Case report

Pathology of mitochondria in MELAS syndrome: an ultrastructural study

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Ultrastructural changes in skeletal muscle biopsy in a 24-year-old female patient with clinically suspected mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) syndrome are presented. We observed proliferation and/or pleomorphism of mitochondria in skeletal muscle and smooth muscle cells of arterioles, as well as in pericytes of capillaries. Paracrystalline inclusions were found only in damaged mitochondria of skeletal muscle. Genetic testing revealed a point mutation in A3243G tRNALeu(UUR) typical for MELAS syndrome. We conclude that differentiated pathological changes of mitochondria in the studied types of cells may be associated with the different energy requirements of these cells.

Key words: MELAS, A3243G mutation, abnormal mitochondria, ultrastructure.

Introduction

Mitochondria are double membrane subcellular organelles, responsible for producing the cellular adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS) [1], and are involved in lipid biosynthesis [2]. They are also the main source of reactive oxygen species (ROS), which can disturb homeostatic signalling pathways, controlling cell differentiation and proliferation [3, 4]. Mitochondria contain their own circular double-stranded DNA (mtDNA) encoding for 13 polypeptides of the electron transport chain (ETC), 22 transfer RNAs, and 2 ribosomal RNAs. Although mtDNA contributes less than 1% to the total cellular nucleic acid content, it is fundamentally important for the function of every human tissue [5].

Mitochondrial function depends on the interaction of many nuclear and mitochondrial genes, and their abnormalities may cause disease [5]. Mitochondrial disorders represent a heterogeneous group of diseases with varying clinical features, showing tissue-specific manifestations and affecting multiple organ systems [4]. These diseases include primary mitochondrial disorders caused by mutations in mitochondrial genes; disorders with mutations in nuclear genes involved in mitochondrial function; and secondary disorders that arise from the accumulation of mitochondrial damage over time frequently involving neurodegenerative pathologies [6]. Among mitochondrial diseases there is a group of neuromuscular disorders with structurally or functionally abnormal mitochondria in the brain and/or muscle known as mitochondrial encephalomyopathies. One of them is mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) syndrome [7, 8]. Clinical features suggesting MELAS were first described in patients with a common characteristic sign, namely the presence of mitochondrial myopathy associated with brain changes, such as mental retardation, seizures, myoclonus, ophthalmoplegia, retinitis pigmentosa, blindness, calcification in basal ganglia and sudden hemiplegia suggestive of stroke [9, 10, 11, 12]. MELAS is a multi-system disease, involving the brain, peripher-

al nervous system, eyes, endocrine glands, heart, guts, kidneys, or dermis [13]. Tissues such as brain, muscle, heart, retina and cochlea, which have enormous energy requirements, are particularly vulnerable to variations in mitochondrial function [6, 14].

MELAS is most frequently caused by mutations in the genes in mitochondrial DNA. The most commonly involved pathogenic point mutations are A3243G and T3271C in the gene encoding tRNALeu [15]. However, the A3243G mutation is responsible for the induction of MELAS in about 80% of remaining patients with MELAS and T3271C for approximately 7.5%, but in up to 10% of patients with MELAS the mutations of mtDNA remain unknown [11]. Molecular consequences of the A3243G mutation in tRNALeu(UUR) has not as yet been completely understood. It is assumed that the point mutation transition A> G at nucleotide position 3243 may affect the stabilization of the tRNA structure, and influence the methylation, aminoacylation and codon recognition [16, 17]. Mutations in mtDNA also contribute to the disruption of ATP synthesis in the process of oxidative phosphorylation in the respiratory chain. Of the five respiratory chain complexes (I-V), four contain polypeptides encoded by both nuclear and mitochondrial DNA. Only complex II is entirely encoded by the nuclear genome [18]. Abnormalities in complexes I and IV are usually attributed to the effects of the A3243G mutation [17].

Investigations of mitochondrial disorders, such as MELAS, require an integrated approach, incorporating clinical, biochemical, genetic, histological and ultrastructural studies to reach a specific diagnosis. The aim of our study is to present the ultrastructural changes in muscle and skin biopsy associated with clinical studies and genetic testing of the material obtained from a patient with MELAS syndrome.

Material and methods

The ultrastructural investigations were performed on the skin and 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 two transmission electron microscopes (TEM), Opton DPS 109 Turbo and JEOL model 1400.

Molecular analysis

DNA was isolated from blood using a standard salting out procedure. Whole mitochondrial DNA (mtDNA) was amplified with TaKaRaLATaq polymerase (Takara Bio Inc., Kusatsu, Japan); primer sequences and PCR conditions are available on request. Libraries for next generation sequencing (NGS) were prepared using the Nextera XT library preparation kit (Illumina Inc., San Diego, CA, USA), indexed and paired-end sequenced (2×150 bp) on the MiSeq Sequencing System (Illumina). Reads were aligned to the mtDNA Revised Cambridge Reference Sequence of the Human Mitochondrial DNA (rCRS); (http://www.mitomap.org/MITOMAP/HumanMitoSeq) with the Burrows-Wheeler Alignment Tool (http://bio-bwa.sourceforge.net/). The level of heteroplasmy was assessed with MToolBox [19] and by read counting in the Integrative Genome Viewer (IGV) [20].

Case report

A 24-year-old female patient was admitted to our Department of Neurology because of suspected mitochondrial encephalopathy. She had experienced her first tonic-clonic seizure in her twenty-third year of life. She was treated with valproate acid. The patient's past medical history revealed that she had suffered from severe migraine headaches for many years. In addition, she had bilateral sensorineural hearing loss corrected: a HiRes 90K implant in the right cochlear, and a left hearing aid. The patient was also diagnosed with kidney stones and asthma. Her parents were non-consanguineous. The family history of her mother revealed bilateral hearing loss and use of hearing aids. The patient's brother was diagnosed with cerebral palsy with paresis of the left limbs. Physical examination of our patient showed short stature (growth 150 cm), atrophied upper and lower limbs, without paresis and with normal muscle tone, diminished tendon reflexes of the upper and lower limbs, and impaired vibration sense of distal lower limbs. Cerebral computed tomography (CT) showed the increased density of tissues of the caudate nuclei head, globus pallidus, thalamus (mostly the posterior part), and slight atrophy of the left cerebellar hemisphere. Magnetic resonance imaging (MRI) of the brain revealed a hyperintense signal on the T2-weighted image (T2WI) within bilateral basal ganglia, putamen and posterior sections of the thalamus. The electroencephalography (EEG) showed abnormal register of general seizure changes. Laboratory tests revealed increased levels of lactate (57.19 mg/dl; norm: 4.5-19.8 mg/dl) and elevated creatine kinase (CK) (265.94 U/l; norm: 25-166 U/l).

Results

An ultrastructural study was performed on mitochondria in the skeletal muscle and small blood vessels.

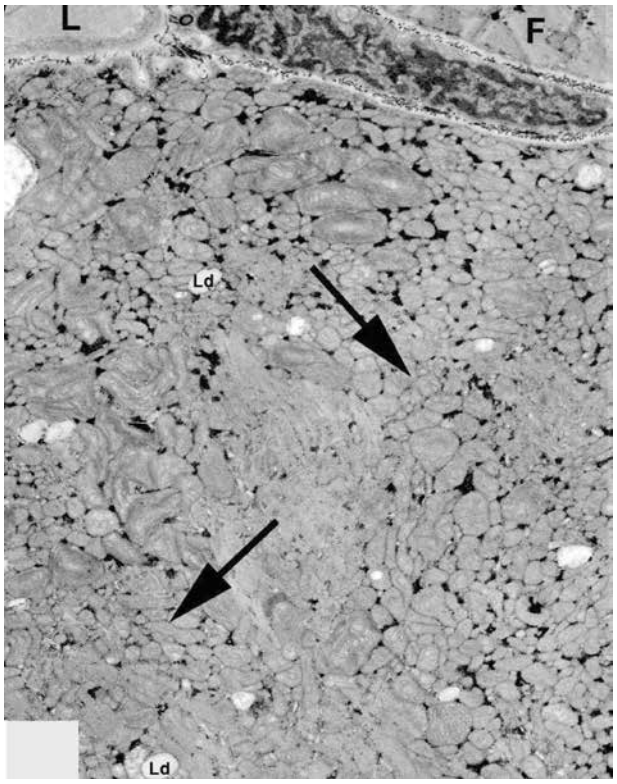

Fig. 1. Proliferation of pleomorphic mitochondria (arrows) in skeletal muscle fibre. In addition to a fragment of the normal muscle fibre (F) and capillary. L – lumen, Ld – lipid droplet. Original magnification 4400×

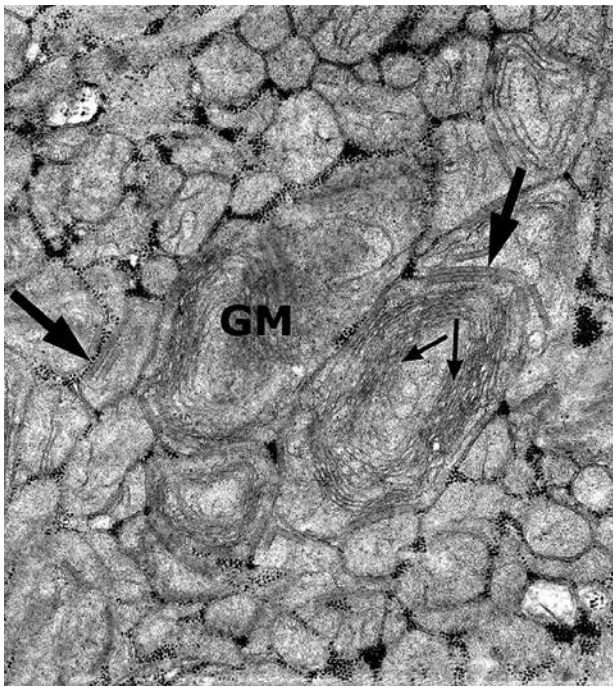

Fig. 2. Giant mitochondria (GM) with concentric cristae (thin arrows) and paracrystalline inclusions (thick arrows) in swollen mitochondria. Original magnification 12 000×

Skeletal muscle

Numerous mitochondria often occurred in clusters, which occupied large areas of fibres (Fig. 1). In these cellular areas we observed proliferation of mitochondria, and some of them were in contact with lipid droplets. Sometimes muscle fibres, filled with clusters of pathological mitochondria, directly bordered with fibres containing normal mitochondria, were regularly distributed in the cell section (Fig. 1). At the ultrastructural level the appearance of abnormal mitochondria, subject to proliferation, was diverse. These mitochondria differed in size, shape and internal structure (Fig. 2). Among them we observed rounded giant mitochondria in which the damaged mitochondrial cristae were often seen as poor or concentric. Inside numerous abnormal mitochondria there were paracrystalline inclusions in the form of bands with a regular pattern, as well as with various lengths and widths in different sections (Fig. 3). Smaller mitochondria were often circular, oval or polygonal on cross-section and often slightly swollen. In these mitochondria a few damaged mitochondrial cristae in a homogeneous electron clear matrix were observed. In some mitochondria electron dense lipid droplets were also sometimes seen (Fig. 3). Apart from the mitochondria of rounded outline, in some muscle fibres

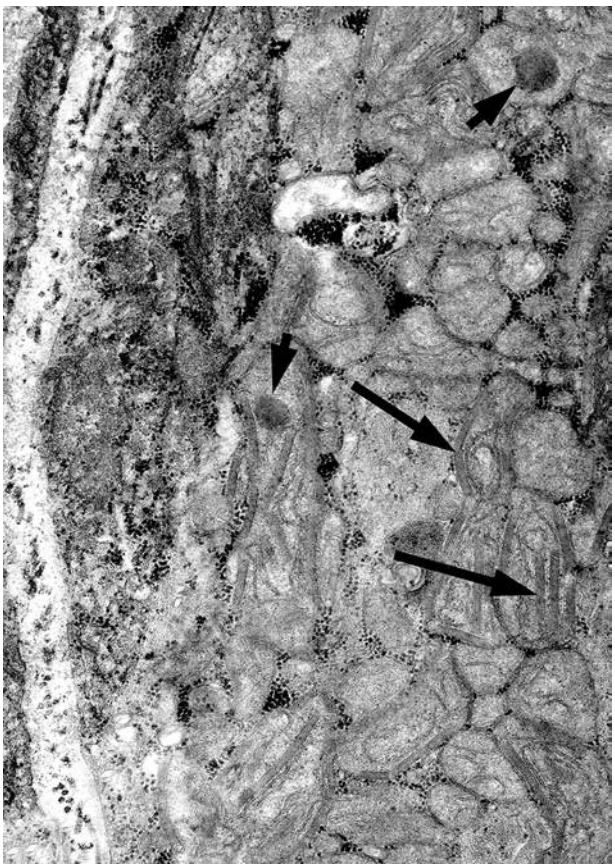

Fig. 3. Paracrystalline inclusions (long arrows) and electron dense lipid droplets (short arrows) within the damaged mitochondria in skeletal muscle fibre. Original magnification 12 000×

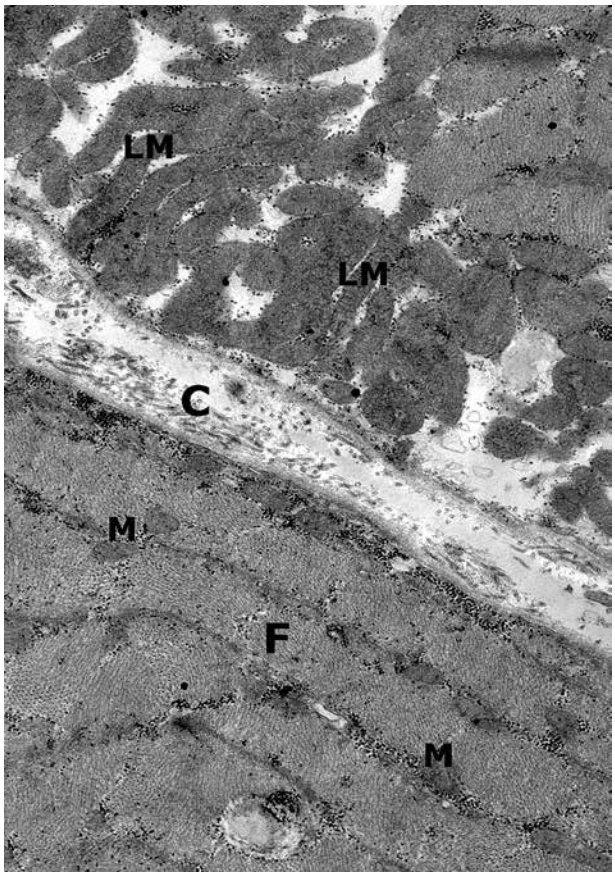

Fig. 4. Very long and twisted damaged mitochondria (LM) from the poorly visible cristae. Near to normal muscle fibre (F). M – mitochondrium, C – collagen fibrils. Original magnification 35 000×

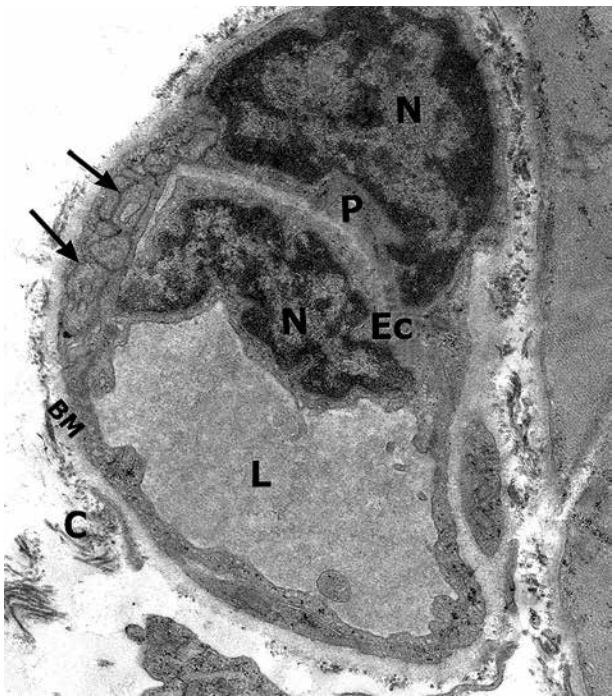

Fig. 6. Capillary. Numerous abnormal mitochondria (arrows) in the cytoplasm of pericytes (P). N – nucleus, L– lumen, EC – endothelial cell, BM – basement membrane, C – collagen fibrils. Original magnification 25 000×

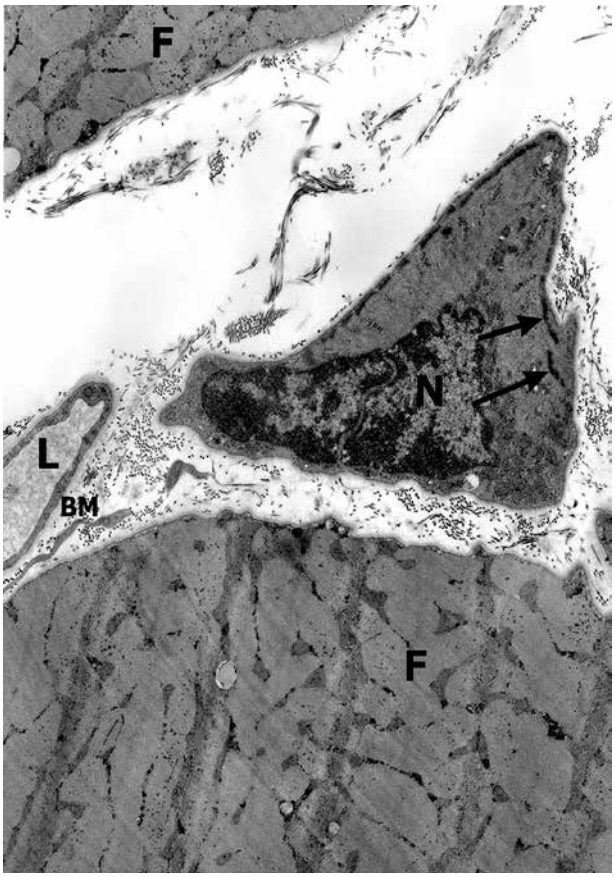

Fig. 5. Atrophic muscle fibre. Nuclei (N) located in the peripheral portion of the small diameter fibre with the damaged structure of the sarcomeres. Z-lines at the periphery of the fibre (arrows). Near to normal fibre (F) and capillary. L – lumen, BM – basement membrane. Original magnification 4400×

damaged mitochondria with elongated shapes and varied morphology were found. Among them we observed enlarged mitochondria, very long and twisted with poorly preserved mitochondrial cristae (Fig. 4). Clusters of these mitochondria were observed in fibres with substantial loss of myofilaments.

Next to skeletal muscle fibres with normal architecture, a few fibres were found to be damaged and deformed (Fig. 5). They were atrophied fibres of small size, with floated structure of sarcomeres and large nuclei concentrated in the peripheral portion of the cell. In atrophic fibres Z-lines were peripherally located near the cell membrane (Fig. 5).

Small blood vessels

Abnormal mitochondria were observed in cells, capillaries and arterioles. In the damaged mitochondria, paracrystalline inclusions in the vascular smooth muscle cells, pericytes and endothelial cells were not observed.

Capillaries

Proliferation and pathological changes in mitochondria were found in capillaries, mostly in the

cytoplasm of pericytes (Fig. 6). These mitochondria occurred in groups and varied in size. Among them there were giant mitochondria with very few and damaged mitochondrial cristae and electron light matrix.

Arterioles

In some arterioles all vascular smooth muscle cells (VSMCs) were filled with very numerous mitochondria, showing varying degrees of damage. In these cells proliferation of mitochondria that filled the whole cells was frequently observed (Fig. 7). The mitochondria were seen in both parts of the peripheral muscle, near the cellular membrane as well as around the centrally located nucleus (Fig. 8). Some of them were in contact with the nuclear envelope. We observed pleomorphism of damaged mitochondria. The mitochondria varied in size. Among them there were giant mitochondria with abnormal cristae arranged concentrically or mitochondria of a very poor system of cristae spaced irregularly. Within the mitochondrial matrix electron light areas were sometimes visible (Fig. 8).

Molecular data

Mutation A2343G tRNALeu(UUR) (MTTL1 gene) in the mitochondrial DNA was revealed in our patient, which confirmed the occurrence of MELAS syndrome. The analysed mitochondrial DNA was collected from peripheral blood. The mutation was found with the mean heteroplasmy level of 40% (36% and 44% as assessed by MToolBox and IGV, respectively). Mean sequencing depth for the whole mitochondrial DNA was 800×, and for position m.3243 it was 1088×. Genetic tests were carried out in the Department of Genetics, Institute of Physiology and Pathology of Hearing, Warsaw/ Kajetany.

Discussion

MELAS syndrome creates a lot of diagnostic difficulties similar to those faced in the majority of mitochondrial encephalomyopathies. They may result from the lack of pathognomonic features typical of this disease.

MELAS syndrome, described in our 24-year-old female patient, is an example of mitochondrial encephalomyopathies, diagnosed clinically and ultrastructurally, and confirmed by genetic testing. Clinical symptoms observed in the patient were consistent with the description corresponding to the full name of the disease, i.e. mitochondrial, encephalomyopathy, lactic acidosis, stroke-like episodes. We also described potential co-existing symptoms, such as hearing loss, visual disturbance, frequent severe migraine, headaches and short stature. Clinical manifestations of MELAS symptoms are quite common in children and young adults (like in our patient), and less common in adults [11, 12]. The wide range of MELAS symptoms and differences in their intensity lead to difficulties in establishing diagnostic criteria. According to the traditional diagnostic criteria established by Hirano, encephalopa-

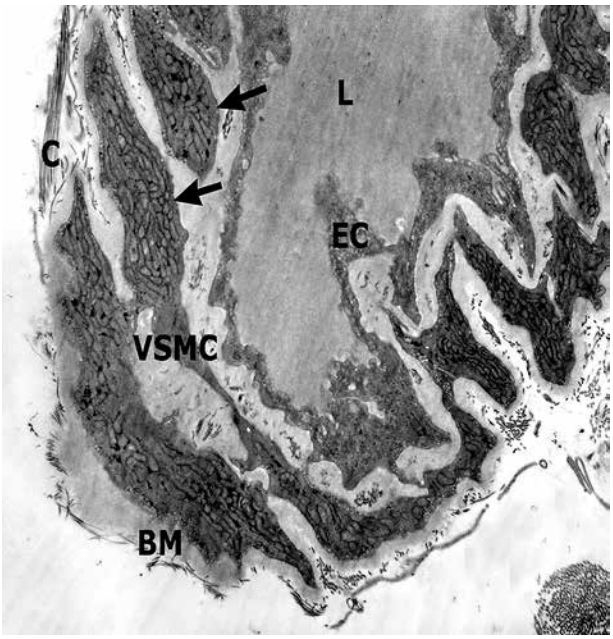

Fig. 7. Arteriole. Proliferation of mitochondria (arrows) in vascular smooth muscle cells (VSMC). L – lumen, EC – endothelial cell, BM – basement membrane, C – collagen fibrils. Original magnification 4400×

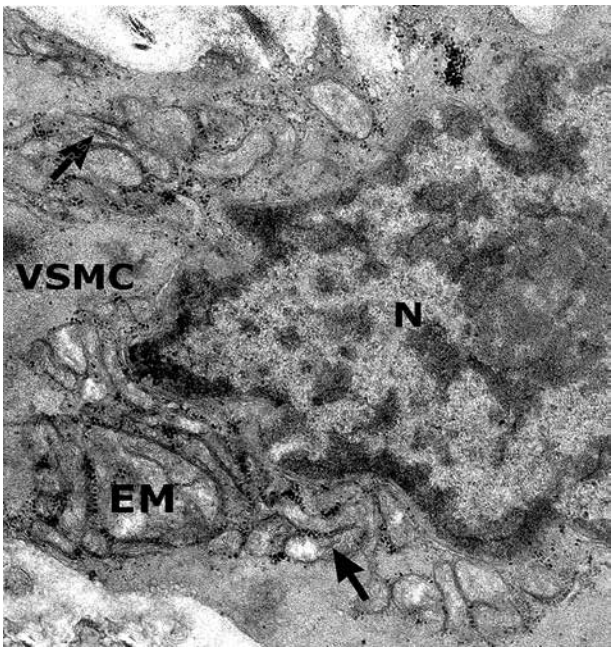

Fig. 8. Enlarged mitochondria (EM) and mitochondria with damaged cristae (arrows) in a vascular smooth muscle cell (VSMC). N – nucleus. Original magnification 50 000×

thy with dementia and/or seizures and the presence of the young age of episodes similar to a stroke are often the major determinants of MELAS [11, 21]. On the other hand, the diagnostic criteria developed by Japanese scientists placed emphasis on the importance of genetic testing to detect mutations responsible for MELAS and indicate the role of mitochondrial dysfunction studies at the ultrastructural level [11, 22].

Genetic testing in our patient confirmed that MELAS syndrome is caused by a point mutation, m.3243A>G in the MTTL1 gene encoding the mitochondrial tRNALeu(UUR) [12, 23]. Although this mutation is the most common genetic cause of MELAS, it may give rise to diagnostic problems. It is responsible for a wide phenotypic diversity and can also cause other mitochondrial encephalomyopathies with different spectra of clinical signs, e.g., myoclonic epilepsy and ragged-red fibres (MERRF), overlap syndrome MELAS/MERRF, Leigh syndrome (LS), chronic external ophthalmoplegia (CEO), and Kearns-Sayre syndrome (KSS) [17, 24, 25, 26, 27]. Another difficulty in identifying the genetic basis of MELAS is the fact that the disease can be caused by other less common genetic mutations in the mitochondrial DNA (mtDNA). The A3252G and C3256T mutations in tRNALeu(UUR) may serve as an example [11]. Moreover, there are also MELAS cases caused by mutations in the nuclear DNA (nDNA) in the POLG1 gene that encodes the gamma polymerase involved in the replication of mitochondrial DNA [28, 29]. Additional diagnostic difficulties arise from the specific features of mitochondrial DNA, such as maternal mode of inheritance, lack of protection by the histones and limited mechanisms of mtDNA repair. Therefore, the frequency of mutation in the mtDNA is 10 to 20 times higher than that of nDNA [4, 30]. In mitochondrial encephalomyopathies, such as MELAS, it is important to estimate the level of heteroplasmy, showing the proportion between the amount of mutant and normal mtDNA in the cell. The levels of heteroplasmy in individual tissues differ and depend on their energy requirements and are high, for example, in muscles, blood vessels and in the urine [17]. The clinical phenotype of the disease visible after exceeding a minimum critical number of mutant mtDNAs is termed "a threshold effect" [31]. In our patient the level of heteroplasmy, as indicated in peripheral blood, was 40% and sufficient for the manifestation of MELAS clinical symptoms.

The presence of pathological changes attributable to the majority of mitochondrial encephalomyopathies caused the difficulty in the ultrastructural estimation of MELAS. The cells derived from skeletal muscle tissues and smooth muscle tissues were examined in TEM. Muscle and nervous tissues, which exhibit very high energy requirements, represent sensitive indicators of ultrastructure disorders in mitochondria. Different types of mitochondrial damage were observed in the cytoplasm of the skeletal muscle fibres, smooth muscle cells of arterioles, and in capillaries in the cytoplasm of pericytes. In the studied cells we observed proliferation of mitochondria, which was most evident in the type I muscle fibres, more rich in mitochondria compared with the type II fibres. However, in the arterioles multiplied numbers of mitochondria in all muscle smooth cells of a single arteriole were commonly observed. Bearing in mind that the pericytes are the counterparts of shrink elements of arterioles, cells of high energy requirements, aggregations of mitochondria, according to our expectations, were also observed in the cytoplasm of pericytes. On the other hand, under physiological conditions, proliferation of mitochondria may result from the organism's adaptation to physical endurance training [32]. The results of laboratory tests of our patient showed a significantly increased level of lactate (up to 57.19 mg/dl) compared to the standard (4.5-19.8). In pathological conditions, resulting in damage to the respiratory chain, alternative metabolic pathways and the conversion of pyruvate to lactate are activated, which is manifested by systemic lactic acidosis. The proliferation of mitochondria can compensate for energy deficiency in the respiratory chain [16, 33].

In the studied cells we also observed pleomorphism of mitochondria. The occurrence of paracrystalline inclusions (PI) was one of its forms found only within the abnormal mitochondria in skeletal muscle fibres. This type of pathological structure was not observed in the cells of small blood vessels, such as smooth muscle cells, pericytes and endothelial cells. Laboratory studies indicated that the creatine kinase (CK) level in our patient was 265.94 U/l, well above the standard (25-166). CK is an enzyme that controls the cellular energy homeostasis [34]. Studies with antibodies against mitochondrial creatine kinase (Mt-CK) in immunofluorescence microscopy revealed that kinase is a major component of PI, which is observed in the intermembrane space of mitochondria [35, 36]. Inside the abnormal mitochondria the amount of Mt-CK increases, and after reaching the critical concentration, the enzyme begins to crystallize [34]. The formation of PI is the result of regulation of the kinase level, which aims to compensate for the energy deficiency. Under these conditions, there is conversion of the octameric form of CK to the dimeric form [32]. Paracrystalline inclusions are found not only in MELAS, but also, for example, in chronic progressive external ophthalmoplegia (CPEO), KSS and MERRF [34]. It is likely that these inclusions are not

an ultrastructural indicator of MELAS, due to their widespread occurrence in mitochondrial encephalomyopathies differing genetically and clinically. The presence of paracrystalline inclusions in the studied mitochondria may be an indicator of abnormal CK levels.

The occurrence of globular osmophilic inclusion bodies in mitochondria was a subsequent manifestation of mitochondrial pleomorphism. Mitochondria with bodies of high electron density were occasionally observed in sarcoplasm of tested skeletal muscle fibres. It is difficult to attribute the presence of these structures only to MELAS, because they are also found in other mitochondrial diseases, such as Luft disease. Osmophilic globular bodies present in the mitochondria may be regarded as structures containing biochemical components of brown adipose tissue [37]. We suggest considering the fact that the main function of brown adipose tissue in the organism is the production of heat; lipid accumulation in the mitochondria in this type of tissue may be associated with disturbed heat management of the organism in mitochondrial disease.

The pathology of mitochondria observed in the skeletal muscle and smooth muscle cells also included sizes achieved by these organelles. Among the mitochondria there were giant mitochondria, round-shaped or very elongated mitochondria, sometimes curved. Mitochondria are highly dynamic organelles. Frequent cycles of their fusion and fission adapt their morphology to the metabolic needs of the cell. Fusion of mitochondria is of particular importance in cells with high respiratory activity. This process allows the spread of metabolites within the mitochondria and serves to optimize their bioenergetic capacity [38]. It is known that high dynamics of mitochondria in the cell under physiological conditions suggests changes in mitochondria also in pathological conditions. Perhaps the disruption of the cellular respiration processes contributes in our case of MELAS to disorders in natural conversion morphology of mitochondria. We suggest that the mitochondria with deformed shapes, substantially enlarged, observed in TEM, may be the result of fusion and fission disturbances in mitochondria, as an adaptation to altered respiratory activity in stressful conditions caused by the disease.

We also observed various types of damage to and deformations of the mitochondrial cristae. It is known that in the fusion and fission processes the adaptation of mitochondrial morphology to the changed bioenergetic conditions of the cell comprises not only changes in the size and shape of mitochondria, but also remodelling of mitochondrial cristae [38].

Sometimes we found swollen mitochondria with electron clear matrix. Different mitochondrial protein precursors involved in the structure and function of mitochondria also include the protein of the mitochondrial matrix. The majority of them are synthesized in the cytosol prior to their import into mitochondria. Undisturbed transport and sorting of these proteins, including matrix proteins, such as Hsp70 and Zim17, are essential for maintaining normal functions of these organelles [39].

In skeletal muscle fibres lipid droplets in contact with mitochondria were observed. In physiological conditions mitochondria display functional heterogeneity with respect to various tissues. For example, mitochondria in skeletal muscle fibres are specialized in fatty acid oxidation [40]. Different stress factors, such as hypoxia or oxidative stress, are conducive to accumulation of lipid droplets and formation of swollen mitochondria. Biogenesis of fatty acids in the form of lipid droplets prevents them from beta-oxidation and protects them under stress conditions [41].

In TEM we observed not only local deformations involving mitochondria damage, but also damage to individual skeletal muscle, such as atrophic fibres of small diameter. We suggest that the disruption of cellular respiration, resulting in the disorder of mitochondrial morphology and, consequently, other organelles, may ultimately cause damage to the entire length of skeletal muscle fibres. It is known that in mitochondrial encephalomyopathies muscle fibres with the proliferation of mitochondria are termed ragged-red fibres (RRF) in histochemical modified Gomori trichrome staining. Mitochondria are visible in the subsarcolemmal space as red granular deposits in skeletal muscle cells exhibiting different degrees of muscle fibre atrophy [42].

Conclusions

In the diagnosis of mitochondrial encephalomyopathies, ultrastructural observations complement clinical and genetic studies. The convergence between clinical, ultrastructural and genetic data in our patient indicates MELAS syndrome.

In TEM, we observed proliferation and/or pleomorphism of mitochondria in skeletal muscle and smooth muscle cells of arterioles, as well as in pericytes of capillaries. Furthermore, we found paracrystalline inclusions in damaged mitochondria, but only in skeletal muscle. This type of pathological structures was not observed in the cells of small blood vessels, such as smooth muscle cells, pericytes and endothelial cells. It appears that the different energy requirements of skeletal muscle compared to the vascular smooth muscle cells may have an impact on the differing requirements of these cells for creatine kinase, which is the ultrastructural de-

terminant of mitochondrial paracrystalline inclusions.

In our opinion, the detailed knowledge of mitochondrial ultra pathomorphology indirectly indicates that in different cell types different adaptive mechanisms are active, and compensate for losses caused by disruption of homeostasis in MELAS syndrome. We hope that our comprehensive research will contribute to a diagnostic algorithm for MELAS and can have a positive impact on the future introduction of new therapeutic methods.

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The authors declare no conflicts of interest.

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Resources

http://www.mitomap.org/MITOMAP/HumanMitoSeq
http://bio-bwa.sourceforge.net/

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