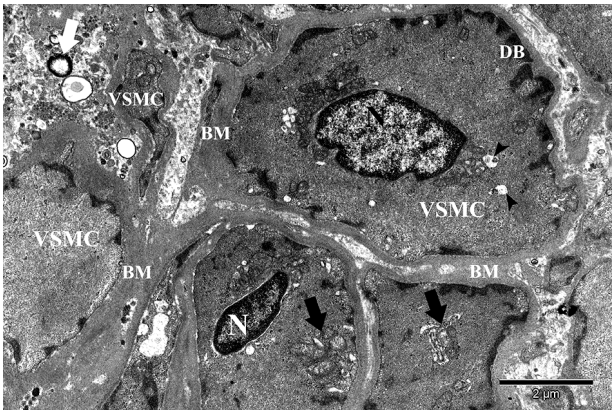

Fig. 12. Vascular smooth muscle cells (VSMC) on cross-sections. Nuclei (N) centrally located, swollen mitochondria (black arrows), vacuoles (arrowheads), and dense bodies (DB). Basement membranes (BM) thickened with structural cavities and oval precipitates of calcium (white arrow). Original magnification 15,000×

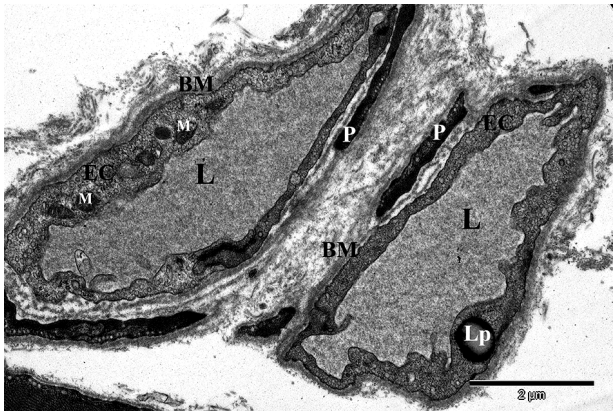

Fig. 10. Capillaries on the cross-sections: L – lumen, EC – endothelial cell, P – pericyte, M – mitochondria, Lp – lipofuscin, BM – basement membrane. Original magnification 20,000×

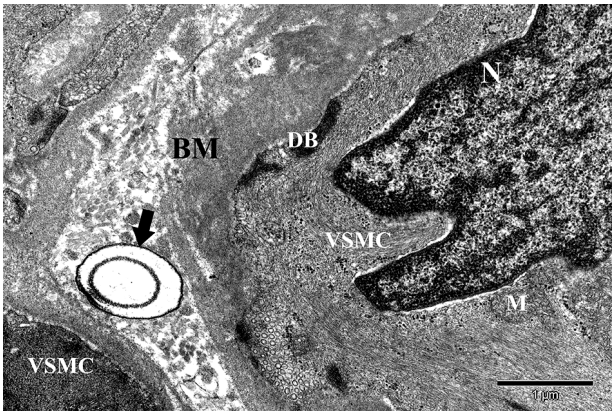

Fig. 13. A fragment of the vascular smooth muscle cell (VSMC) with cell nucleus (N) and dense bodies (DB). An enlarged basement membrane (BM) with visible precipitated calcium in the form of concentric circles (arrow). M – mitochondria. Original magnification 30,000×

with significantly enlarged sizes, the so-called giant or gigantic mitochondria (GM). These mitochondria took various shapes (Fig. 3). The cross-sections were: elongated, oval, bent, trapezoidal, or rounded. The loss of myofilaments around these organelles was compensated by glycogen grains, and the interior of the mitochondria was occupied by crystalline inclusions of type I, in varied amounts, observed in different cross-sections. Mitochondria were also found enlarged with a multiplied number of internal membranes arranged in regular concentric patterns or irregular figures. These membrane assemblies filled the entire mitochondria or occurred only in their periphery. The interior of the mitochondria was occupied by crystalline inclusions of type I or vacuoles of different sizes (Figs. 2, 3).

Some giant mitochondria were surrounded by double membranes, but without cristae and filled with homogeneous contents. Type I mitochondrial inclusions were usually found in type I fibres rich in mitochondria. These inclusions were characterised by a regular "squared pattern" of crystalline inclusions (Fig. 4). The cross-sections of type II mitochondrial inclusions (Fig. 5) took on the appearance of long, "tape-like" structures of dense, strongly osmophilic pattern. The remains of the mitochondrial cristae were preserved in the terminal sections of these organelles. Type II mitochondrial inclusions, in comparison with the first type, were less frequent and were observed mainly in the second type of skeletal muscle fibres, which were poorer in the mitochondria.

In the vicinity of fibres with the correct structure of myofilaments and mitochondria, damaged fibres were found (Figs. 6-9). In muscle fibres with a blurred myofilament structure, where cell nuclei often occupy central positions, osmophilic families were observed (Fig. 6).

The observation shows that the degree of degeneration of the skeletal muscle fibres was different: partial in most of the fibres tested (Figs. 6, 7) or total in some fibres (Figs. 8, 9). Fibril fragments were observed in which there was no regular myofilament system; these areas were filled with glycogen grains. In these damaged fibres there were swollen mitochondria with a very poor system of cristae or without cristae with an electron-light matrix. Cell nuclei in such altered fibres were divided into irregularly shaped fragments (Fig. 7). The muscle fibres with the maintained myofilament system were bordered by the areas of electron-light fibres, with thinning contents of myofilaments and clusters of mitochondria almost devoid of cristae (Fig. 8). Next to them there were residual fibres of small diameter, bounded by clearly visible muscle membrane, and the interiors were electron clear, devoid of myofilaments, unstructured, and occupied by numerous "vesicular mitochondria". Some of these mitochondria showed a homogeneous structure, while others contained vacuoles or osmophilic crystal inclusions. Mitochondrial crystalline inclusions of both type I (Fig. 9) and type II were also found outside the mitochondrial cavity, in a bright space of degenerate fibre bounded by the basement membrane.

2. Small blood vessel

In small blood vessels the distribution and morphology of mitochondria in capillary and arteriolar cells, as well as the range of lesions and types of calcium deposits in the arteriolar walls, were investigated.

In the endothelium (Fig. 10) and the pericytes of the capillaries the mitochondria with visible cristae were observed in the cytoplasm. These mitochondria were characterised by a higher electron density compared to the surrounding cytoplasm filled with pinocytic vesicles. In the cytoplasm of capillary endothelium, single deposits of lipofuscin were often found (Fig. 10).

Compared to capillaries, the mitochondria in arterioles were also observed in the endothelial cytoplasm. More often than in the endothelium, the mitochondria were present in the smooth muscle of the vessels, where the central and clustered positions usually occupied the cells (Fig. 11). The shapes of these mitochondria were oval or elongated or branched, depending on the section (Fig. 11). Within the inner and middle membranes of the arterioles examined, damage to the internal elastic lamina (IEL) and smooth muscle walls was observed. These sites were visible as electronically clear defects in the structure of the walls that were occupied by calcium precipitates (Fig. 12). Calcium deposits varied in size, shape, and electron density. Small, rounded grainy deposits and larger ones with dark rims of different thickness were found on the cross-sections. Some of the curves looked like concentric circles (Fig. 13).

Discussion

Our comprehensive studies were focused on Myoclonic Epilepsy with Ragged Red Fibers (MERRF), also called Fukuhara's disease [17, 18]. In the examined case of MERRF syndrome, skeletal muscle pathology was compared with the pathology of small blood vessels, capillaries, and arterioles, at the ultrastructural level. In our research were analysed damage to muscle fibres: local, involving damage to individual mitochondria, and extensive, involving damage to fragments or whole muscle fibres. In skeletal muscle fibres, proliferation and pleomorphism of mitochondria were observed and multiplied amounts of mitochondria were found in both subsarcolemmal and intermyofibrillar areas of muscle fibres. The accumulated mitochondria were characterised by phenotypic diversity. Among them there were often mitochondria

enlarged by the so-called giant mitochondria (GM): elongated, rounded, or irregularly shaped.

Although the mitochondrial pleomorphism in encephalomyopathies has long been studied, the origin and significance of these ultrastructural changes in the mitochondria has not yet been elucidated. One of the frequently observed mitochondrial pathologies in MERRF is the presence of concentrically arranged membranes instead of mitochondrial cristae. Such pathological mitochondria have also been documented with other encephalomyopathies, for example: Chronic Progressive External Ophthalmoplegia (CPEO) [23] and Kearns-Sayre Syndrome (KSS) [24].

Another example of the damage to mitochondria in the examined case of MERRF was the mitochondrial inclusion of different structures and localisations in the skeletal muscle fibres. Mitochondrial inclusions, similarly to those in our case of MERRF, are described for the CPEO [23]. The structure of mitochondrial inclusions has been thoroughly examined. There are two types of true crystal that occur in different regions of the intermembrane compartment and in different muscle fibre types [25, 26]. The type I crystals occur in the intra-crystal spaces in the type I fibres rich in mitochondria. The type II crystals are present in the intermembrane space between the outer and inner membrane in the type II fibres poor in mitochondria [26]. The occurrence of these forms of inclusion in various mitochondrial diseases is not specific for one disease, but it is generalised. This generalisation also applies to the chemical component of these inclusion bodies.

In addition to the ultrastructural impact assessment of mitochondrial creatine kinase (MtCK) distribution in mitochondrial myopathies, the level of cytoplasmic CK isoforms in laboratory tests was also evaluated. The level of CK in our patient was 132 U/l and was within the normal range: 25-166, despite the clinical and genetic markers of the MERRF disease.

Myopathies are a heterogeneous group of disorders that can be challenging to diagnose. CK is an extremely useful laboratory study for the evaluation of patients with a suspected myopathy. CK is elevated in the majority of patients with muscle disease but may be normal in slowly progressive myopathies [27]. Moreover, CK levels vary significantly by gender and race. Possible reasons include differences in muscle mass or total body mass and inherited differences in the permeability of the sarcolemma to CK. There is also a small reduction in CK levels as people age [28].

The CK levels are reported to be around 70% higher in healthy black people, as compared to white people. As serum CK in healthy people occur from a proportional leak from normal tissues, the black population subgroup has a generalised higher CK activity in tissues. Furthermore, there are differences in CK-dependent ATP buffer capacity in tissue between black and the white people, which may become apparent with high energy demands [29].

Although at the TEM level, mitochondrial damage is phenotypically very different, attempts have been made to classify these lesions. In addition to the above-described concentric membrane systems and the presence of mitochondrial inclusion, currently, in mitochondrial diseases, the following are also distinguished: linearised cristae membranes, compartmentalisation, nanotunneling, hyperbranching, and donut mitochondria. These phenotypes of variations are described in the examples of diseases, such as CPEO and MERRF [30]. The presented classification probably does not exhaust the spectrum of changes in mitochondrial structure in disease conditions; however, it orders the observed abnormalities of the mitochondria. In addition, it promotes the understanding of the relationship between the biochemical function of mitochondrial proteins and their ultrastructural presentation in pathology.

Besides local lesions, extensive damage to the muscle fibres was observed in the patient, phenotypically it was very diverse and nonspecific. Mechanisms that control the sequence of changes visible in the muscle fibres affected by mitochondrial diseases were sought and studied. It should be assumed that regardless of the type of mechanism leading to cell death (e.g. apoptosis, autophagy), the biological sense of these changes consists of limiting losses resulting from the size of the damage.

In mitochondrial diseases the presence of apoptotic features associated with specific phenotypes and a high percentage of mutated mitochondrial genomes was observed [31]. Skeletal muscle cells are fully differentiated and multinucleated. Apoptosis has been described in developing myoblasts but also in mature myotubes. However, the involvement of apoptosis in skeletal muscle pathologies is unclear [32]. In mitochondrial diseases apoptotic features were documented in muscle fibre of single mtDNA deletions above 40% and of tRNA point mutations above 70%. In patients with dysfunction of complexes I and IV respiratory chain, muscle fibres are more prone to undergo apoptosis [31].

Mitochondrial diseases are usually associated with skeletal muscle fibre dysfunction. Muscle pathology with damaged mitochondria seems to be an important feature of mitochondrial myopathy at the ultrastructural level. However, careful studies of mitochondrial encephalomyopathy show that damage may also affect blood vessels, including small blood vessels.

Our observations show that the number of mitochondria in endothelial cells and pericytes of capillaries was generally lower than in vascular smooth

muscle cells (VSMCs) of intramuscular arterioles. Due to the limited occurrence of mitochondria, especially in capillaries, it was difficult to assess the degree of damage to their mitochondrial membranes at the ultrastructural level. On the other hand, it is known that our patient has suffered from severe migraine headaches with vomiting for several years. Such symptoms may be a clinical manifestation of microangiopathy [14]. The pathogenesis of migraine-like headache is poorly understood, but some studies have indicated vasculopathy in MELAS and MERRF. Vascular pathology is characterised by episodic changes in the diameter of small cerebral arteries. According to the vascular hypothesis of migraine, it is assumed that initially there is vasoconstriction followed by vasodilation. Activation of the calcitonin-related peptide gene is responsible for hyperperfusion and migraine. Increased influx of calcium increases oxidative stress; therefore, muscle biopsy of patients with migraine may show mitochondrial abnormalities [14].

Although there was no mitochondrial proliferation in small blood vessels in the presented MERRF in the patient, the pathology included, among others, the proliferation of individual endothelial cells, most visible in the arterioles.

The endothelium is a monolayer of cells covering the vascular lumen. It is a multifunctional organ, critically involved in modulating vascular tone and structure. Endothelial function is important for the homeostasis of the body, and its dysfunction is associated with diseases, such as atherosclerosis, hypertension, and diabetes [33].

Compared to the mitochondria of skeletal fibres, mitochondrial inclusion was not found in the mitochondria of vascular cells: endothelium, pericytes, and VSMCs, which corresponds to the literature data. The lack of CK crystals in vascular mitochondria may indicate that the origin and ways of energy expenditure in both skeletal muscles and vessels are different.

The creatine kinase/phosphocreatine (CK/PCr) system seems to play a complex role in cellular energy homeostasis. The low content of mitochondria in ECs may indicate that the dependence of mitochondria on oxidative phosphorylation is not that important for energy supplement in those cells. ECs obtain a large proportion of their energy from the anaerobic glycolytic metabolism of glucose [34].

Another pathology of small blood vessels observed in our patient, as well as proliferation of endothelial cells, was the presence of calcium deposits in the vessel wall of arterioles. In addition, damage to VSMCs was observed.

Primary familial brain calcification (PFBC) is a neuropsychiatric disorder with calcium deposits in the brain, especially in basal ganglia, cerebellum, and subcortical white matter. Secondary brain calcification might also occur in several conditions, such as mitochondrial encephalomyopathy, MELAS, and MERRF. In general, the accumulation of granular material, mostly calcium salts, around the walls of capillaries, small arteries, and veins of the affected brain regions have been demonstrated [35].

In our study, calcification was observed in the vessel wall of muscular arterioles. In VSMCs there were sometimes losses of myofilaments, and there were vesicles and vacuoles of various sizes. Some mitochondria in VSMCs were swollen. Considering the significance of VSMCs in the vascular calcification process, it is important to understand the origin of the observed calcium precipitates and the mechanism of their formation.

Vascular calcification is an actively regulated form of matrix mineral metabolism resulting in the deposition of calcium phosphate in the vessel wall. Under normal vascular conditions, calcium phosphate deposition in the vessel wall is a thermodynamically unfavourable reaction, and pyrophosphate inhibits the formation of crystals [36].

Our ultrastructural observations show that calcium precipitates in vessel wall were regular oval structures of different sizes. Moreover, the construction of calcium deposits in the vessel wall resembles in a simplified manner the structure of osteons in cross-sections. The presence of concentric circles in some calcium deposits and in bone osteons is a common feature. Perhaps, their biochemical composition, as known from the literature, and the manner of deposition of calcium phosphate determine the appearance of these structures.

Sometimes we observed the collagen fibrils in the vicinity of calcium deposits in the vascular basement membrane. The deposition of calcium on collagen fibres in the extracellular matrix is typically initiated by a family of phosphoproteins secreted by fibroblasts or in bone by osteoblasts which play important roles in the regulation of matrix calcification. This process occurs in both normal woven bone and pathological calcification. The formation of precipitates of calcium is regularly referred to as "laminar calcification", hence the term "laminar calcium deposits" [37]. Various sizes of calcium deposits observed in our study may indicate an active remodelling process, as in the case of the bone.

Conclusions

Our studies focused on the skin-muscle biopsy in a patient with clinically suspected Myoclonic Epilepsy with Ragged Red Fibres (MERRF) syndrome, in whom the genetic tests showed point mutation in the gene m.8344A>G in tRNALys.

The pathology of skeletal muscles and small blood vessels (capillaries and arterioles) is ultrastructurally

demonstrated. Increased and differentiated mitochondrial lesions in skeletal muscle fibres were observed, including the presence of two types of mitochondrial inclusions and lesions of varying severity of fibre fragments or whole muscle fibres.

In small blood vessels, mild mitochondrial damage was found, capillary and arteriole endothelial cell proliferation, vascular smooth muscle cell damage, and the presence of calcium deposits in the vascular walls. In MERRF, the differences in mitochondrial damage in skeletal muscles compared to blood vessels may be related to the different origins and expenditure of useful biological energy in these cells, indicating the role of oxidative phosphorylation in mitochondria of muscle fibre and the role of cytoplasmic glycolysis in vascular cells.

This work was supported by the statutory fund No. 501-071-18021 and by "Digital Brain – digital collection of the Institute of Psychiatry and Neurology" (Project No. POPC.02.03.01-00.0042/18). The authors are grateful to the First Polish Brain Bank in the Institute Psychiatry and Neurology, Warsaw, Poland.

The authors declare no conflict of interest.

References

1. Koopman WJH, Beyrath J, Fung CW, et al. Mitochondrial disorders in children: toward development of small-molecule treatment strategies. EMBO Mol Med 2016; 8: 311-327.
2. Wallace DC. Mitochondrial diseases in man and mouse. Science 1999; 283: 1482-1488.
3. Gupta S. Molecular steps of death receptor and mitochondrial pathways of apoptosis. Life Sci 2001; 69: 2957-2964.
4. DiMauro S. Schon EA. Mitochondrial respiratory-chain diseases. N Engl J Med 2003; 348: 2656-2668.
5. Houshmand M. Role of mitochondria in epilepsy. In: Novel Treatment of Epilepsy. Foyaca-Sibat H. (ed) First Edition. Intech Open 2011; 37-58.
6. Anderson S, Bankier AT, Barrell BG, et al. Sequence and organization of the human mitochondrial genome. Nature 1981; 290: 457-465.
7. Filosto M, Tomelleri G, Tonin P, et al. Neuropathology of mitochondrial diseases. Biosci Rep 2007; 27: 23-30.
8. Kang H-C, Lee Y-M, Kim HD. Mitochondrial disease and epilepsy. Brain Dev 2013; 35: 757-761.
9. Schapira AH. Mitochondrial disease. Lancet 2006; 368: 70-82.
10. Chinnery PF. Mitochondrial disorders overview. In: Gene Reviews. Adam MP, Ardinger HH (eds) Seattle (WA) University of Washington, Seattle 2014; 1993-2018.
11. Zeviani M, Di Donato S. Mitochondrial disorders. Brain 2004; 127: 2153-2172.
12. Longo N. Mitochondrial encephalopathy.Neurol Clin 2003; 21: 817-831.
13. Finsterer J. Mitochondriopathies. Eur J Neurol 2004; 11: 163-186.
14. Finsterer J, Zarrouk-Mahjoub S. Mitochondrial vasculopathy. World J Cardiol 2016; 8: 333-339.
15. Riggs JE, Schochet SS, Fakadej AV, et al. Mitochondrial encephalomyopathy with decreased succinate-cytochrome c reductase activity. Neurology 1984; 34: 48-53.
16. Leonard JV, Schapira AH. Mitochondrial respiratory chain disorders I: mitochondrial DNA defects. Lancet 2000; 355: 26-99-304.
17. Fukuhara N, Tokiguchi S, Shirakawa K, et al. Myoclonus epilepsy associated with ragged-red fibers (mitochondrial abnormalities): disease entity or a syndrome. J Neurol Sci 1980; 4791: 117-133.
18. Tsairis P, Engel WK, Kark P. Familial myoclonic epilepsy syndrome associated with skeletal muscle mitochondrial abnormalities. Neurology 1973; 23:408.
19. Sirtoli R. Myoclonic epilepsy with ragged-red fibers (MERRF). The Winnower 2015; 24: 1-12.
20. Chan DC. Mitochondria: dynamic organelles in disease, aging, and development. Cell 2006; 125: 1241-1252.
21. Lorenzoni PJ, Scola RH, Kay CS, et al. When should MERRF (myoclonus epilepsy associated with raggerd-red fibers) be the diagnosis? Arq Neuropsiquiatr 2014; 72: 803-811.
22. Rahman S. Mitochondrial disease and epilepsy. Dev Med Child Neurol 2012; 54: 397-406.
23. Dias-Tosta E. Chronic progressive external ophthalmoplegia II. A qualitative and quantitative electronmicroscopy study of skeletal muscles. Arq Neuro-Psiquiat 1988; 46: 143-155.
24. Schwartzkopff B, Frenzel H, Breithardt G, et al. Ultrastructural findings in endomyocardial biopsy of patients with Kearns-Sayre Syndrome. J Am Coil CardioI 1988;12: 1522-1528.
25. Stadhouders AM, Sengers RCA. Morphological observations in skeletal muscle from patients with a mitochondrial myopathy. J Inher Metab Dis 1987; 10: 62-80.
26. Farrants GW, Hovmoller S, Stadhouders ADM. Two types of mitochondrial crystals in diseased human skeletal muscle fibers. Muscle and Nerve 1988; 11: 45-55.
27. Barohn RJ, Dimachkie MM, Jackson CE. A pattern recognition approach to the patient with a suspected myopathy. Neurol Clin 2014; 32: 569-593.
28. Moghadam-Kia S. Approach to asymptomatic creatine kinase elevation. Cleve Clin J Med 2016; 83: 37-42.
29. Brewster LM, Coronel CMD, Sluiter W, et al. Ethnic differences in tissue creatine kinase activity: an observational study. PLoS one 2012; 7: 1-3.
30. Vincent AE, Ng YS, White K, et al. The spectrum of mitochondrial ultrastructural defects in mitochondrial myopathy. Sci Rep 2016; 6: 1-11.
31. Mirabella M, Di Giovanni S, Silvestri G, et al. Apoptosis in mitochondrial encephalomyopathies with mitochondrial DNA mutations: a potential pathogenic mechanism. Brain 2000; 123: 93-104.
32. Liu CC, Ahearn JM. Apoptosis of skeletal muscle cells and the pathogenesis of myositis: a perspective. Curr Rheumatol Rep 2001; 3: 325-333.
33. Sena CM, Pereira AM, Seica R. Endothelial dysfunction-a major mediator of diabetic vascular disease. Biochim Biophys Acta. 2013; 1832: 2216-2231.
34. Tang X, Luo Y-X, Chen H-Z, et al. Mitochondria, endothelial cell function, and vascular diseases. Front Physiol 2014; 5: 1-17.
35. Taglia I, Bonifati V, Mignarri A, et al. Primary familiar brain calcification: update on molecular genetics Neurol Sci 2015; 36: 787-794.
36. Hofmann Bowman MA, McNally EM. Genetic pathways of vascular calcification. Trends Cardiovasc Med 2012; 22: 93-98.
37. Cheville NF. Calcification In: Ultrastructural Pathology The Comparative Cellular Basis of Disease. Cheville NF (ed.). 2nd ed. Wiley-Blackwell, Iowa, USA 2009: 797-828.

Resources
https://www.omim.org/entry/545000

Address for correspondence

Paulina Felczak
Department of Neuropathology
Institute of Psychiatry and Neurology
Sobieskiego 9
02-957 Warsaw, Poland
tel.: + 48 22 45 82 646
e-mail: felczak.p@wp.pl