REVIEW

Variability of Clinical Phenotypes Caused by Isolated Defects of Mitochondrial ATP Synthase

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Summary
Disorders of ATP synthase, the key enzyme in mitochondrial energy supply, belong to the most severe metabolic diseases, manifesting as early-onset mitochondrial encephalo-cardiomyopathies. Since ATP synthase subunits are encoded by both mitochondrial and nuclear DNA, pathogenic variants can be found in either genome. In addition, the biogenesis of ATP synthase requires several assembly factors, some of which are also hotspots for pathogenic variants. While variants of *MT-ATP6* and *TMEM70* represent the most common cases of mitochondrial and nuclear DNA mutations respectively, the advent of next-generation sequencing has revealed new pathogenic variants in a number of structural genes and *TMEM70*, sometimes with truly peculiar genetics. Here we present a systematic review of the reported cases and discuss biochemical mechanisms, through which they are affecting ATP synthase. We explore how the knowledge of pathophysiology can improve our understanding of enzyme biogenesis and function.

Keywords
Mitochondrial diseases • ATP synthase • Nuclear DNA • Mitochondrial DNA • TMEM70

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ATP synthase

Mammalian F_1F_o-ATP synthase (complex V) is mitochondrial enzyme, that is responsible for the production of more than 90 % of cellular ATP. To produce ATP, F_1F_o-ATP synthase operates in synthetic mode. In this mode, energy from the proton gradient, generated by the respiratory chain complexes, is used for phosphorylation of ADP to ATP. However, since it belongs to the family of ATPases, it can also catalyze ATP hydrolysis. In the so-called reverse mode, hydrolysis of ATP to ADP powers the proton pumping from mitochondrial matrix to intermembrane space [1]. Human ATP synthase is composed of 18 different subunits, encoded by both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Structurally it can be divided into three parts (Fig. 1A). Subunits α and β form $\alpha_3\beta_3$ hexamer, where the ADP↔ATP conversion takes place, and together with γ, δ and ε subunits, they constitute F_1 catalytic part. Membrane-embedded F_o part is composed of a bunch of small subunits, including *e*, *f*, *g*, DAPIT (diabetes-associated protein in insulin sensitive tissues), MLQ, 8 copies of subunit *c* (c_8 ring), and two mtDNA-encoded subunits *a* and A6L. Proton translocation across the inner mitochondrial membrane occurs within the F_o domain, on the interface between the ring of subunits *c* and subunit *a*. The third structural component, so-called peripheral stalk, contains subunits *b*, *d*, F_6 and OSCP (oligomycin-sensitivity conferring protein) [2]. The peripheral stalk is stationary and immobilizes the $\alpha_3\beta_3$ hexamer. In contrast, the central stalk (composed of γ, δ, and ε subunits) and c_8 represent the rotor – it transfers the torque between F_1 and F_o domains and, depending on the direction, allows ADP phosphorylation or ATP hydrolysis. The last subunit associated with F_1F_o-ATP synthase is inhibitory factor IF_1. IF_1 binds to the F_1 part under certain circumstances, for example, under low pH or during assembly, and inhibits the hydrolytic activity of ATP synthase [3,4].

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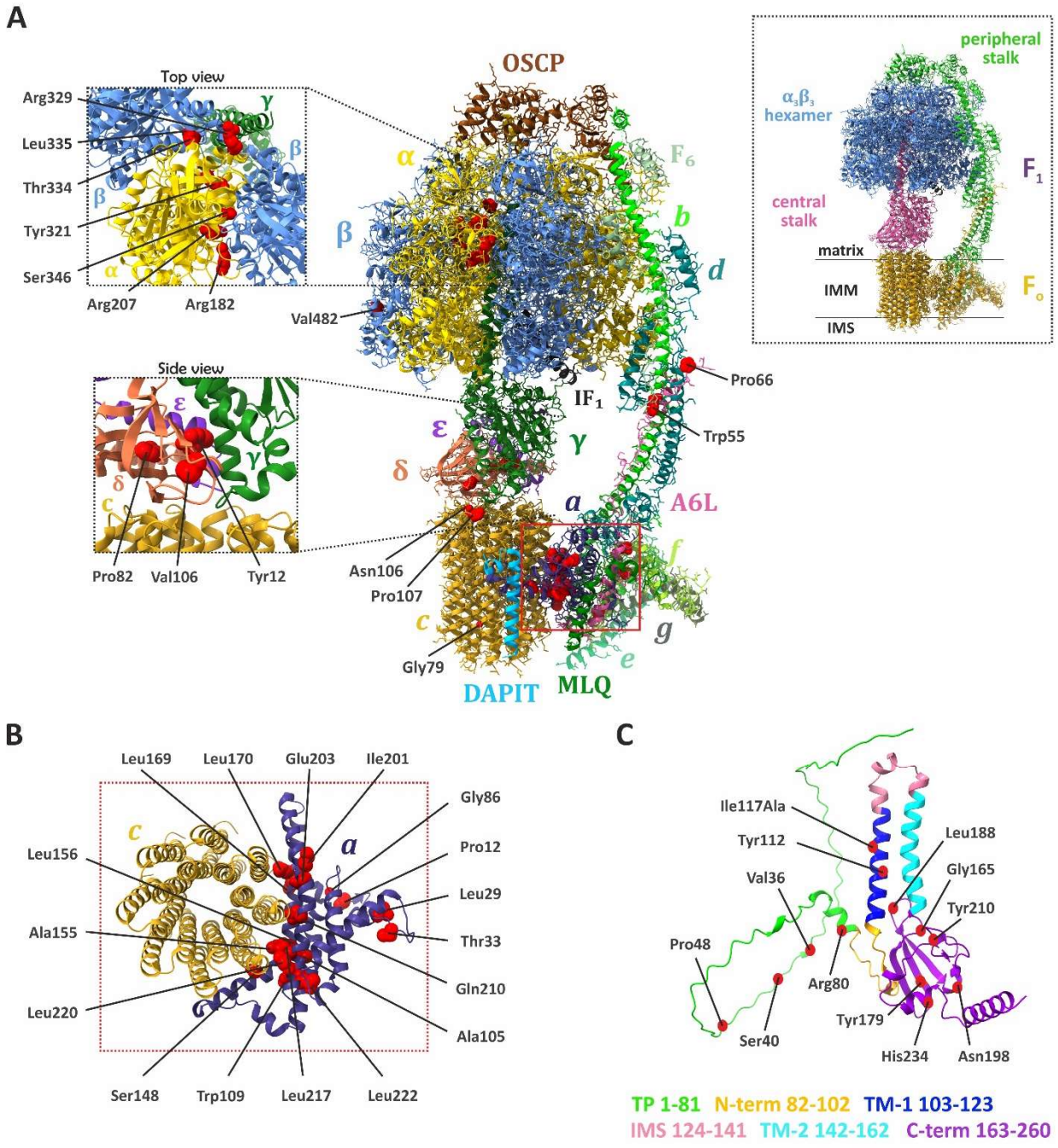

Fig. 1. Human ATP synthase and its pathogenic variants. **(A)** Structure of human ATP synthase, created in UCSF ChimeraX version 1.7.1, adapted from cryo-EM structure, model 8h9s Human ATP synthase state 1 (combined), https://doi.org/10.2210/pdb8H9S/pdb [2]. DAPIT protein is added from the cryo-EM structure of bovine F_o part, model 6zbb Bovine ATP synthase Fo domain, https://doi.org/10.2210/pdb6ZBB/pdb [184]. C-terminal part of subunit A6L (AA 52-68) is from AlphaFold computed structure, model AF-P03928-F1 [185,186]. Subunits of ATP synthase affected by both nDNA or mtDNA pathogenic variants are in color – α (yellow), β (blue), δ (coral), ε (purple), OSCP (brown), *c* (gold), DAPIT (cyan), *a* (dark blue) and A6L (pink). The remaining subunits are in various shades of green. Affected amino acids (red) are in sphere mode, remaining residues in stick mode. Only name and number of the residue is listed, for details about AA change and respective pathogenic variant see Table 2+3. Pathogenic variants of α, β, δ, and ε subunits are visualized in detail in black dashed boxes on the left (top view for α and β; side view for δ and ε). Black dashed box on the right: ATP synthase complex divided into structural parts: $α_3β_3$ hexamer (blue) together with central stalk (pink) form F_1 catalytic part; peripheral stalk (green); F_o membrane-embedded part (gold). IMM – inner mitochondrial membrane, IMS – intermembrane space. Pathogenic variants of subunit *a* (red box) are visualized in detail in red dashed box in part **(B)** of the figure (top view). Affected regions of DAPIT and OSCP subunits are not visualized since they lead to the loss of full-length mature protein. **(C)** Structure of human TMEM70 protein and its pathogenic variants. AlphaFold computed structure, model AF-Q9BUB7-F1 [185,186]. Transit peptide (TP) in green, N-terminal part (N-term) in yellow, transmembrane domain 1 (TM-1) in dark blue, intermembrane space loop (IMS) in pink, transmembrane domain 2 (TM-2) in light blue, C-terminal part (C-term) in purple. Affected positions labeled with red bal. Only name and number of the residue is listed, for details about amino acid change and respective pathogenic variant see Table 4.

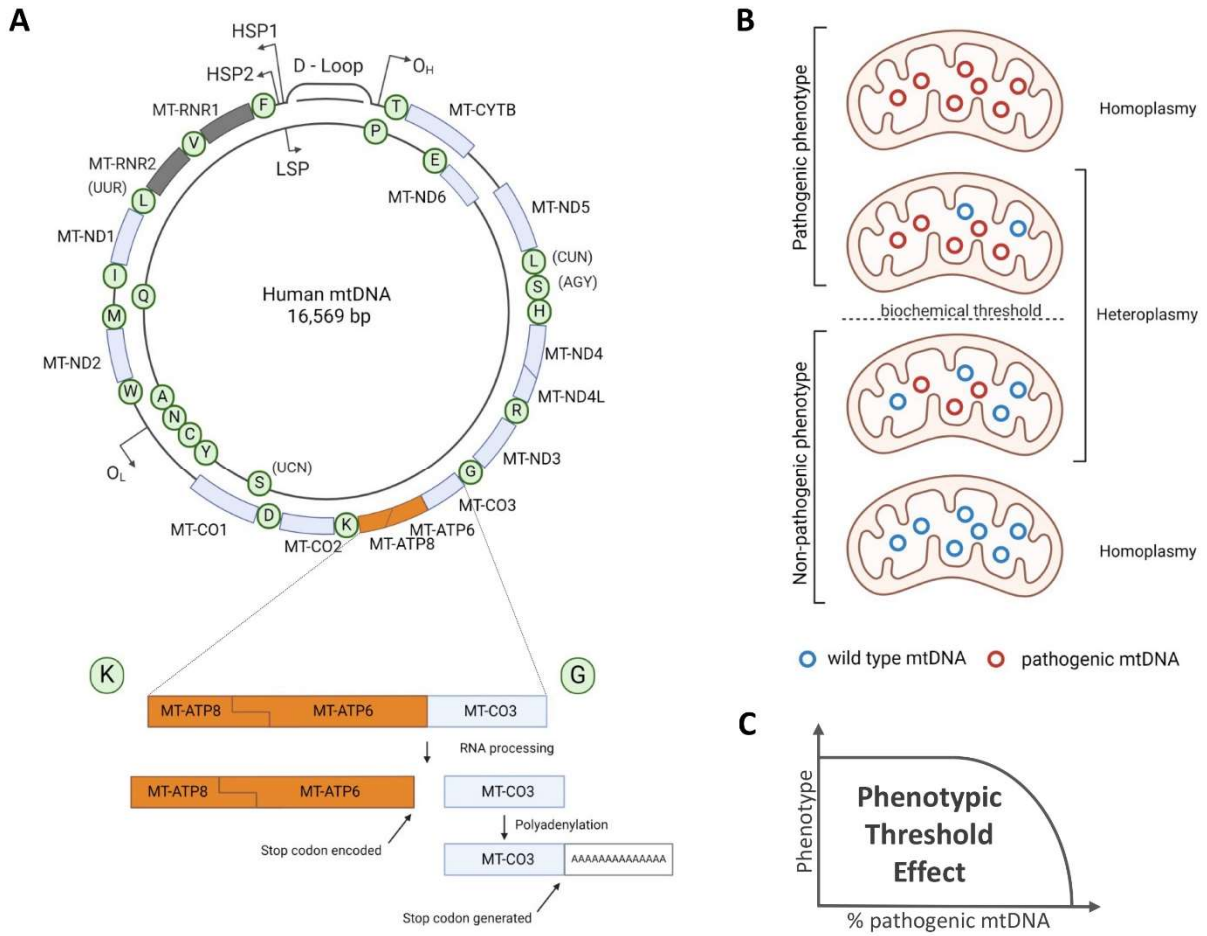

Fig. 2. (A) Human mitochondrial DNA encodes for 13 protein coding genes (light blue), the 22 tRNAs (green) and 2 rRNAS (grey). The origin of outer heavy strand (OH) and inner light strand (OL) are depicted. Transcription is bidirectional, initiated in D-loop control region from three promoters HSP1, HSP2 and LSP. Scheme of polycistronic MT-ATP8/MT-ATP6/MT-CO3 mRNA transcript is shown in detail (adapted from Ng 2022, 36399564). **(B)** Scheme of mtDNA heteroplasmy. The mtDNA in mitochondria are homoplasmic when all mtDNA copies are either wild type or mutant, the percentage of pathogenic mtDNA determines the mtDNA heteroplasmy. Biochemical threshold indicates the limiting amount of the pathogenic mtDNA above which the pathogenic phenotype manifests **(C).** Part **(A)** and **(B)** Created with BioRender.com. Part **(C)** adopted from Rossignol et al. [25].

The biogenesis and assembly of F_1F_o-ATP synthase is a complex process, due to its multisubunit composition, localization in the inner mitochondrial membrane, and two genomes-origin. In yeast, numerous assembly factors were described, with only a subset of them having mammalian homologs. Those conserved from yeast to humans include ATPAF1 and ATPAF2 (ATP11 and ATP12 in yeast), which are necessary for $\alpha_3\beta_3$ hexamer formation [5], and FMC1 homolog (or c7orf55, homolog of yeast FMC1 protein), which was suggested to play a role in this process as well [6]. On the other hand, two described assembly factors are specific for higher eukaryotes. TMEM70 and TMEM242 proteins are involved in the assembly of c_8 ring and its association with ATP synthase complex. However, the exact mechanism of their function is still not completely explained [7,8]. The last factor presumed to be involved in the assembly of ATP synthase in mammals is Prickle planar cell polarity protein 3 encoded by *PRICKLE3* gene [9].

Mitochondrial diseases

From an organismal perspective, mitochondria are the predominant source of ATP. Glycolysis can substitute for mitochondrial ATP production, but only in an organ or time limited fashion. Therefore, mitochondrial dysfunctions manifest in the tissues with high energy demands such as heart, brain, liver, or skeletal muscle, and frequently lead to metabolic diseases. Another consequence of defective oxidative phosphorylation system (OXPHOS) is increased oxidative stress, a condition also linked with human pathologies [10]. As a consequence of this, mitochondrial defects are associated with a broad range of clinical phenotypes. They range from

early-onset, severe and devastating encephalo-cardiomyopathies to late-onset and milder forms of mitochondrial diseases. However, they also include polygenic neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis; motor neuron diseases (amyotrophic lateral sclerosis); metabolic disorders, such as obesity and diabetes; metabolic syndrome; cancer; and aging [10-18]. Due to the dual genetic origin of OXPHOS system, mitochondrial diseases can be caused by pathogenic variants/mutations of either nuclear or mitochondrial genomes. Historically, maternally transmitted mtDNA (Fig. 2A) was more accessible for genetic studies, which led to extensive characterization of its mutational landscape. To date, over a thousand pathogenic variants in mtDNA have been described (MITOMAP: A Human Mitochondrial Genome Database http://mitomap.org, 2023, [19]) and their detection have become rather routine task [20,21]. However, according to current estimates, mtDNA variants are responsible for only ~15 % of mitochondrial diseases in the pediatric population [22]. Consequently, pathogenic variants of nuclear genes are more prevalent, while their identification and especially functional validation still represent a challenge.

Pathogenic variants of genes associated with isolated ATP synthase deficiency are summarized in Table 1. Approximately 60 % (38 out of 63) of the variants are encoded by nDNA, but this may be skewed by the fact, that only truly pathogenic variants of mtDNA are considered (for criteria, please see section "ATP synthase deficiencies associated with pathogenic variants of mtDNA"). Of these, 20 are localized in *TMEM70* gene, which encodes ATP synthase assembly factor, representing a slight majority of nDNA pathogenic variants. The remaining nDNA pathogenic variants are in genes encoding seven structural subunits, three of which are directly involved in the production of ATP. This means

Table 1. The number of pathogenic variants of genes related to isolated ATP synthase deficiency. For mtDNA genes encoding structural subunits of ATP synthase, the number of pathogenic variants discussed in this review is given, along with the total number of variants reported according to MITOMAP in parentheses. *MT-ATP8* variants include one in *MT-ATP8/MT-ATP6* overlapping region affecting only A6L subunit, similarly *MT-ATP6* variants include one in *MT-ATP8/MT-ATP6* overlapping region affecting only subunit *a*, *MT-ATP8/MT-ATP6* variants include those affecting both subunits. For nDNA genes, the total number of pathogenic variants reported is given, with compound heterozygous combinations counted as one variant (or being counted as a single variant).

mtDNA: structural subunits		**nDNA: structural subunits**		**nDNA: assembly factors**	
Gene	**No. of pathogenic variants (MITOMAP)**	**Gene**	**No. of pathogenic variants**	**Gene**	**No. of pathogenic variants**
MT-ATP8	1 (13)	*ATP5F1A*	5	*ATPAF1*	0
MT-ATP8/MT-ATP6	3 (5)	*ATP5F1B*	3	*ATPAF2*	1
MT-ATP6	21 (78)	*ATP5F1C*	0	*TMEM70*	20
		ATP5F1D	2	*TMEM242*	0
		ATP5F1E	1	*FMC1*	0
		ATP5PB	0		
		ATP5PD	0		
		ATP5PF	0		
		ATP5PO	2		
		ATP5MC	3		
		ATP5ME	0		
		ATP5MF	0		
		ATP5MG	0		
		ATP5MJ	0		
		ATP5MK	1		
		ATP5IF1	0		

that to date, no pathogenic variant has been described for nine nDNA genes encoding structural subunits of ATP synthase. We can only speculate, whether this suggests greater level of sequence flexibility for structural subunits compared to catalytic ones or whether pathogenic variants in the remaining subunits will be identified in the future. However, in the case of the only two mtDNA-encoded structural subunits of ATP synthase, it seems that the functional versus structural significance could really be the key factor. For *MT-ATP6* gene, which encodes the subunit directly involved in the production of ATP, approximately 80 variants have been described to date. However, the majority of these have not yet been confirmed as pathogenic. In contrast, for *MT-ATP8* gene, which encodes a subunit with an unknown function, only 13 variants with a predominantly unclear pathogenicity have been described. Moreover, in the case of *MT-ATP6*, pathogenic variants are distributed throughout the entire sequence, while *MT-ATP8* variants with the strongest pathogenic potential are localized at the end of the gene. This suggests that the C-terminal part of this subunit requires more specific fold without the room for any wobble. It is also noteworthy that five pathogenic variants, affecting both subunits, have been reported in *MT-ATP8/MT-ATP6* overlapping region (Fig. 2A).

Mitochondrial DNA pathogenic variants

In pediatric cases, pathogenic variants in mtDNA are often associated with severe metabolic defects, and to a lesser extent also with neurodegenerative syndromes, deafness, optic neuropathy, and other diseases. Approximately 550 of mtDNA pathogenic variants in structural genes for subunits of respiratory complex I, III and IV, as well as of ATP synthase, and more than 450 pathogenic variants in tRNAs or rRNAs, have been described. These include point substitutions and simple deletions or insertions (MITOMAP). The majority of mtDNA pathogenic variants are single nucleotide substitutions that result in a missense codon and replacement of respective amino acid (AA) with a different one. Less often, a premature stop codon is created, or a shift in the reading frame may occur due to nonsense or frameshift variants. In addition to maternally inherited point variants, more than a hundred mainly somatic deletions or insertions of large mtDNA regions were described (MITOMAP).

Clinical and biochemical phenotypes of the patients with mtDNA pathogenic variants can vary considerably between individuals harboring the same variant. This may reflect different nuclear genetic background of the patients since similar symptoms or group of symptoms can be observed within one family but vary between different families. Physiological variability in mtDNA may also influence the clinical presentation of a pathogenic variant in either way. Some mtDNA variants may serve as disease modifiers in a negative [23] or positive [24] manner. It is not uncommon for members of the same family carrying the same variant(s) to present with phenotypes ranging from none or very mild to severe. This variability can be explained by the phenomenon of mtDNA heteroplasmy, which refers the percentage of pathogenic mtDNA within a single cell, which typically contains several thousands of copies of mtDNA (Fig. 2B). In many cases, the degree of mutational load determines the pathogenic phenotype. In the most severe cases, all copies of mtDNA are pathogenic (also known as mtDNA homoplasmy). It is important to note that the relationship between heteroplasmy and disease progression is often not linear and exerts a phenotypic threshold effect when the disease starts to manifest (Fig. 2C) [25]. The key factor may not be just the level of heteroplasmy itself, but rather its variability across tissues. Individuals carrying similar mutational loads throughout the whole body, as well as patients with a broad span of heteroplasmy levels between tissues, are well described. For instance, m.8618-8619insT, which was identified in two patients exhibiting a predominantly neuro-muscular phenotype, was detected at relatively low levels in the blood (approximately 20 %), yet heteroplasmy levels of 65 % and 85 % were observed in skeletal muscle [26,27]. Another aspect blurring the relationship between heteroplasmy levels and the severity of the symptoms is the determination of mutational load itself. Here, we face the problems of methodological limitations, tissue availability for analysis, but also features of the analyzed material. For example, very rare m.9205delTA variant of *MT-ATP6* gene was reported only in the two cases with completely different phenotypes but both patients were originally reported as homoplasmic in fibroblasts [28,29]. Prolonged cultivation of the patients' fibroblasts with very mild phenotype revealed that the variant was present as heteroplasmic, albeit at a very high mutational load. Therefore, the mild phenotype may be explained by the selection against the pathogenic variant in different tissues (which were not available for analysis) [30].

ATP synthase deficiencies associated with pathogenic variants of mtDNA

Of the two ATP synthase subunits encoded by mtDNA, pathogenic variants of subunit *a* (*MT-ATP6*) are much more frequent, while subunit A6L (*MT-ATP8*) is only rarely affected (Table 1). The most prevalent are missense variants of mtDNA *MT-ATP6* gene. Altogether, around 80 disease-causing variants of *MT-ATP6* have been described, including small-scale deletions and insertions. These variants were associated with a range of severe disorders affecting brain, heart, and muscles, but also with deafness, multiple sclerosis, autism, optic neuropathy, and diabetes, usually in combination with other mtDNA variants (MITOMAP). Twelve variants of *MT-ATP8* gene were associated with heart and brain defects, but they were also linked to type II diabetes mellitus, again typically in combination with other possible pathogenic variants. Finally, eight cardiomyopathic or neuromuscular variants of *MT-ATP8*/*MT-ATP6* overlapping region (Fig. 2A) affecting either both genes or only *MT-ATP8* were described. Taken together, approximately 100 variants of mtDNA genes coding for ATP synthase subunits were reported to be (potentially) linked with human diseases (reported vs confirmed pathogenicity). Due to the high number of cases, in the following section, we will discuss only mtDNA variants with known phenotype that 1) are confirmed as disease-causing in MITOMAP, 2) were found in more than one patient as the single variant, or 3) were associated with aberrant ATP synthase structure and function as a possible explanation of the disease phenotype (Table 2).

Table 2. Single *MT-ATP8/MT-ATP6* and *MT-ATP6* pathogenic variants, associated with isolated ATP synthase disorders. In the case of substitutions in position 8993, only papers describing specific phenotype as the first, and studies describing unusual phenotypes, are listed.

Gene Variant (Protein)	Clinical phenotype	Biochemical phenotype	Refs.
MT-ATP8/MT-ATP6			
m.8528T>C (p.Trp55Arg/p.Met1Thr)	**HCMP/biventricular hypertrophy**, 3-MGA, HA, LA, hyperketonemia, LVNC, arrythmia, WPW, HF, PAH, hypotonia, exercise intolerance, myopathy, anemia, thrombocytopenia, PMR, FTT	↓↓↓ synthesis of both *a* and A6L subunits, ↓↓↓ cV complex levels, ↓↓↓ synthesis of ATP	[31-34]
m.8529G>A (p.Trp55Ter/-)	HCMP, neuropathy, ataxia, ophthalmoplegia, PMR (1P)	↓ cV complex levels, ↑↑ F_1 subcomplexes	[35]
m. 8561C>T (p.Pro66Ser/p.Pro12Leu)	Hypotonia, microcephaly, ataxia, lesions in the basal ganglia, bilateral retinal hypoplasia, PMR (1P)	↓ cV complex levels, ↑↑ F_1 subcomplexes, ↓ ATP hydrolytic activity	[37]
m. 8561C>G (p.Pro66Ala/p.Pro12Arg)	**hypergonadotropic hypogonadism**, **ataxia**, **neuropathy**, **brain atrophy,** sensorineural hearing impairment	↑ F_1 subcomplexes	[36]
MT-ATP6			
m.8611_8612insC (p.Leu29ProfsTer36)	LA, gallstones, ataxia, encephalopathy, myopathy, PMR (1P)	↓↓↓ content of subunit *a,* ↓↓↓ complex V stability, ↓↓↓ ATP hydrolytic activity	[92]
m.8612T>C (p.Leu29Pro)	HSP, LS, volatile anesthetic hypersensitivity, PMR	n.a.	[86,93]
m.8618-8619insT (p.Thr33HisfsTer63)	**cerebellar atrophy, hearing loss, PMR,** NARP, impaired renal function, myopathy, ophthalmologic defects, diabetes, FTT	↓↓↓ ATP hydrolytic activity, ↓↓ cV complex levels, ↑ F_1 subcomplexes, ↑ ROS	[26,27]
m.8782G>A (p.Gly86Ter)	IUGR, **cerebellar ataxia, hearing loss,** myoclonic epilepsy, short stature, kidney disease, diabetes, speech impairment	↓↓ ATP synthase activity, ↓↓ basal OCR, ↑ ROS, ↑↑ F_1 subcomplexes	[26]
m.8839G>C (p.Ala105Pro)	NARP (1P)	↓↓ $\Delta\Psi_m$	[88]

m.8851T>C (p.Trp109Arg)	BSN, LS, microcephaly, brain malformations, encephalopathy, stroke, myopathy, hypotonia, neuropathy	↓↓ cV complex levels	[41,87,94]
m.8969G>A (p.Ser148Asn)	MLASA, LA, HCMP, WPW, patent foramen ovale, epilepsy, brain atrophy, myoclonic seizures, IgA nephropathy, liver steatosis, hypospadias, hearing impairment, PMR, FTT	Normal cV complex levels, ↓↓ ATP synthase activity, ↑ ROS	[98,99, 101,102]
m.8989G>C (p.Ala155Pro)	NARP (1P)	↓↓ ATP hydrolytic activity	[89]
m.8993T>C (p.Leu156Pro)	LS, NARP, neuropathy, ataxia, brain atrophy, pyramidal signs, dystonia, muscle weakness, optic atrophy, hearing impairment	↓ ATP synthase activity, ↑↑ ROS	[21,40,47, 50,51,53,55, 60,61,112]
m.8993_8994TG>CA (p.Leu156Pro)	developmental delay and myopathy (1P)	n.a.	[41]
m.8993T>G (p.Leu156Arg)	LS, NARP, HCMP, seizures, ataxia, muscle weakness, glomerular proteinuria, sensorineural hearing loss, PMR	↓↓↓ ATP synthase activity, ↑↑ ROS	[39,42,43, 45,49,51,53, 54,56-58,62, 63,66,112]
m.9032T>C (p.Leu169Pro)	NARP, LA, epilepsy, ataxia, cerebellar atrophy, microcephaly, sensorineural hearing loss, PMR	↓ ATP synthase activity, ↑ ROS, ↑ F_1 subcomplexes	[90,97]
m.9035T>C (p.Leu170Pro)	**Ataxia,** LS, cerebellar atrophy, neuropathy, dystonia, hypotonia, hypertonicity, visual impairment, retinitis pigmentosa, learning disability, PMR	↓↓ ATP hydrolytic activity, ↑↑↑ ROS	[51,84,95, 96]
m.9127-9128delAT (p. Ile201ProfsTer2)	NARP (1P)	↓↓ ATP hydrolytic activity	[91]
m.9134A>G (p.Glu203Gly)	IUGR, LA, HCMP, hypotonia (1P)	↓↓ ATP hydrolytic activity	[100]
m.9155A>G (p.Gln210Arg)	**MIDD**, metabolic syndrome, focal segmental glomerulosclerosis	Normal cV complex levels and activity	[103,104]
m.9176T>C (p.Leu217Pro)	FBSN, LS, CMT, HSP, LA, HCMP, ataxia, dystonia, external ophthalmoparesis, episodic muscle weakness and neuropathy, PMR	Normal cV complex levels, ↓↓ ATP synthase activity, ↑ ROS	[38,47,48, 51,53,67,73, 77,79,81,82]
m.9176T>G (p.Leu217Arg)	LS, seizures, epilepsy, ataxia, spastic paraparesis, polyneuropathy, PMR	↓↓↓ ATP synthase activity	[57,68,69]
m.9185T>C (p.Leu220Pro)	LS, LS-like, NARP, CMT, WPW, HCMP, arrhythmia, brain atrophy, epilepsy, ataxia, hypotonia, neuropathy, neuropathy, muscle weakness, respiratory and renal failure, ptosis, sensorineural hearing loss, learning difficulties	↓ cV complex levels, ↓ ATP hydrolytic activity, ↑ ROS	[47,51-53, 55,70-76, 78,80,83,84]
m.9191T>C (p.Leu222Pro)	LS (1P)	↓ ATP hydrolytic activity	[76]
m.9205delTA (Ter-Met)	transient LA with seizures, severe encephalopathy and LA	↓↓↓ cV complex levels	[28,29]

1P – data available for 1 patient only; n.a. – biochemical phenotype for the patient is not available, in the case of m.8993_8994TG>CA expected to be similar to m.8993T>C, resulting in the same amino acid exchange; $\Delta\Psi_m$ – mitochondrial membrane potential; cV – complex V (ATP synthase); OCR – oxygen consumption rate; ROS – reactive oxygen species. Clinical phenotype in bold – shared symptoms if more than one patient; 3-MGA – 3-methylglutaconic aciduria; CMT – Charcot-Marie-Tooth disease; DF – dysmorphic features; (F)BSN – (familiar) bilateral striatal necrosis; FTT – failure to thrive; HCMP – hypertrophic cardiomyopathy; HA – hyperammonemia; HF – heart failure; HSP – hereditary spastic paraplegia; IUGR – intrauterine growth restriction; LA – lactic acidosis; LS – Leigh syndrome; LVNC – left ventricular non-compaction; MIDD – maternally inherited diabetes and deafness; MLASA – mitochondrial myopathy, lactic acidosis and sideroblastic anemia; NARP – neurogenic muscle weakness (neuropathy), ataxia and retinitis pigmentosa syndrome; PAH – pulmonary arterial hypertension; PMR – psychomotor retardation (in general, details in references and text); WPW – Wolf-Parkinson-White syndrome.

MT-ATP8/MT-ATP6 pathogenic variants

As previously stated, pathogenic variants of *MT-ATP8* gene are extremely rare with only a few patients described for each variant. This renders the discussion about their potential pathogenicity rather unsatisfactory. The majority of patients carry m.8528T>C missense variant of *MT-ATP8/MT-ATP6* overlapping region, which affects both subunits. This variant replaces highly conserved tryptophan 55 to arginine in A6L subunit and methionine 1 to threonine in the case of subunit *a*, potentially disrupting the initiation of translation of the subunit [31]. Hypertrophic cardiomyopathy (HCMP) or biventricular hypertrophy was found in almost all patients, with an early onset from prenatal to five months of life. Other life-threatening symptoms, including heart failure, metabolic crises, hypotonia, failure to thrive (FTT), and feeding difficulties, developed, and two patients died within a few months of life. The variant is typically present at a high heteroplasmy level, exceeding 90 % [31,32]. However, in the blood of one patient with HCMP, only 59 % of pathogenic mtDNA was detected [33].

Zigman *et al.* [34] described another patient with myocardial hypertrophy carrying homoplasmic m.8528T>C variant, who suffered from severe neonatal hyperammonemia requiring hemodialysis during the first days of life. The mother of the patient was the first asymptomatic carrier with high heteroplasmy (82 % in the blood) of m.8528T>C variant described while in the previous cases, very low levels of the variant were found in patients' relatives. This pathogenic variant was shown to cause impaired synthesis of both subunits *a* and A6L, with low levels of complete ATP synthase detected in patient samples. Based on the experience with m.9205delTA *MT-ATP6* variant [30], one would expect that the lack of subunit *a* is the primary driver of diminished assembly/stability of complex V in the case of m.8528T>C substitution. However, in a patient with similar phenotype of HCMP, neuropathy, ataxia, ophthalmoplegia, and psychomotor retardation carrying m.8529G>A variant (one base next to the previous one), only subunit A6L (p.Trp55Ter) is affected. Still, this leads to the decreased stability of ATP synthase complex [35]. As illustrated in Figure 1A, all A6L pathogenic variants described in this review are situated in the extra-membrane part of the protein, where it interacts with subunits of the peripheral stalk. Consequently, these variants can influence the connection between membrane part and the peripheral stalk. Similar to previously discussed cases, m.8529G>A variant exhibited a high degree of heteroplasmy (over 90 %), yet the disease course was rather milder, with later onset at 4 years of age. Excessive mutational load was determined also in patients with other two variants in *MT-ATP8/MT-ATP6* overlapping region, affecting the stability of ATP synthase, both in position 8561. These patients presented with neurological symptoms, including hypotonia, ataxia, microcephaly/brain atrophy, and neuropathy, without cardiac involvement [36,37]. In addition, variant m.8561C>G was associated with a very unusual mitochondrial disease phenotype of hypergonadotropic hypogonadism [36].

Most frequent MT-ATP6 pathogenic variants

Contrary to the above-mentioned variants, which affect both subunit *a* and A6L, variants affecting only *MT-ATP6* gene are predominantly linked with neurodegenerative phenotypes. The most common is devastating Leigh syndrome (LS), subacute, necrotizing encephalopathy characterized by bilateral symmetrical necrotic lesions of grey matter nuclei in the basal ganglia, diencephalon, cerebellum, or brainstem. The onset of the disease is typically in early infancy, and patients manifest a heterogeneous set of symptoms, including regression or psychomotor delay, optic atrophy, ophthalmoplegia, ptosis, nystagmus, respiratory abnormalities due to brainstem dysfunction, and pyramidal signs. In addition, patients may exhibit signs of dystonia, ataxia, peripheral neuropathy, and intention tremor associated with lactic acidosis (LA) in the blood, cerebrospinal fluid, or urine [38]. The second relatively common presentation of *MT-ATP6* pathogenic variants is less severe neurogenic muscle weakness (or neuropathy), ataxia, and retinitis pigmentosa (NARP) syndrome. However, the clinical picture of *MT-ATP6* patients is not limited only to these two syndromes.

The most prevalent is m.8993T>G missense variant, which was first described by Holt *et al.* in 1990. This variant replaces highly conserved leucine 156 with arginine [39]. To date, several hundred or even thousands of patients with m.8993T>G variant have been diagnosed. At the same position, another two pathogenic variants were identified. The second most common disease-causing variant of *MT-ATP6* gene is m.8993T>C, which changes leucine 156 to proline [40]. In a single patient, m.8993_8994TG>CA was also observed, resulting in the same amino acid substitution [41].

In accordance with the prevailing consensus, patients exhibiting heteroplasmy levels of up to ~70 % are typically asymptomatic or present with relatively mild symptoms. Heteroplasmy levels between 70 % and 90 %

are associated with NARP, while levels above 90 % are associated with the typical presentation of LS [39,40,42-49]. The m.8993T>G substitution typically presents in the first months of life, while in the patients with m.8993T>C, the onset is delayed, with a milder disease course [47,50-53]. However, while this is the general pattern, exceptions can be found. Thus, there are reports of patients with heteroplasmy above 90 % or even homoplasmy manifesting a milder phenotype than LS [41,51,53-57]. Conversely, individuals suffering from LS but harboring heteroplasmy under 90 % have been documented [51,53]. Another group includes patients with late or adult onset of the disease that harbor both very low or very high heteroplasmy [42,45,51,53,58,59] or mainly adults carrying m.8993T>C with a phenotype similar to NARP but possessing ophthalmologic disorders [60,61]. Exceptionally, m.8993T>G and m.8993T>C variants have been linked with a number of conditions, including renal disease [62], sensorineural hearing loss/deafness [21,50,63-65], epilepsy [65], and cardiomyopathy [66].

In addition, common *MT-ATP6* disease-causing variants include transitions at positions 9176 and 9185: m.9176T>G (p.Leu217Arg), m.9176T>C (p.Leu217Pro), and m.9185T>C (p.Leu220Pro). The clinical picture of the patients encompasses mainly neurodegenerative phenotypes with rather late onset, but the symptoms differ more than in the case of 8993 variants. The severity of symptoms is not heteroplasmy dependent, and almost all the patients possess very high mutational loads ranging from 90 % to homoplasmy. Once again, the most severe presentation is LS [38,47,48,51-53,67-75]. In some patients, the LS was accompanied by cardiac phenotype, epileptic seizures, renal failure [70,71,75], or with some features of poliodystrophy [68]. Interestingly, three patients with mild and reversible phenotype of LS were described [74,76].

Conversely, the Charcot-Marie-Tooth syndrome represents a condition of minimal severity. It is characterized by (but not limited to) muscle weakness and neuropathy, often associated with T>C transition at positions 9176 and 9185 [77-79]. In some patients, it is accompanied by ataxia or sensorineural hearing loss [70,78,80].

Another group of patients presents with milder symptoms, exceptionally linked with T>C substitutions. These include NARP [51,53,71], familial bilateral striatal necrosis (FBSN), which is characterized by developmental regression, choreoathetosis and dystonia progressing to spastic quadriparesis [81], and hereditary spastic paraplegia (HSP), a group of inherited disorders that involves weakness and spasticity [82]. FBSN and HSP were described only in patients with m.9176T>C [81,82]. Symptoms mimicking periodic paralyses due to channelopathies or spinal muscular atrophy, but were ultimately diagnosed as mitochondriopathy due to m.9185T>C transition, were described in few patients [55,83].

A significant proportion of patients carrying one of the common pathogenic variants present with a spectrum of neurological and neuromuscular disorders that do not fit into any specific syndrome. These include hypotonia, muscle weakness, neuropathy, ataxia, paresis, epilepsy, seizures, ptosis, and pyramidal signs, with varying severity [51,53,55,57,65,68,70,71,80,84,85], sometimes accompanied by mental retardation [67,68], LA, and cerebellar atrophy [83]. For all the common pathogenic variants, carriers with lower mutational load [38,51,53,60,69,74,78] as well as asymptomatic individuals with high heteroplasmy or even homoplasmy have been described [51,53,67,71,76]. However, they could just be identified before the disease onset since patients with very late onset of the disease are occasionally described for all the variants discussed above [58,61,74,82].

Less frequent MT-ATP6 pathogenic variants

The clinical presentation of remaining *MT-ATP6* pathogenic variants is highly diverse, encompassing the entire spectrum of symptoms commonly associated with mitochondrial diseases. LS is relatively rare presentation observed in patients with m.8612T>C, m.8851T>C, m.9035T>C, and m.9191T>C transitions [51,76,86,87]. Similarly, NARP is associated with m.8618-8619insT, m.8839G>C, m.8989G>C, m.9032T>C, and m.9127–9128delAT variants [27,88-91]. Still, the neurological signs such as encephalopathy, brain atrophy, epilepsy, stroke, and microcephaly, or neuromuscular symptoms of ataxia, hypotonia, BSN, neuropathy, and myopathy in general are the main clinical characteristics of rare *MT-ATP6* variants m.8611_8612insC, m.8612 T>C, m.8851T>C, and m.9035T>C [41,84,92-96]. In addition to the previously mentioned symptoms, various visual and hearing defects are also observed in individuals with m.8618-8619insT, m.8782G>A, m.9032T>C, and m.9035T>C variants, in the first two also kidney disease was reported [26,96,97]. The patient carrying m.8611_8612insC insertion was originally diagnosed at the age of four months with gallstones that persisted during

follow-up [92]. The severity of the disease course does not seem to be heteroplasmy dependent since the majority of patients have very high heteroplasmy above 90 % or even homoplasmy.

Slightly different spectrum of symptoms was found in the group of patients with m.8969G>A and one patient with m.9134A>G. In one case, MLASA plus syndrome was diagnosed (mitochondrial myopathy, LA and sideroblastic anemia plus developmental delay, sensorineural hearing loss, epilepsy, agenesis of the corpus callosum, FTT, and stroke-like episodes, [98]). Similarly, another individual exhibited epileptic episodes and decreased muscle strength, brain atrophy, severe hearing impairment together with kidney disease (IgA nephropathy) and cardiac involvement (Wolf-Parkinson-White syndrome) [99]. Remaining patients suffer from various combinations of cardiac symptoms (HCMP, patent foramen ovale), LA, anemia, hypotonia, myoclonic seizures, facial dysmorphism, hypospadias, tortuosity of the retinal vessels, liver steatosis, and psychomotor retardation [100-102].

Only two patients were described carrying m.9205delTA, two-base deletion that affects not only ATP synthase but also complex IV (cytochrome *c* oxidase). This mutation results in the removal of the stop codon of *MT-ATP6* gene, thereby altering the processing of MT-ATP8/MT-ATP6/MT-CO3 polycistronic transcript (Fig. 2A). Consequently, the biosynthesis of both subunit *a* of ATP synthase and of subunit Cox3 of cytochrome *c* oxidase is markedly diminished [30]. Transient LA and seizures were the only symptoms in the first patient with more than 90 % heteroplasmy [28], while the second homoplasmic patient suffered from severe encephalopathy and LA [29]. Finally, two adult patients were described with the m.9155A>G variant. They presented with maternally inherited diabetes and deafness syndrome (MIDD), with one of them also having metabolic syndrome and focal segmental glomerulosclerosis [103,104].

The specific type of the disease based on mitochondrial dysfunction is Leber's hereditary optic neuropathy (LHON), maternally inherited disease that can lead to acute bilateral blindness due to the loss of the optic nerve and papillomacular bundle nerve fibers, predominantly in young men [105]. The patients usually carry one of the so-called primary mutations in mtDNA genes encoding subunits of complex I. However, they may also carry additional, so-called secondary mutations that modify the disease course. Such secondary variants can be identified in the *MT-ATP6* gene as well. In sporadic cases, patients with LHON were described who carried only the substitution in *MT-ATP6* as a single variant (e.g. m.8836A>G and m.9101T>C). These variants were possibly causal for the disease development [106-108].

Functional and structural consequences of MT-ATP6 pathogenic variants

As the key subunit for proton translocation, subunit *a* plays a pivotal role in ATP synthesis and ATP synthase stabilization [30,109]. Missense variants of *MT-ATP6* typically affect the efficiency of proton translocation, resulting in reduced ATP production and eventually to increased reactive oxygen species (ROS) production [68,76,88-90,95,97,99,101,110-113]. In some cases, when ATP synthase activity is only slightly affected, increased ROS production itself may serve as the underlying pathological mechanism [55,82,95,112]. In contrast to the synthetic activity of ATP synthase, the hydrolytic activity of this enzyme is not altered in most patient samples. The reason for this is, that the reverse reaction of ATP hydrolysis is not dependent on the proton flux. Moreover, it was demonstrated that in the reverse mode, protons could be transported normally even when a pathogenic variant of subunit *a* was present [114]. ATP hydrolytic activity is therefore an inadequate diagnostic tool for isolated ATP synthase deficiency caused by pathogenic variants of mtDNA. Nevertheless, in certain set of mutations, even ATP hydrolysis has been observed to be reduced [70,76,91,100]. The literature contains conflicting reports regarding the impact of the most common variant, m.8993T>G, and the relatively rare m.9035T>C variant on ATP hydrolytic activity. Some studies have documented a reduction in ATP hydrolysis in a few patients [95,113,115], while others have observed no change [42,46,96,116]. The discrepancies observed may be attributed to differences in methodology and the use of different assay protocols, which can lead to disparate outcomes. Moreover, the hydrolytic activity specific for ATP synthase is typically determined as the oligomycin-sensitive portion of the total hydrolytic activity of the sample. It is questionable how variants of subunit *a* can alter the sensitivity of ATP synthase to oligomycin, which is bound to subunit *c* in close proximity to subunit *a*. Some studies suggest that m.8993T>G variant may result in increased sensitivity to oligomycin [116,117]. However, in the case of m.9035T>C, total hydrolytic activity was found to be decreased to the same extent as the oligomycin-sensitive portion [95]. Another aspect to consider is the

stability of the enzyme. In condition of activity measurement, F_1 can be (partially) dissociated from F_o, which results in a loss of sensitivity to oligomycin. For instance, some studies have indicated that normal ATP hydrolytic activity in muscle was preserved in some patients, yet it was decreased in fibroblasts of the same patient [45,65]. This suggests that the enzyme in fibroblasts may be more fragile. A reduction in ATP hydrolysis can be causally linked with a reduction in the levels of ATP synthase complex [70,76,78].

Previously, disease-causing substitutions and potential mechanism by which they can affect ATP synthase function were discussed in the context of available yeast [118] or bovine [65] structures. When we take into consideration the recently reported cryo-EM structure of human ATP synthase [2], we can now map all the reported variants on it (Fig. 1A), as recently demonstrated here [119]. It is evident that the majority of the disease-causing variants of *MT-ATP6* discussed here are changing residues around c_8 ring, in proximity with residues crucial for proton translocation (Fig. 1B). Residues 169, 170 and 203 are located in the region of subunit *a*, involved in the entry of protons from intermembrane space, while 109, 217, 220 and 222 are close to the exit site towards matrix. Positions 105, 155, 156, and 148 are in close proximity to residues, where exchange of protons between subunits *a* and *c* occur [118]. Residue 210 is rather far from active sites, yet its involvement in the proton flow through subunit *a* is probable. Transitions at positions 12 and 29 were accompanied by higher levels of F_1 part, suggesting that these residues could be involved in the connection of subunit *a* with peripheral stalk of ATP synthase complex.

In the case of frameshift variants and one nonsense variant creating premature stop codon, ATP production is strongly reduced. In addition, ATP synthase complex is significantly more labile due to the lack of full-length subunit *a* [26,27,29,92]. Decreased complex stability leads to higher levels of free F_1 part of the enzyme, which still possesses hydrolytic activity and may further exacerbate the biochemical phenotype.

Nuclear DNA pathogenic variants

As mentioned above, nuclear DNA variants are a frequent cause of mitochondrial disorders. Since the advent of next-generation sequencing (NGS), the number of recognized nuclear disease-causing genes has increased rapidly, and nuclear genetic defects in all respiratory chain complexes, including ATP synthase, have been reported. In most cases, defects in OXPHOS complexes present as autosomal recessive traits [120]. In other words, the patients are homozygous for one pathogenic variant, or compound heterozygotes carrying two different pathogenic variants of the same gene, one in each allele. The parents of the patients are usually healthy heterozygous carriers of the variant. Pathogenic variants have been identified either in genes encoding structural proteins or in the biogenetic and regulatory factors of OXPHOS machinery – so-called “direct and indirect hits” [121]. The spectrum of affected pathways, which ultimately result in the defect of OXPHOS apparatus is very broad. Pathogenic variants have been described in proteins involved in mtDNA stability, replication, and expression. Similarly, proteins involved in the metabolism of cofactors and toxic compounds may also affect OXPHOS system, and finally, proteins involved in mitochondrial dynamics, homeostasis, and quality control represent another broad group of targets [120].

Nuclear DNA pathogenic variants associated with isolated deficiency of ATP synthase

The first evidence that a nuclear gene variant may be associated with inborn ATP synthase dysfunction was published by Holme *et al.* [122], who described a child with cardiomyopathy, LA, persisting 3-methylglutaconic aciduria (3-MGA) and severely decreased activity of ATP synthase without any underlying mtDNA variant. A few years later, another patient with early onset neonatal and fatal LA, cardiomyopathy, and hepatomegaly was reported by Houštěk *et al.* [123]. In patient tissues, an isolated 70 % decrease of ATP synthase complex was found. However, transmitochondrial cybrids containing patients' mtDNA contained normal levels of the complex, confirming the nuclear origin of the disease. A number of similar patients with isolated ATP synthase deficiency lacking pathogenic mtDNA variants were later described [124]. However, the pathogenic variant of *ATPAF2* gene encoding ATP synthase assembly factor ATP12 was identified in only one of these patients [125]. In 2008, using the homozygosity mapping and sequencing of candidate genes in the group of 24 patients with ATP synthase deficiency, severe neonatal LA and encephalo-cardiomyopathy, *TMEM70* was identified as another disease-causing gene [126]. Since then, the number of new patients carrying *TMEM70* pathogenic variants has steadily increased, with more than 80 cases described to date. Apparently, *TMEM70* gene is highly susceptible to mutagenesis, and

this type of rare mitochondrial disease has rather frequent incidence.

The first pathogenic variant in ATP synthase structural gene was described two years later, when disease-associated variant of *ATP5F1E* gene encoding ε subunit was found in the patient with milder mitochondrial disease phenotype [127]. In contrast, four patients with two different *ATP5F1A* (α subunit) pathogenic variants with fatal disease course and premature death in early childhood (one week or several months) were reported at the same time [128,129]. With the development of sequencing techniques, several novel pathogenic variants of ATP synthase structural genes have recently been characterized. These include variants of genes encoding α, β, δ, ε, *c*, DAPIT and OSCP subunits [130-137]. It is noteworthy that a considerable number of the recently identified pathogenic variants of ATP synthase structural genes are heterozygous, yet still result in the manifestation of disease phenotypes. The autosomal dominant mode of inheritance is rather unusual in the context of mitochondrial diseases. Similarly, two heterozygous patients with *PEX14* pathogenic variant were recently described with a peroxisomal disorder, a condition normally linked with the autosomal recessive mode of inheritance [138]. These findings indicate the necessity for a change in the approach to the diagnostic process for genetic diseases, with a greater focus on these dominant pathogenic variants. Pathogenic variants of ATP synthase structural genes and their associated phenotypes are summarized in Table 3 and visualized in Fig. 1A. The severity of the clinical manifestation and biochemical consequences are highly variable, ranging from asymptomatic patients to premature death.

Although the number of genes involved in inborn and isolated ATP synthase deficiency has increased significantly, the pathogenic variants of structural genes still represent a very rare cause of the disease. In the following section, we will examine pathogenic variants and their clinical manifestations. For the purposes of this review, we have divided these rare pathogenic variants according to the severity of the phenotypes observed in a small group of patients. While this classification makes easier the discussion of phenotypes in the context of this review, it should be kept in mind, that it is highly subjective. It should be noted, that the current prevailing consensus is to see the severity as a continuous spectrum, where even the same pathogenic variant can present with different degree of severity in two different patients.

nDNA pathogenic variants in structural genes of ATP synthase with mild phenotype

In general, the less severe phenotype can be observed in the individuals carrying heterozygous substitutions in *ATP5F1A* [130,131], *ATP5F1B* [133], and *ATP5MC3* [131,135] genes.

Recently, *de novo* heterozygous pathogenic variant c.620G>A (p.His207Arg) of *ATP5F1A* gene coding for α subunit was found in four independent patients [130,131]. All the patients suffered from early-onset mitochondrial disease and presented with psychomotor delay, failure to thrive and LA, accompanied by feeding intolerance, chronic diarrhea, anemia, hyperammonemia, and in some cases, acute encephalopathy. Although the initial manifestation suggests a typical mitochondrial disease course with metabolic imbalance, the severity of the phenotype for this variant is rather mild, and the major clinical findings recovered by late infancy in all the cases.

Table 3. Nuclear DNA pathogenic variants of structural genes for ATP synthase subunits associated with isolated deficiency of ATP synthase.

Gene Variant (Protein)	Clinical phenotype	Biochemical phenotype	No. of patients	Refs.
ATP5F1A				
c.545G>A Ht (p.Arg182Gln)	ataxia, spastic paraparesis, dystonia, PMR	n.a.	1	[131]
c.620G>A Ht (p.Arg207His)	**LA, FTT**, HA, anemia, metabolic imbalance, encephalopathy, feeding intolerance, PMR, #	Normal cV complex levels, ↓↓↓ ATP hydrolytic activity, ↓ OCR	4	[130, 131]
c.962A>G Hm (p.Tyr321Cys)	**microcephaly, hypotonia,** IUGR, HF, encephalopathy, seizures, pulmonary hypertension, FTT	mtDNA depletion, ↓↓ ETC activity	2	[128]

c.985C>T + c.-49+418C>T* (p.Arg329Cys + *)	**severe encephalopathy, small renal cysts hypoplastic lungs**	↓↓↓ cV complex levels, ↓↓ ATP hydrolytic activity, ↓ OCR	2	[129]
c.1037C>T Ht (p.Ser346Phe)	LA, cerebral palsy, dystonia, spastic tetraparesis, absent speech, PMR	n.a.	1	[131]
ATP5F1B				
c.1000A>C Ht (p.Thr334Pro)	generalized dystonia	Normal cV complex levels, ↓↓↓ ATP hydrolytic activity, ↓ $\Delta\Psi_m$	3 (+2)	[133]
c.1004 T>C Ht (p.Leu335Pro)	hyperphagia, tachypnea, episodic hyperthermia, euthyroid hypermetabolism, FTT, PMR	↑ basal OCR, ↓basal $\Delta\Psi_m$, ↓ coupling of RCC and cV	2	[132]
c.1445T>C Ht (p.Val482Ala)	generalized dystonia	Normal cV complex levels, ↓ ATP hydrolytic activity, ↓↓↓ $\Delta\Psi_m$	1 (+1)	[133]
ATP5F1D				
c.245C>T Hm (p.Pro82Leu)	3-MGA, LA, HA, dilated CMP, rhabdomyolysis, PMR	↓ cV complex levels, ↓↓↓ ATP hydrolytic activity	1	[134]
c.317T>G Hm (p.Val106Gly)	3-MGA, HA, ketoacidosis, mild exercise intolerance, PMR	↓↓ cV complex levels, ↓↓ ATP hydrolytic activity	1	[134]
ATP5F1E				
c.35A>G Hm (p.Tyr12Cys)	**LA, PMR**, 3-MGA, HCMP, ataxia, seizures, hypotonia, dystonia, neuropathy, visual and hearing deficit, #	↓↓↓ cV complex levels, ↓↓↓ ATP synthase activity, ↓OCR	3	[127]
ATP5PO				
c.34C>T + c.329-20A>G (p.Gln12Ter + §)	LA, seizures, microcephaly, brain atrophy, dystonia, hypotonia encephalopathy, restlessness, sleep disturbances, PMR	↓↓ cV complex levels, ↑↑ F_1 subcomplexes	1	[131]
c.87+3A>G Hm (exon 2 skipped)	**LS**, **HCMP**, **hypotonia**, **hypospadias**, 3-MGA, bradycardia, seizures, epilepsy, hypothrophic muscles, renal insufficiency, DF, cryptorchidism, PMR	↓↓ cV complex levels, ↑↑ F_1 subcomplexes, ↓↓↓ ATP hydrolytic activity	3	[137]
ATP5MK				
c.87+1G>C Hm (exon 3 skipped)	**LS, febrile illness,** HCMP, accessory spleen, testicular atrophy, fatty liver	normal cV monomer levels but no dimer, ↓↓ ATP synthase activity	4	[136]
ATP5MC3				
c.236G>T Ht (p.Gly79Val)	LA, lower-extremity spasticity, PMR	↓↓ cV complex levels, ↓↓ ATP hydrolytic activity	1	[131]
c.318C>G Ht (p.Asn106Lys)	dystonia [dystonia or HSP]	↓ ATP synthase activity	2 [+17]	[131, 135]
c.319C>G Ht (p.Pro107Ala)	pyramidal signs, generalized dystonia, PMR	n.a.	1	[131]

Hm – homozygous variant; Ht – heterozygous variant; * – pathogenic substitution in the first intron disrupting expression of *ATP5F1A* gene from this variant; § – loss-of function allele associated with abnormal gene splicing and reduced ATP5PO mRNA levels; # – major clinical findings recovered during life; ΔΨm – mitochondrial membrane potential; cV – complex V (ATP synthase); ETC – electron transport chain; OCR – oxygen consumption rate; RCC – respiratory chain complexes. No. of patients in round brackets means healthy individuals carrying the pathogenic variant, in square brackets family members suffering from dystonia of HSP supposed to have the same *ATP5MC3* pathogenic variant. Clinical phenotype in bold – shared symptoms if more than one patient; 3-MGA – 3-methylglutaconic aciduria; DF – dysmorphic features; FTT – failure to thrive; (H)CMP – (hypertrophic) cardiomyopathy; HA – hyperammonemia; HF – heart failure; HSP – hereditary spastic paraplegia; IUGR – intrauterine growth restriction; LA – lactic acidosis; LS – Leigh syndrome; PMR – psychomotor retardation (in general, details in the references and text).

Heterozygous disease-causing variants have also been reported in *ATP5F1B* gene encoding β subunit, bringing new and exciting insights into the pathogenesis of isolated ATP synthase deficiency. Two of them, c.1000A>C (p.Thr334Pro) and c.1445T>C (p.Val482Ala) were recently described by Nasca *et al.* in two unrelated families [133]. All the patients presented with isolated and slowly progressive dystonia without any additional neurological or systemic features, with early or late onset, ranging from infancy to adolescence. Although dominant, these variants have incomplete penetrance, as asymptomatic carriers (aged 22, 57, and 76 years) were identified in both families.

Similarly, the very mild course of the disease, which preferentially affects muscle tissue, has been associated with heterozygous pathogenic variants of *ATP5MC3* gene coding for subunit *c* [131,135]. Variant c.318C>G (p.Asn106Lys) was found in two unrelated individuals, one patient, carrying *de novo* substitution, presented with late onset (7 years) isolated dystonia, while the second patient presented with earlier onset (2 years) generalized dystonia, and the family history shows autosomal dominant form of the disease [135]. Adult-onset cases tend to develop predominantly uncomplicated, gradually progressive HSP, early childhood-onset patients have severe progressive generalized dystonia, and later childhood cases have intermediate segmental dystonia or spasmodic dysphonia. Three branches of the family show a tendency for successive generations to develop earlier and more severe symptoms than their affected parents (genetic anticipation) [139]. *De novo* heterozygous substitution in the next bp, c.319C>G (p.Pro107Ala), was described in the patient who presented with milestone delay and pyramidal signs in addition to generalized dystonia, but with late onset of 7 years [131]. Finally, the c.236G>T (p.Gly79Val) *ATP5MC3* variant was reported in one patient with delayed psychomotor development, lower-extremity spasticity, and LA [131].

nDNA pathogenic variants in structural genes of ATP synthase with moderate phenotype

Mild to moderate severity of the phenotype is associated with other heterozygous variants of *ATP5F1A* [131] and *ATP5F1B* gene [132], but also with homozygous variants of *ATP5F1D* [134] and *ATP5F1E* gene [127,131].

Within multicenter collaboration and community data sharing, Zech *et al.* [131] found and described two additional patients carrying *de novo* heterozygous substitution in *ATP5F1A*, c.545G>A (p.Arg182Gln) and c.1037C>T (p.Ser346Phe). Patients carrying these variants presented with psychomotor delay, dystonia, spastic paraplegia or tetraparesis, intellectual disability, ataxia, or cerebral palsy, the later also with swallowing problems and LA. At the last follow-up, the patients were 17 and 12 years old, respectively.

ATP5F1B de novo heterozygous c.1004T>C variant (p.Leu335Pro) was found in monozygotic twin boys, who were born with intrauterine growth restriction (IUGR) and developed failure to thrive at two months of age, later accompanied by hyperphagia, developmental delay, euthyroid hypermetabolism characterized by excessive caloric intake, inability to gain weight, and tachypnea [132]. Both patients had unexplained episodic hyperthermia, a condition similar to Luft syndrome [140,141].

Two homozygous substitutions with biparental inheritance were found in *ATP5F1D* gene encoding δ subunit, c.245C>T (p.Pro82Leu) and c.317T>G (p.Val106Gly), each in one patient [134]. Both patients underwent episodes of metabolic decompensations with LA, 3-MGA and hyperammonemia, both suffered from muscle weakness and their psychomotor development tended to be slightly delayed. They differ in the onset of the disease, c.245C>T manifested on the second day of life, with additional phenotypes of rhabdomyolysis and dilated cardiomyopathy (normalized between first and fourth years of life), whereas in the case of c.317T>G, the patient was healthy until almost five years of age, with no further cardiologic or neurologic symptoms until the last follow-up at six years of age.

The first nuclear pathogenic variant of ATP synthase structural gene was identified in a patient who was 22 years old at the time of diagnosis (P13 in [124]). This patient developed early-onset LA and 3-MGA but without cardiac involvement. This was followed by mild mental retardation, exercise intolerance, ataxia, peripheral neuropathy, and dystonia. Targeted sequencing of *ATP5F1E* gene encoding ε subunit revealed homozygous missense variant *c.35A>G* replacing tyrosine 12 with cysteine [127]. The same substitution was later found in two unrelated patients [131]. A 13-year-old girl presented with a similar phenotype of intellectual disability, developmental delay, LA, ataxia, seizures, peripheral neuropathy, and transient respiratory failure. Severe respiratory distress at birth was one of the initial phenotypes of the third patient, along with vomiting, seizures, and LA, followed by developmental delay, progressive generalized dystonia, and visual and hearing

deficits. In all three patients, the metabolic abnormalities improved or normalized later in life [131].

nDNA pathogenic variants in structural genes of ATP synthase with severe phenotype

Remaining homozygous pathogenic variants of structural genes of ATP synthase subunits, namely of *ATP5F1A* [128,129], *ATP5MK* [136], and *ATP5PO* [131,137] manifested with severe, usually lethal phenotype, and most of the patients died within the first year of life (mainly *ATP5F1A* and *ATP5PO* patients), or during childhood (*ATP5MK* patients).

First disease-causing variants of *ATP5F1A* gene were described almost simultaneously in 2013, with both papers describing unexpected features. The first study found *ATP5F1A* variant in a patient who presented with microcephaly, pulmonary hypertension, hypotonia, and heart failure at birth and died at three months of age [128]. The authors identified homozygous c.962A>G substitution (p.Tyr321Cys) in *ATP5F1A* gene in the index patient and her older sister, who previously died at 15 months of age with a diagnosis of microcephaly, hypotonia, and seizures, while their mother was healthy carrier of the variant. The study of Jonckheere *et al.* [129] describes two siblings with severe neonatal encephalopathy, both died in the first week of life. In this case, compound heterozygous variants c.985C>T + c.-49 + 418C>T of *ATP5F1A* were found. The missense variant c.985C>T leads to the replacement of arginine 329 with cysteine. However, this replacement in heterozygous form cannot explain the severity of the patients' phenotype, since their father was heterozygous healthy carrier of the variant. Indeed, the authors found that healthy mother of the patients carried heterozygous substitution c.-49 + 418C>T localized in the first intron of *ATP5F1A* gene resulting in the decreased levels of ATP5F1A mRNA (60 % of controls) in her fibroblasts. This suggests that the synthesis of α subunit from this variant is partially disrupted, and only pathogenic allele is expressed in both patients.

Four patients from three unrelated families were described by Barca *et al.* with c.87+1G>C pathogenic variant of *ATP5MK* gene encoding DAPIT protein [136]. All of them were diagnosed with severe LS between 6 and 18 months of age. The disease manifested with developmental delay and ataxia in all patients, other symptoms included movement disorders, various types of brain lesions, ophthalmoplegia, accessory spleen, testicular atrophy, fatty liver, and HCMP. Two patients died at six and nine years of age, while the other two aged six years were still alive at the time of reporting. This splice site variant resulted in the loss of DAPIT protein due to skipping of exon 3.

Last subunit of ATP synthase, whose variants have been associated with ATP synthase deficiency, is subunit OSCP. Zech *et al.* described one patient with compound heterozygous variant of *ATP5MO* gene, c.34C>T + c.329-20A>G [131]. As a result of c.34C>T nonsense variant, 12 AAs long OSCP protein is produced, representing only the first half of transit peptide, while c.329-20A>G splice site variant resulted in the skipping of exon 5 and exon 4 plus 5 and to a drastic reduction in ATP5MO mRNA levels. The patient with neonatal onset died at age of six years after several epileptic episodes. She suffered from severe symptoms including fever-induced partial seizures, hypotonia, LA, acquired microcephaly, global developmental delay, dystonia, progressive brain atrophy, seizure deterioration, epilepsy, restlessness, sleep disturbances, and speech abnormality. Another splice site c.87+3A>G variant of *ATP5MO* gene, resulting in the skipping of exon 2 of OSCP protein, has been reported in three LS patients from two unrelated families. Since exon 2 encodes the last 11 AA (out of 23) of transit peptide, this variant is associated with drastic reduction of OSCP levels in the mitochondria. Two male patients died in the first six months of life, while the only female patient was three years old at the time of publication [137]. They all shared common symptoms of LS, such as hypotonia, developmental delay, HCMP, LA, progressive epileptic encephalopathy, and progressive brain atrophy. In addition, facial dysmorphism was present in two siblings, and both boys had hypospadias, in one of them accompanied with cryptorchidism and enlarged kidneys.

Functional consequences of structural subunits pathogenic variants

Huge variability is associated not only with the clinical features of patients, but also with the underlying biochemical phenotype. Some variants cause defects in the structure and stability of the enzyme, accompanied with severe reduction of ATP synthase levels and activity, while others affect only the activity, but not the content of the enzyme. In some cases, however, the relevant biochemical data are not available, and in others they are scarce. Significantly decreased activity of ATP synthase, mainly determined as hydrolytic activity, but normal levels of ATP synthase have usually been found in the tissues of patients with milder or reversible phenotype, namely in

patients with heterozygous substitutions in *ATP5F1A* (c.620G>A) [130] and in *ATP5F1B* (c.1000A>C and c.1445T>C) [133], but also in patient with homozygous c.87+1G>C variant of *ATP5MK* suffering from Leigh syndrome [136]. Strong reduction of both activity and content of ATP synthase complex has been found in samples from patients with severe or even lethal phenotype, carrying pathogenic variants of *ATP5F1A* [129] or of *ATP5PO* [131,137], but it has also been described in case of *ATP5F1E* [127,131], *ATP5F1D* [134], and *ATP5MC3* [131] variants, resulting in mild phenotype.

In the case of pathogenic *ATP5MK* and *ATP5PO* variants, the affected proteins are key structural ATP synthase subunits, DAPIT and OSCP, respectively. OSCP is essential for the assembly of the peripheral stalk, which stabilizes enzyme structure, but also regulates the activity of catalytic F_1 part [142]. It is highly probable that aberrant or completely missing OSCP protein has a strong effect on the whole enzyme. Indeed, the levels of ATP synthase complex were drastically reduced in fibroblasts of patients, causing a strong decrease in hydrolytic activity of ATP synthase [131,137]. DAPIT protein has been shown to affect dimerization of ATP synthase complex [143], but its exact role in enzyme function is not known because it does not form dimerization interface between ATP synthase monomers [2,65]. In patient fibroblasts, the absence of DAPIT protein biochemically resulted in a severe reduction of ATP levels and the absence of dimeric form of ATP synthase, altering mitochondrial cristae structure [136].

Not surprisingly, all the missense variants that manifest as the ATP synthase defects affect highly conserved residues. Most of them are located in genes for subunits of F_1 domain (Fig. 1A). As already mentioned, homozygous variants of *ATP5F1D* and *ATP5F1E* (δ and ε subunits, respectively) share the same biochemical phenotype with significant and rather severe reduction of ATP synthase content and thus with reduced activity of the complex, resulting in similar mild and rather non-progressive disease course [127,131,134]. Together with γ subunit, they form central stalk that stabilizes γ subunit connection with the *c* ring and they are important for the complex stability [2]. Both *ATP5F1D* pathogenic variants lead to changes in the predicted protein structure of δ subunit, associated with its inability to properly bind other F_1 subunits. This results in reduced assembly of ATP synthase complex, but normal levels of free δ subunit [134]. In contrast, amount of ε subunit is significantly reduced as a result of pathogenic *ATP5F1E* variant, and the remaining ε subunit is assembled into ATP synthase complex without affecting its stability, even when pathogenic [127].

Since α subunit forms catalytic $\alpha_3\beta_3$ hexamer, the severe phenotype of homozygous variant c.962A>G in *ATP5F1A* is expected. Furthermore, this substitution is located in highly conserved region associated with mitochondrial genomic integrity (*mgi*) in yeast [144]. Although the levels of ATP synthase in patient samples are not discussed in the original report, based on the yeast model the authors suggest that this variant led to reduced synthesis of all OXPHOS mtDNA-encoded subunits, and thus to decreased membrane potential and uncoupling of ATP synthase. This is in accordance with significant decline in activity of complex I, III and IV in the patient's muscle tissue and with mitochondrial DNA depletion in muscle and liver (app. 40 % of controls) [128]. Similarly, in patients with compound heterozygous c.985C>T + c.-49 + 418C>T *ATP5F1A* variant, where only pathogenic variant of the subunit is produced, the interaction between α and β subunits is disturbed, leading to drastic reduction in the level of assembled ATP synthase and fatal course of the disease [129].

On the contrary, the heterozygous variants of α or β subunits genes result in decreased activity but normal levels of ATP synthase complex, leading to mild or moderate disease phenotype. All three substitutions in *ATP5F1A*, c.545G>A, c.620G>A and c.1037C>T, are localized at the interface between α and β subunits (Fig. 1A), as clearly shown by authors using 3D modelling [130,131]. Since ATP synthesis takes place at α/β interface [145], these variants may affect assembly/stability of $\alpha_3\beta_3$ hexamer and of the whole complex, but also functional properties of the enzyme. However, decreased hydrolytic activity of ATP synthase but normal levels of ATP synthase complex were found only in the case of c.620G>A variant, since it was not analyzed in detail in the others. At the α/β interface and close to the pocket for γ subunit, heterozygous c.1000A>C *ATP5F1B* substitution is also localized (Fig. 1A). Interestingly, c.1445T>C variant has exactly the same clinical presentation as c.1000A>C, butt c.1445T>C is not located at α/β interface, but on the outer surface of β subunit (Fig. 1A), far away from any contact site [133]. All these heterozygous substitutions affect synthesis of ATP everywhere, but only specific cell types and tissues, such as neurons and muscle, appear to be sensitive to this mild energetic deprivation [133]. Since heterozygous variants produce about 50 % of defective subunit (α or β) and one ATP synthase complex

contains three copies of each in its F_1 domain, it is questionable how many pathogenic copies are present in one fully assembled enzyme and thus how the enzyme activity is affected. Interestingly, in patients with c.620G>A variant of *ATP5F1A*, the symptoms remitted and patients later developed normally [130,131], suggesting that cells can either adapt their metabolism to reduced ATP supply or to assemble the enzyme preferentially using the non-defective subunits.

Slightly exceptional are the patients with heterozygous *ATP5F1B* variant, c.1004T>C, who present with more severe Luft-like phenotype [132]. Luft syndrome is a very rare disease, belonging to the category of "mitochondrial uncoupling syndromes" presenting with high caloric intake and hyperthermia caused by uncoupled proton translocation and ATP synthesis, with only a few patients described worldwide [140,141]. Analysis of patient fibroblasts confirmed that the mitochondrial respiration is not fully coupled to ATP synthesis, and membrane potential is dissipated in the form of heat. It is of interest that this variant lies in the region of *mgi* variants in yeast, as discussed above [132].

Another example of pathogenic heterozygous variant in a subunit present in multiple copies per enzyme monomer are substitutions in *ATP5MC3* gene encoding subunit *c*. It plays a crucial role in energy transduction, since *c* ring harnesses the energy released from the translocation of protons across the mitochondrial inner membrane (from intermembrane space to matrix) and couples it to its rotation. Subunit *c* is tightly connected with γ subunit, which fits between α and β interface and the rotation results in structural changes in $\alpha_3\beta_3$ hexamer necessary for binding and phosphorylation of ADP (and P_i) and release of generated ATP. Variant c.236G>T affects amino acid at the interface of the two adjacent helixes (Fig. 1A), potentially disturbing its interaction [131]. c.318C>G and c.319C>G affect highly conserved loop structure that interacts directly with the F_1 subunits (Fig. 1A) [131,135]. Biochemically, all these variants lead to decreased hydrolytic activity of ATP synthase and, in the case of c.236G>T, to a significant decrease in the amount of fully assembled ATP synthase. For the remaining two variants, the level of assembled ATP synthase was not determined in the original report, so no general conclusions can be drawn. However, even in the cells completely lacking all three isoforms of subunit *c*, vestigial complex remains present in the membrane [146]. An interesting aspect is, that subunit *c* is encoded by three genes *ATP5MC1, ATP5MC2,* and *ATP5MC3*, which do not show any strong tissue-specific expression and ultimately produce identical mature protein. The requirement for the presence of 8 copies of subunit *c* per assembled *c* ring may explain the dominant presentation of the heterozygous *ATP5MC3* variant even under such conditions. However, the low penetrance of the c.318C>G variant is peculiar. It would be attractive to speculate that non-symptomatic carriers express subunit *c* from its other isoforms.

nDNA pathogenic variants in assembly factors of ATP synthase

ATPAF2

In 2004, De Meirleir *et al.* [125] described a patient with severe neonatal encephalopathy harboring missense variant of ATPAF2 protein, an assembly factor essential for incorporation of α subunit into F_1 domain of ATP synthase. A homozygous c.280T>A substitution in *ATPAF2* gene was found in a girl with dysmorphic features, cortical-subcortical brain atrophy followed by basal ganglia atrophy and metabolic acidosis, who died at the age of 14 months. The TGG>AGG transition caused replacement of evolutionary conserved neutral tryptophan at position 94 to a basic arginine (Table 4), probably affecting the interaction of ATPAF2 with α subunit and leading to a severe reduction of native complex V without accumulation of F_1-containing subcomplexes.

Table 4. Nuclear DNA pathogenic variants in ATP synthase assembly factors associated with isolated deficiency of ATP synthase

Gene Variant (Protein)	Clinical phenotype	Biochemical phenotype	No. of patients	Refs.
ATPAF2				
c.280T>A Hm (p.Trp94Arg)	encephalopathy, 3-MGA, LA, DF	↓↓↓ levels of cV complex, ↓↓ ATP hydrolytic activity	1	[125]

TMEM70				
c.105dupT Hm (p.Val36CysfsTer52)	**IUGR, LA, HCMP, hypotonia, DF**, 3-MGA, HA, hypoglycemia, hypercitrullinemia, encephalopathy, buphthalmos, PMR, FTT, gastrostomy	n.a.	2	[161]
c.141delG/c.316+1G>A (p.Pro48ArgfsTer2/ exon 2 skipped)	**LVNC, PMR**, 3-MGA, LA, metabolic acidosis, HA, hypoglycemia, hyperketonemia, tachypnea, hernia, undescended testicle, FTT	↓↓↓ levels of cV complex	2	[166]
c.211–450_317–568del Hm (2290bp deletion – exon 2)	IUGR, 3-MGA, LA, HCMP, PMR	↓↓↓ levels of cV complex	1	[150]
c.238C>T Hm (p.Arg80Ter)	oligohydramnios, **IUGR, 3-MGA, LA, HA, encephalopathy**, **hypotonia,** metabolic acidosis, HCMP, multiorgan failure, DF, PMR, FTT, apnea	↓↓ ATP hydrolytic activity	2	[154,156, 159]
g.2436–3789 in-frame deletion Hm (exon 2 skipped)	oligohydramnios, IUGR, LA, HA, HHH-syndrome, HCMP, encephalopathy, ataxia, ptosis, PMR, FTT, gastrostomy	↓↓↓ levels of cV complex	1	[160]
c.316+1G>T Hm (exon 2 skipped)	IUGR, in utero cardiac thickening, **oligohydramnios, LA**, **encephalopathy, hypotonia**, **hypospadias**, **DF**, aminoaciduria, HCMP, HF, hernia, PMR, FTT, feeding difficulties, intestinal pseudo-obstructions and delayed gastric emptying	n.a.	2	[154,159]
c.317–2A>G Hm (exon 2 skipped)	oligohydramnios, IUGR, **3-MGA, LA**, **HA**, **HCMP/NCCM/LVNC**, **hypotonia**, **DF, PMR**, **FTT**, HF, hypoglycemia, cardiomegaly, WPW, tachycardia, epilepsy, PPHN/AH, microcephaly, ataxia, brain atrophy, extrapyramidal signs, liver failure, hepatomegaly, microphthalmia, cataract, ptosis, strabismus, hypospadias, cryptorchidism, hernia, feeding difficulties, apnoea	↓↓↓ levels of cV complex, ↓↓↓ ATP synthase activity	52	[123,124, 126, 148-158]
c.3172A>G/c.118_119insGT (exon 2 skipped/ p.Ser40CysfsTer11)	oligohydramnios, poor fetal movement, **3-MGA, HCMP**, LA, hernia, diastases recti, DF, FTT	↓↓↓ levels of cV complex, ↓↓↓ ATP hydrolytic activity	2	[126,148, 154,164]
c.317–2A>G/c.251delC (exon 2 skipped/n.a.)	late-onset epilepsy and mild intellectual disability	n.a.	1	[154]
c.317–2A>G/c.349_352del (exon 2 skipped/ p.Ile117AlafsTer36)	oligohydramnios, IUGR, LA, HA, HCMP, epilepsy, hypotonia, DF, PMR, apnea	↓↓↓ levels of cV complex, ↓↓ ATP hydrolytic activity	1	[156]
c.317–2A>G/c.470T>A (exon 2 skipped/n.a.)	n.a.	n.a.	1	[154]
c.317–2A>G/c.494G>A (exon 2 skipped/p.Gly165Asp)	3-MGA, LA, HA, Reye-like syndrome, tachycardia, HCMP, brain abnormalities, exercise intolerance	↓↓↓ levels of cV complex	1	[165,170]
c.317–2A>G/c.628A>C (exon 2 skipped/p.Tyr210Pro)	**3-MGA, LA, HA, hypotonia, PMR, growth retardation**, patent foramen ovale, HCMP, pneumothorax, PPHN, WPW	↓↓↓ levels of cV complex, ↓↓ ATP synthase activity	2	[153]
c.317–2A>G/c.783A>G (exon 2 skipped/p.Ter261Trpext17)	oligohydramnios, IUGR, LA, HCMP, epilepsy, DF, PMR, apnea	↓↓↓ levels of cV complex, ↓	1	[156]

		ATP hydrolytic activity		
c.336T>A Hm (p.Tyr112Ter)	oligohydramnios, IUGR, in utero cardiac thickening, LA, HA, HCMP, hypertonia, limb deformations, hernia, undescended testicles, hypospadias, DF, PMR, FTT	↓↓↓ ATP hydrolytic activity	1	[159]
c.359delC Hm (n.a.)	n.a.	n.a.	1	[154]
c.535C>T Hm (p.Tyr179His)	IUGR, 3-MGA, HA, LA, HCMP, hypotonia, DF, hernia, bilateral cataract, undescended testis, hypospadias, PMR	n.a.	1	[154,162]
c.563T>C Hm (p.Leu188Pro)	IUGR, 3-MGA, LA, HCMP, LVNC, dilated aortic root + sinotubular junction + ascending aorta, hypotonia, DF, FTT	n.a.	1	[163]
c.578_579delCA Hm (p.Asn198Ter)	**IUGR, 3-MGA, LA, HCMP, encephalopathy, hypotonia, cataract, DF, hypospadias, PMR, FTT,** ataxia, brain atrophy, epilepsy, hernia, vomiting, undescended testis, delayed gastric emptying,	↓↓↓ ATP hydrolytic activity	2	[154,159]
c.701A>C Hm (p.His234Pro)	oligohydramnios, IUGR, **3-MGA, LA, HCMP, hypotonia, PMR,** HA, encephalopathy, epileptic spasm, PPHN, leukoencephalopathy, DF, FTT, feeding difficulties, apnea	↓↓ ATP hydrolytic activity	2	[152,156]

Hm – homozygous variant, Ht – heterozygous variant; as – altered splicing; cV – complex V (ATP synthase). n.a. – data not available. Clinical phenotype in bold – symptoms described in most of the patients if more than one; 3-MGA – 3-methylglutaconic aciduria; AH – arterial hypertension; DF – dysmorphic features; FTT – failure to thrive; HA – hyperammonemia; HCMP – hypertrophic cardiomyopathy; HF – heart failure; HHH-syndrome – hyperornithinemia-hyperammonemia-homocitrullinuria syndrome; HSP – hereditary spastic paraplegia; IUGR – intrauterine growth restriction; LA – lactic acidosis; LS – Leigh syndrome; LVNC – left ventricular non-compaction; NCCM – non-compaction cardiomyopathy; PPHN – persistent pulmonary hypertension of the newborn; PMR – psychomotor retardation (in general, details in the references and text).

TMEM70

Pathogenic variants of *TMEM70* gene, which encodes TMEM70 protein, represent the most common cause of isolated ATP synthase deficiency in newborns (Table 4). *TMEM70* was first described in 2006 by Calvo *et al.* [147] as a gene encoding potentially mitochondrial protein. TMEM70 protein was recognized as a new biogenetic factor of ATP synthase based on the findings in cells of patients carrying *TMEM70* pathogenic variants, which in most cases led to a drastic reduction in the level of fully assembled ATP synthase with a slight accumulation of F_1 subassemblies [126]. First two pathogenic variants of T*MEM70* gene were found in the group of patients with severe neonatal LA and encephalo-cardiomyopathy.

c.317–2A>G: the most common TMEM70 pathogenic variant

Homozygous splice site c.317–2A>G variant at the end of the second intron of *TMEM70* gene, which prevents the synthesis of TMEM70 protein, has been found in 24 cases including the first patient reported in 1999 [123,126,148]. Later on, another 28 patients carrying this variant in bi-allelic form were described [149-158]. The absence of TMEM70 protein in patients typically presented prenatally as IUGR, sometimes accompanied with oligohydramnios. Alternatively, symptoms may appear soon after birth. Early phenotypes include severe metabolic disbalance with LA, 3-MGA, hyperammonemia, hypotonia, and HCMP. During the life, other typical symptoms develop, less severe malformations and abnormalities such as facial dysmorphism, hernias (including the most severe diaphragmatic hernia), hypospadias and cryptorchidism in boys, but also neuromuscular disorders (ataxia, extrapyramidal signs, ptosis), strabismus, hepatomegaly, brain defects including encephalopathy, atrophy, microcephaly, and epilepsy, and general developmental delay with failure to thrive and psychomotor retardation. In a subset of patients, the disease course was accompanied with persistent pulmonary artery hypertension in the neonatal period [149,152-154]. Although the metabolic crisis is usually one of the initial phenotypes, Baban *et al.* described 3 patients suffering from early-onset metabolic

cardiomyopathies, one with *TMEM70* c.317–2A>G variant and with 3-MGA development only at later stage [158]. Rarely, the cardiac presentation is not limited to HCMP, but also leads to another type of cardiomyopathy, left ventricular non-compaction (LVNC) [155], and in one case in combination with HCMP, progressing to dilated form [158]. Heart rate abnormalities are also uncommon, presenting mainly as tachycardia or Wolf-Parkinson-White syndrome [149,152-154,157,158]. Six patients out of 51 were not diagnosed with any form of cardiac involvement [148]. As mitochondrial defects can be associated with ophthalmologic defects, these were found also in patients carrying *TMEM70* c.317–2A>G variant, except for strabismus as a rather rare condition of chronic progressive external ophthalmoplegia, cataract and microphthalmia [148,152-154].

More than half of the patients died, usually from a severe acute metabolic crisis or heart failure. They can be divided into two groups according to the age at death – most of the patients died in the first few days or months of life at most, and the rest between one and five years. Interestingly, some patients can survive significantly longer, with one of them reaching 12 years and two reaching 13 years when they were last reported [148,150,155]. As pointed out by Honzík *et al.* in a detailed retrospective clinical study, if the patients survive the critical postnatal period of the first weeks and months of life, the metabolic problems and cardiac disorders may at least partially recede [148]. Interestingly, none of the TMEM70 patients with neonatal onset surviving to the age five years, nor any of the patients with later onset, have died to date.

Other TMEM70 pathogenic variants

Discovery of *TMEM70* as disease-causing gene in patients with a previously unknown cause of isolated ATP synthase deficiency of nuclear origin allowed the diagnosis of other patients, and indeed the patients carrying the novel *TMEM70* pathogenic variants were reported. In most cases, the clinical picture shared the same typical features as the common variant, but the genetic background was different.

Three homozygous pathogenic variants have been reported, skipping exon 2 of *TMEM70* gene and resulting in putative aberrant TMEM70 protein lacking functional N-terminal targeting sequence, affecting import of TMEM70 into mitochondria or its membrane assembly. Patients presented with typical features described above, but without 3-MGA in most of them. *TMEM70* c.316+1G>T splice site variant was found in two siblings, who died at ten days and five months of age, respectively [159]. In contrast to these patients, the patient carrying g.2436–3789 in-frame deletion as well as the patient with c.211–450_317–568del variant (who presented also with 3-MGA), survived much longer, being reported at six and seven years of age, respectively [150,160].

Synthesis of potentially truncated TMEM70 has been reported to be associated with four additional homozygous nonsense or frameshift variants, creating premature stop codon. Two siblings harbored frameshift c.578_579delCA deletion resulting in a putative 197 AA long protein lacking almost two thirds of the C-terminus with remarkably different survival; one is 24 years old while the second died at 3.5 years [159]. Three *TMEM70* variants predicted to result in very short TMEM70 protein (112 AA at maximum) have been described in five patients. One patient with nonsense c.336T>A variant was identified at 11 months of age [159], while of the two patients with nonsense c.238C>T variant, one died at seven days postnatally [159] and the second was reported at one year of age [156]. Frameshift c.105dupT variant has been described in two siblings with transiently elevated levels citrulline [161], a condition previously reported only in two living TMEM70 patients with the common variant [148].

So far, only three homozygous missense variants have been described. The first is c.535C>T substitution that changes the highly conserved tyrosine to a histidine at position 179 at the beginning of the C-terminus [162]. The second missense variant c.701A>C changes conserved histidine 234 to proline [152,156]. Both patients presented with typical phenotypes such as cardiomyopathy, hypotonia, and metabolic crisis, and less frequently arterial hypertension. Recently, third missense c.563T>C *TMEM70* variant, changing highly conserved leucine 188 to proline, was described in a boy with HCMP and LVNC, moderately dilated aortic root, severely dilated sinotubular junction, and severely dilated ascending aorta, failure to thrive, facial dysmorphism, and hypotonia. Although cardiomyopathy was present at birth, the patient did not show typical early-onset metabolic decompensation, but rather later decompensation triggered by infectious disease [163].

The common c.317–2A>G variant can also be found as compound heterozygous in combination with other *TMEM70* pathogenic variants. Two patients with typical TMEM70 symptoms but in a milder form have been described to carry c.317–2A>G and c.118_119insGT

frameshift variant resulting in premature stop codon and truncated TMEM70 protein p.Ser40CysfsTer11 [126,164]. Two combinations of common c.317–2A>G and missense variants have been reported. Clinical outcome of the patient with c.494G>A variant, which changes neutral glycine 165 to acidic aspartate at the C-terminus, was mild with Reye-like syndrome and long survival of at least 14 years [165], while both patients reported by Torraco *et al.* are severely affected by metabolic acidosis and cardiomyopathy based on the combination of c.317–2A>G and c.628A>G, which changes highly conserved threonine 210 to proline [152,153]. Two other compound heterozygous variants affecting the C-terminus of TMEM70 protein have been found in combination with the common variant [156]. Frameshift microdeletion c.349_352delC is predicted to change isoleucine 117 to alanine and result in a truncated protein shortened to 36 AA, while missense variant c.783A>G is predicted to change the stop codon to tryptophan, resulting in an aberrant protein with 17 extra AA at the C-terminus. Although the patients suffer from severe encephalo-cardiomyopathy without 3-MGA, the onset of the disease was quite late (eight months and two years, respectively). A unique combination of the novel c.141delG *TMEM70* deletion with c.316+1G>A variant previously described as homozygous was reported by Hirono *et al.* being the first *TMEM70* bi-allelic variant in Japanese patients [166]. Both variants are predicted to result in the loss-of-function phenotype. Two brothers were diagnosed with LVNC and psychomotor delay, the younger also with LA, 3-MGA, inguinal hernia, undescended testis, and failure to thrive, typical clinical picture of TMEM70 patients.

In the study of Magner *et al.* [154], several novel *TMEM70* pathogenic variants were mentioned, including homozygous c.359delC microdeletion, and compound heterozygotes, harboring two novel variants in combination with the common one: c.251delC microdeletion and c.470T>A substitution. No specific information about the patients was given, only the fact that the patient carrying c.317–2A>G and c.251delC was 25 years old with very mild phenotype of epilepsy and mild intellectual disability. The last five TMEM70 patients not mentioned before, are those reported by Brambilla *et al.* [167], diagnosed with primary mitochondrial disorder, all with cardiovascular involvement. However, information on the clinical course or type of *TMEM70* variant was lacking (these patients are not included in Table 4).

Pathogenic *TMEM70* variants found in patients with isolated ATP synthase deficiency can also be distinguished according to the sequence regions affected. First are variants localized in transit peptide [126,159,161,166] or in the first transmembrane domain [156,159] of the protein, generating premature stop codon, with the longest version of the protein consisting of 112 AA, containing only N-terminus and approximately half of the first transmembrane domain (Fig. 2C). The second group represents variants affecting TMEM70 C-terminus, mostly altering highly conserved residues [152,153,156,162,163,165] or creating premature stop codon where the majority of the C-terminus is missing [159]. Remaining variants, including the most common c.317–2A>G variant, are splice site variants that skip exon 2 (coding for AA 71-105), usually resulting in the loss of TMEM70 transcript and thus of the protein [126,150,159,160]. Taken together, C-terminus of TMEM70, which is directed into the mitochondrial matrix, is most likely the key part of protein that functions in the assembly of ATP synthase complex. This is consistent with the findings that TMEM70 is involved in the incorporation of subunit *c* into inner mitochondrial membrane, in the formation of c_8 ring, and in its association with F_1 part of the complex [7,8]. Furthermore, its role in translation and membrane insertion of mtDNA encoded subunits of ATP synthase cannot be excluded [8].

Discussion and Conclusions

Since mitochondria play a crucial role in energetic metabolism of the living cells, defects of their function are linked to many pathologies. In most of them, for example in neurodegenerative diseases such as Parkinson and Alzheimer, or systemic diabetes mellitus, as well as in cancer, mitochondrial dysfunction is not the only underlying mechanisms, but the basis of these polygenic diseases is often a combination of more pathological factors. Moreover, in these pathologies the mitochondrial function is only partially affected and usually linked with another condition, such as increased ROS production [10]. Unsurprisingly, strong reduction in ATP levels is usually embryonically lethal and thus cannot represent relevant pathological mechanism in these diseases. On the other side of the spectrum are rare inherited isolated defects of oxidative phosphorylation apparatus including disorders of the mitochondrial ATP synthase, where profound dysfunction of mitochondrial energy provision is the driver of pathological presentation. NGS strategies highly accelerated the discovery of new disease-causing genes and the diagnosis of clinically affected patients with

mitochondrial disease, but causative and effective treatment of mitochondrial disorders with high heterogeneity of symptoms still does not exist yet. Thus, the therapy remains largely symptomatic, although, in many cases, it is crucial for the survival and quality of life of the patients.

In large number of cases with proven mitochondrial disorder the affected gene remains unknown, and the diagnostic and screening depend on clinical and biochemical phenotyping. It would be nice to have some specific marker for ATP synthase diseases, which could reveal the defect before first symptoms occur (if the onset is not at birth), but unfortunately, any such marker doesn´t exists. In 2014, Mori *et al.* described a homoplasmic patient with LS started at 4 months of age, but the initial sign was hypocitrullinemia, which was revealed during neonatal screening [49]. The mother of the patient with lover levels of pathogenic variant and diagnosed with NARP syndrome at age 24 years had low levels of citrulline as well. These findings strengthen the suggestion of Rabier *et al.* [168] that hypocitrullinemia should be used as a marker of mitochondrial disorders and patients can benefit from early diagnosis. Elevated levels of ammonia in the blood are a common biochemical finding in patients suffering from mitochondrial diseases of ATP synthase. The majority of cases are attributed to pathogenic variants of *TMEM70* (Table 2-4). Hyperammonemia may therefore be regarded as a further potential marker for this disorder. Both citrulline synthesis and detoxification of ammonium are linked with the urea cycle, which occurs in mitochondria [169]. Given that carbamoylphosphate synthetase 1 is a urea cycle enzyme that depends on ATP, it can be postulated that ATP synthase defects can result in hypocitrullinemia and hyperammonemia. Nevertheless, there are at least three clearly documented cases of TMEM70 patients with hyperammonemia accompanied by hypercitrullinemia during metabolic crises, yet with normal citrulline levels at follow-up [148,161], questioning the real diagnostic utility of this marker. The combination of hyperammonemia and hypercitrullinemia may be explained by a defect in argininosuccinate synthase 1, another urea cycle ATP-dependent enzyme that converts citrulline to arginine in the cytosol [148,161,169]. The next potential marker is 3-methylglutaconic acid aciduria (3-MGA), as elevated levels of this compound are frequently associated with nDNA pathogenic variants of ATP synthase-linked genes [125,127,134,137,150,154,156,161-163,166,170]. There are two classes of 3-MGA [171]. Primary 3-MGA is caused directly by defects in the leucine catabolism pathway. Secondary 3-MGA is not linked with defective catabolism of leucine; however, a pathogenic variant in some mitochondrial gene was identified in nearly all cases. It was proposed that defects in the OXPHOS pathway led to the inhibition of the TCA cycle. Consequently, acetyl-CoA is unable to enter the TCA cycle, resulting in the accumulation of this intermediate metabolite. This accumulation activates a divergent pathway that ultimately leads to the synthesis of 3-MGA [171]. However, not every patient with mitochondrial disease, even with isolated ATP synthase deficiency, has elevated levels of 3-MGA, and patients with delayed increase of 3-MGA are reported [163]. Furthermore, 3-MGA aciduria is linked with metabolic diseases in general, not only with defective mitochondrial metabolism [172].

As summarized in this review, clinical pictures of the patients with isolated ATP synthase deficiency represent a diverse spectrum of phenotypes, even if the same gene is affected. At the same time, the symptoms range from very mild to fatal, making the situation even more complicated. Moreover, some of the patients display atypical clinical features [173], and mitochondrial disorders mimicking others (usually neuromuscular), and *vice versa*, were described [55,83,174]. On the other hand, the knowledge of the disease-causing genes in combination with initial disease phenotype can help the clinicians to predict the disease course and better target the therapy. In any case, identification of the causative variant even in the genes that are known to be linked with mitochondrial disorders is a long-distance run. Thanks to the advances in methodology of NGS, namely whole exome sequencing (WES) and whole genome sequencing (WGS), revealing of mitochondrial disease-causing genes became a lot easier. In 2012, a key study of Calvo *et al.* [175] described the use of WES in a clinical trial, and they identified several disease-causing genes not to be linked with mitochondrial diseases before. This study was, however, limited only to “Mitoexome”, it means only to the genes that were known to be coding for mitochondrial proteins. From that time, several other studies on cohorts of patients suffering from mitochondrial diseases with unknown underlying genetic variant were performed, describing novel genes involved in these disorders [173,176,177], in some cases encoding non mitochondrial proteins [174], or mitochondrial proteins not included in Mitocarta database [178]. During eight years, from 2012 till 2020, the number of mitochondrial disease-related genes increased from about 100 to more than 300 [179]. It

is obvious, that even if NGS technology itself is more and more accessible for clinicians methodologically, and cost-effective methods and kits are developed [180], still the key aspect is the setup of the studies. It means not only the criteria according to which the patients are selected for the study, but especially bioinformatic restrains defined in the pipeline (how to further sort the patients, which gene database to use, how to confirm the pathogenicity, etc.) [179]. It is obvious, that freely accessible and up to date databases of genes, linked with mitochondrial diseases, could significantly increase the chances of mitopatients to effective therapy. It can help to associate patients with similar phenotypes for therapeutic trials and speed up the diagnosis of those still waiting for it. Such databases could also help with the proper classification of the variants according to their pathogenicity, as many of the variants are undergoing reclassification [181].

Another interesting question is the possibility to prevent mitochondrial diseases by genetic counselling. Of course, in the families with known pathogenic variants of nuclear origin, prenatal diagnostics is commonly used. Regarding the mtDNA variants, the prenatal diagnosis is much more difficult. In cases of inherited variants, the risk for the disease prevalence is very high, because of the bottleneck phenotype [182]. In the study of Sallevelt *et al.* [183], several families with previous appearance of *de novo* mtDNA *MT-ATP6* pathogenic variants were described. Using prenatal diagnosis in the following pregnancies the authors suggested that the risk that defect will occur *de novo* again is very low, and all the families with negative findings of the mutation in second pregnancy had healthy children.

Abbreviations

3-MGA, 3-methylglutaconic aciduria; AA, amino acid(s); DAPIT, diabetes-associated protein in insulin sensitive tissues; (F)BSN, (familial) bilateral striatal necrosis; FTT, failure to thrive; HCMP, hypertrophic cardiomyopathy; HSP, hereditary spastic paraplegia; IUGR, intrauterine growth restriction; LA, lactic acidosis; LHON, Leber's hereditary optic neuropathy; LS, Leigh syndrome; LVNC, left ventricular non-compaction; mtDNA, mitochondrial DNA; NARP, neurogenic muscle weakness (or neuropathy), ataxia and retinitis pigmentosa syndrome; nDNA, nuclear DNA; NGS, next-generation sequencing; OSCP, oligomycin-sensitivity conferring protein; OXPHOS, oxidative phosphorylation system; PMR, psychomotor retardation; ROS, reactive oxygen species; WES, whole exome sequencing; WGS, whole genome sequencing.

Conflict of Interest

There is no conflict of interest.

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