

Review

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Riboflavin metabolism: role in mitochondrial function

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Abstract

Riboflavin, known as vitamin B2, a water-soluble vitamin, is an essential nutrient in vertebrates, hence adequate dietary intake is imperative. Riboflavin plays a role in a variety of metabolic pathways, serving primarily as an integral component of its crucial biologically active forms, the flavoenzymes flavin adenine dinucleotide and flavin mononucleotide. These flavoenzymes ensure the functionality of numerous flavoproteins including dehydrogenases, oxidases, monooxygenases, and reductases, which play pivotal roles in mitochondrial electron transport chain, β -oxidation of fatty acids, redox homeostasis, citric acid cycle, branched-chain amino acid catabolism, chromatin remodeling, DNA repair, protein folding, and apoptosis. Unsurprisingly, impairment of flavin homeostasis in humans has been linked to various diseases including neuromuscular and neurological disorders, abnormal fetal development, and cardiovascular diseases. This review presents an overview of riboflavin metabolism, its role in mitochondrial function, primary and secondary flavoenzyme defects associated with mitochondrial dysfunction, and the role of riboflavin supplementation in these conditions.

Keywords: Mitochondria, riboflavin, flavoenzymes, primary flavoenzyme defects, secondary flavoproteome defects, riboflavin responsive disorders



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INTRODUCTION

Riboflavin is the precursor of metabolically active flavoenzymes which are utilized as cofactors for approximately 90 flavoproteins in numerous enzymatic reactions as either flavin adenine dinucleotide (FAD) (84%) or flavin mononucleotide (FMN) (16%)^[1]. These flavoproteins (enzymes using flavoenzymes) including dehydrogenases, oxidases, monooxygenases, and reductases play critical roles in mitochondrial electron transport chain, mitochondrial and peroxisomal β -oxidation of fatty acids, citric acid cycle, redox homeostasis, nitric oxide synthases, and branched-chain amino acid (BCAA) catabolism^[2,3]. They are also involved in chromatin remodeling, DNA repair, protein folding, apoptosis^[2] biosynthesis or regulation of other essential cofactors and hormones, including coenzyme A, coenzyme Q, heme, pyridoxal 5'-phosphate, steroids, and thyroxine; and metabolism of other B vitamins (folate, pyridoxine, and niacin) and P450 enzymes^[1,4].

Riboflavin, as with other water-soluble B vitamins, must be obtained through diet as mammals have lost the ability to synthesize this molecule. Endogenous synthesis of riboflavin by microflora in the large intestine may contribute but to a lesser extent^[5]. Hence, adequate dietary intake from major sources such as milk and dairy products, eggs, seafood, poultry, lean meat, cereals, and vegetables is imperative. The recommended daily allowance of riboflavin on average is 1.3 mg/day for adult men and 1.1 mg/day for women, with variations depending on age and reproductive status including pregnancy and lactation^[5].

RIBOFLAVIN METABOLISM AND TRANSPORT

Riboflavin ingested in diet exists either as free riboflavin, predominantly found in milk and eggs, or its protein bound form as flavoproteins including FAD and FMN, which must be released from the carrier proteins to which they are bound. The latter occurs through dietary protein denaturation in the stomach and subsequent hydrolysis to free riboflavin by alkaline phosphatases and FMN/FAD pyrophosphatases in the ileal brush border to be absorbed in the small intestine^[6] [Figure 1]. Next, free riboflavin is transported into the enterocytes via carrier-mediated uptake by RFVT3 (previously hRFT2, encoded by *SLC52A3*), which functions primarily to absorb riboflavin from dietary intake^[7]. This saturable uptake process occurs at the apical membrane and is reported to be linear up to approximately 30 mg riboflavin per meal^[8], following which little additional absorption of riboflavin occurs^[9,10].

After cellular uptake, free riboflavin undergoes adenosine triphosphate (ATP)-dependent phosphorylation by riboflavin kinase (RFK) (EC 2.7.1.26), a ubiquitous rate-limiting flavokinase, to form FMN, which is consequently adenylated to FAD by FAD synthase (FADS) (EC 2.7.7.2). Riboflavin may subsequently be released into the portal blood and to the liver in its free form or as FMN after being transported by RFVT1 (previously hRFT1) and RFVT2 (previously hRFT3), encoded by *SLC52A1* and *SLC52A2*, respectively, and embedded within the basolateral membrane of the enterocytes^[5]. Apart from being expressed in the gastrointestinal system, RFVT1 is also detected in the placenta where it transports maternal riboflavin to the fetus. RFVT2-mediated transport allows riboflavin uptake into the brain where it is highly expressed, and additionally in endocrine organs such as the pancreas, liver, and muscle^[7].

Circulating plasma riboflavin is either bound to albumin and immunoglobulins or is converted into its coenzyme forms in erythrocytes or leukocytes. Unbound flavins are rapidly hydrolyzed to free riboflavin and excreted in urine. There is little or no storage of riboflavin in the body; hence, any intake in excess of tissue requirements or which surpasses renal reabsorption is eliminated in the urine as riboflavin or its catabolites 7- α -hydroxy riboflavin, 10-hydroxyethylflavin, and lumiflavin^[5,11]. As a result, riboflavin has a relatively low toxicity even at supra-pharmacological doses^[5].

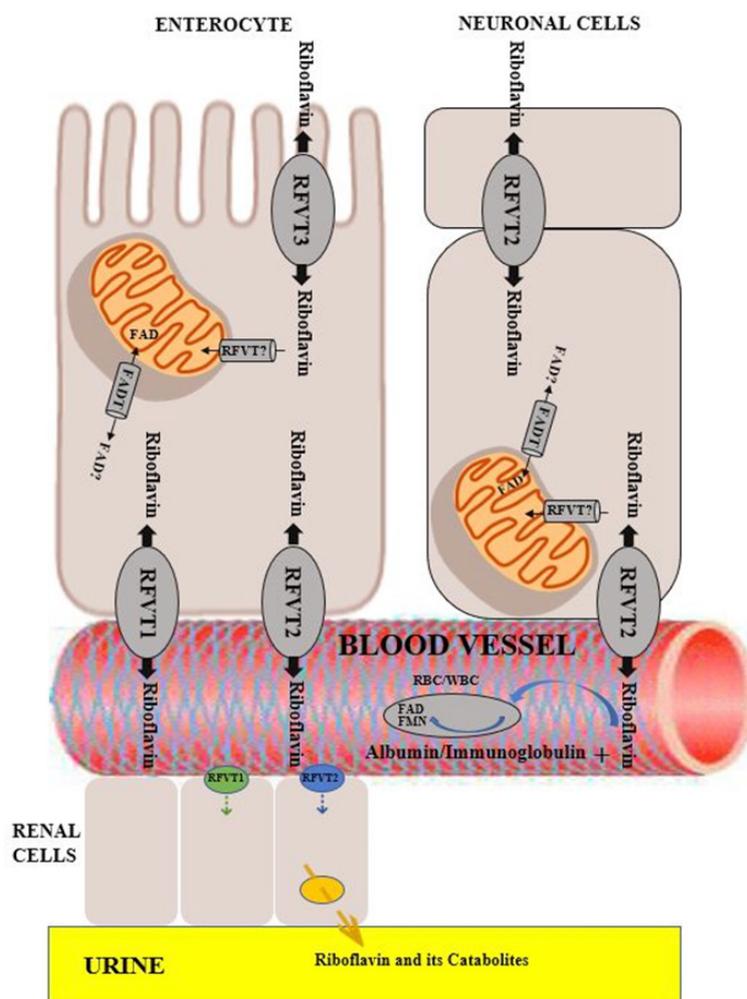


Figure 1. Metabolism and transport of riboflavin and flavocoenzyme. Dietary FAD and FMN are converted to riboflavin by non-specific hydrolases on the brush-border membrane of ileal enterocytes and are subsequently absorbed into the intestine via apically expressed RFVT3. Inside the enterocytes, riboflavin can either be further metabolized to FMN by riboflavin kinase and subsequently to FAD by FAD synthase or released into portal blood by basolaterally expressed RFVT1 and RFVT2. Circulating plasma riboflavin associates with albumin or globulins or is converted into a coenzyme form in erythrocytes or leukocytes. RFVT2-mediated transport allows riboflavin uptake into the brain where it is highly expressed, and additionally into endocrine organs, such as pancreas, liver, and muscle tissue. The mechanism of import of riboflavin into the mitochondrial matrix has not been precisely elucidated to date. "RFVT?" is depicted as a putative riboflavin transporter responsible for this step. The mitochondrial FADT imports FAD from the cytosol into the mitochondria. The question mark indicates that FADT-mediated efflux of FAD from the mitochondrial matrix to the cytosol remains to be established (see also Barile *et al.*^[5] 2016 and Balasubramaniam *et al.*^[3] 2019). FAD: flavin adenine dinucleotide; FMN: flavin mononucleotide; FADT: flavin adenine dinucleotide transporter

MITOCHONDRIA-THE CELL'S POWERHOUSE

Mitochondria are maternally inherited multifunctional double-membrane, highly dynamic cytoplasmic organelles, ubiquitously present in all cells, except erythrocytes. The mitochondrion is composed of several compartments that carry out specialized functions. These include the outer membrane, inner membrane, the intermembrane space, and cristae, which are the in-folding of the inner membrane and matrix. Mitochondria execute myriad vital cellular processes including fatty acid oxidation, urea cycle, Krebs cycle, biosynthesis of heme and steroids, maintenance of calcium homeostasis, caspase-dependent apoptosis, reactive oxidant species (ROS) generation, and heme and steroid synthesis^[12,13]. In addition to these functions, the hallmark of mitochondria is the pivotal role it plays in aerobic cellular energy generation via oxidative phosphorylation (OXPHOS).

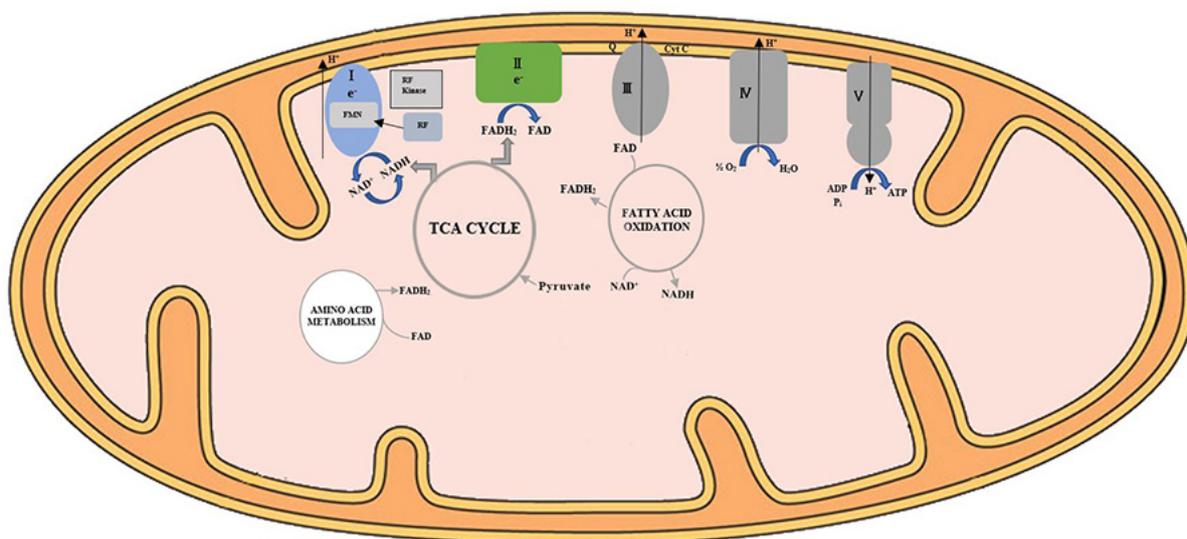


Figure 2. Schematic diagram of flavocoenzymes in mitochondrial energy metabolism. The OXPHOS system is a 5-enzyme complex which encompasses the mitochondrial respiratory chain (Complexes I-IV), Complex V, and two mobile electron shuttles (coenzyme Q10 and cytochrome c). Electrons derived from oxidation of pyruvate mediated by pyruvate dehydrogenase (PDH) and fatty acid oxidation are transferred via NADH to Complex I (FMN-dependent NADH-ubiquinone oxidoreductase), while electrons from succinate in the Krebs cycle, amino acid metabolism, and fatty acid oxidation are transferred to Complex II (FAD-dependent succinate-ubiquinone oxidoreductase) via FADH₂. Electrons are subsequently transferred to ubiquinone (Coenzyme Q10) and then to Complex III (reduced CoQ-cytochrome c reductase), and via cytochrome c to cytochrome c oxidase (COX) (Complex IV), the terminal oxidase of the RC before finally reducing molecular oxygen to water. The free energy liberated during this sequential electron transfer is used to generate an electrochemical gradient of protons, which is finally used by Complex V (ATP synthase or F1F0 ATPase) to drive ATP synthesis from ADP and inorganic phosphate. RF: Riboflavin; RF kinase: riboflavin kinase; Q: Coenzyme Q10; CytC: cytochrome c oxidase; FMN: flavin mononucleotide; FAD: flavin adenine dinucleotide

Riboflavin is a key component of the mitochondrial respiratory chain (RC), hence is pivotal for ATP generation and sustaining energy-dependent cellular functions. The OXPHOS system is a 5-enzyme complex which encompasses the mitochondrial respiratory chain (Complexes I-IV), Complex V, and two mobile electron shuttles (coenzyme Q10 and cytochrome c)^[14] [Figure 2]. Electrons derived from oxidation of pyruvate and fatty acids are transferred via NADH to Complex I (FMN-dependent NADH-ubiquinone oxidoreductase), while electrons from succinate in the Krebs cycle are transferred to Complex II (FAD-dependent succinate-ubiquinone oxidoreductase) via FADH₂. Electrons are subsequently transferred to ubiquinone (Coenzyme Q10) and then to Complex III (reduced CoQ-cytochrome c reductase), and via cytochrome c to cytochrome c oxidase (COX) (Complex IV), the terminal oxidase of the RC before finally reducing molecular oxygen to water. The free energy liberated during this sequential electron transfer is used to generate an electrochemical gradient, by pumping protons from the matrix to the intermembrane space at three coupling sites (Complexes I, III, and IV). This proton gradient is used by Complex V (ATP synthase or F1F0 ATPase) to drive ATP synthesis from ADP and inorganic phosphate.

PATHOGENESIS OF RIBOFLAVIN OR FLAVOCOENZYME DEFICIENCY AND MITOCHONDRIAL DYSFUNCTION

Riboflavin deficiency or defects in the production of its flavocoenzymes FAD and FMN can lead to disruption of the RC, consequently mitochondrial dysfunction, and increased production of ROS, overwhelming the cellular antioxidant mechanisms. Exposure to excessive ROS, including free radical superoxide O₂⁻, hydrogen peroxide, and hydroxyl radicals, favors oxidative stress, alters mitochondrial Ca²⁺ homeostasis, triggers membrane lipid peroxidation and potentially induces nuclear and mtDNA damage^[15]. Sustained elevations in intracellular Ca²⁺ concentrations ultimately cause neuronal degeneration and cell

death by initiating apoptosis^[16]. All these perturbations are implicated in mitochondrial dysfunction, hence strategies to mitigate this may pose an important therapeutic avenue.

CLASSIFICATION OF DEFECTS IN RIBOFLAVIN METABOLISM ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION

Simplistically classified in this review, the primary defects of flavocoenzyme metabolism include disorders of riboflavin or flavocoenzyme transport and enzymes in the synthetic pathway of FMN and FAD. Secondary flavoproteome defects include FMN- and FAD-dependent reactions resulting in functional disruption of the cellular flavoproteins, but do not arise as a direct result of defects in the synthesis and transport of riboflavin, FMN, or FAD.

Secondary flavoproteome defects can be further subcategorized into primary mitochondrial disorders (PMD) and secondary mitochondrial dysfunction (SMD)^[17]. PMD are genetic disorders that directly impair OXPHOS proteins or its function by impacting the complex machinery involved in the OXPHOS process. SMD can be caused by germline mutations in non-OXPHOS genes, accompany various hereditary non-mitochondrial diseases, or may be acquired secondary to adverse environmental factors which can cause oxidative stress. A summary of disorders of flavocoenzymes and flavoproteins associated with primary and secondary mitochondrial dysfunction is presented in [Table 1](#).

PRIMARY DISORDERS OF FLAVOCOENZYME METABOLISM ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION

Disorder of Riboflavin Transport

Riboflavin Transporter Deficiency Neuronopathy (OMIM #614707, OMIM# 211500, and OMIM #211530) – previously known as *Brown-Vialetto-Van Laere* and *Fazio-Londe syndrome*

Human riboflavin transporters, RFVT1, RFVT2, and RFVT3, are encoded by their respective genes, *SLC52A1*, *SLC52A2*, and *SLC52A3*^[18-21]. These transporters with different tissue expressions transport riboflavin across plasma membranes and maintain the supply of flavins to the cells.

Mutations in the *SLC52A2* and *SLC52A3* have been reported to cause *Brown-Vialetto-Van Laere* (BVVL) and *Fazio-Londe* (FL)^[22,23]. Both conditions are now considered as a single disease entity^[24], with progressive sensorimotor and cranial neuropathy in both and absent sensorineural hearing loss in FL as primary manifestations. A new nomenclature was proposed to clarify the specific disease mechanism and both conditions were renamed to riboflavin transporter deficiency (RTD)^[25], with mutations in *SLC52A2* and *SLC52A3* causing RTD2 and RTD3, respectively^[26]. A recent review identified 109 RTD patients (52 RTD2, 56 RTD3, and 1 RTD2/3)^[26]. The overall mean age of onset of these patients was 5.3 years (range: 0-5 years). The predominant clinical features were hearing loss and muscle weakness. Some characteristics of patients with RTD3 included a later onset of presentation (> 10 years to third decade) and bulbar symptoms. The common presenting features of patients with RTD2, on the other hand, was abnormal gait and/or ataxia^[26]. Plasma acylcarnitine profiles prior to riboflavin supplementation was suggestive of multiple acyl-CoA dehydrogenase defect (MADD)^[22]. Urine organic acids commonly showed ethylmalonic aciduria suggesting impaired fatty acid, methionine, and/or isoleucine oxidation^[26]. Both plasma acylcarnitine and urine organic acids, however, have been observed to be normal in nearly half of RTD patients^[26].

Effective doses of riboflavin varied 10-80 mg/kg/day with over 70% of patients demonstrating improvements in muscle strength, motor abilities, respiratory function, and/or cranial nerve deficits^[26]. The pathomechanism of the specific vulnerability of neurons in RTD might be due to mitochondrial dysfunction and impairment in the clearance of reactive oxygen species^[26].

Table 1. Overview of disorders of flavocoenzymes and flavoproteins associated with primary and secondary mitochondrial dysfunction

Disease name	Gene	Phenotype OMIM number	Primary clinical phenotype	Biochemical findings	Riboflavin responsiveness
Riboflavin transporter deficiency	<i>SLC52A2</i>	614707	Sensorimotor and cranial neuropathy Sensorineural hearing loss	Multiple acyl Co-A dehydrogenase defect (MADD) profile on acylcarnitine and ethylmalonic aciduria	Yes
	<i>SLC52A3</i>	211500	Sensorimotor and cranial neuropathy		
		211530	Sensorimotor and cranial neuropathy Sensorineural hearing loss		
FAD transporter deficiency	<i>SLC25A32</i>	616839	Neuromuscular e.g. exercise intolerance, ataxia, muscle weakness	MADD profile Complex II deficiency	Yes
FAD synthase deficiency	<i>FLAD1</i>	255100	Fatal infantile onset hypotonia, swallowing difficulties, respiratory insufficiency, cardiomyopathy Late onset lipid storage myopathy	MADD profile Combined respiratory chain deficiencies	Yes
Mitochondrial complex I deficiency, nuclear type 20	<i>ACAD9</i>	611126	Early onset and lethal hypertrophic cardiomyopathy, encephalopathy Later presentation with myopathy, e.g., exercise intolerance and muscle weakness	Complex I deficiency	Yes
Mitochondrial complex I deficiency, nuclear type 19	<i>FOXRED1</i>	618241	Leigh syndrome Encephalomyopathy	Complex I deficiency	Limited data (<i>n</i> = 2)
COQ10 deficiency-6	<i>COQ6</i>	614650	Steroid resistant nephrotic syndrome, sensorineural hearing loss	COQ10 deficiency	No data
Myopathy, mitochondrial progressive, with congenital cataract, hearing loss, and developmental delay	<i>GFER</i>	613076	Neuromuscular e.g. hypotonia, muscle weakness, psychomotor retardation, muscle weakness, Cataracts, hearing loss	Isolated Complex IV deficiency or combined deficiencies	No data
	Auditory neuropathy and optic atrophy <i>FDXR</i>	617717	Auditory neuropathy and optic atrophy, encephalopathy	Combined respiratory chain deficiencies	No data
Mitochondrial Complex I deficiency, nuclear type 4	<i>NDUFV1</i>	618225	Encephalopathy, motor delay Neurodevelopmental regression, Oculomotor impairment	Complex I deficiency	Limited data (<i>n</i> = 7), riboflavin administered with other supplements ^[94,97,98,101]
Mitochondrial Complex I deficiency, nuclear type 7	<i>NDUFV2</i>	618229	Hypertrophic cardiomyopathy Encephalopathy Leigh syndrome	Complex I deficiency	Limited data (<i>n</i> = 1), riboflavin administered with other supplements ^[113]
Mitochondrial Complex II deficiency	<i>SDHA</i>	252011	Leukoencephalopathy, Leigh syndrome, cardiomyopathy, Cancer susceptibility (gastrointestinal stromal tumours, paraganglioma/pheochromocytoma, pituitary adenoma and renal carcinoma)	Complex II deficiency	No response (<i>n</i> = 2) ^[133]
Multiple acyl-coenzyme A dehydrogenase deficiency (MADD)	<i>ETFA, ETFB, ETFDH</i>	231680	Neonatal onset- with (type I) or without congenital anomalies (type II) and early onset encephalopathy and cardiomyopathy Late onset myopathy (type III)	Increase in short, medium and long chain acylcarnitines Characteristic MADD pattern in urinary organic acids	YES - established for late onset myopathy ^[138]
Dihydropyrimidinase deficiency (E3)	<i>DLD</i>	246900	Early onset encephalopathy, Primary liver involvement, Myopathy	Elevated lactate, pyruvate, alpha ketoglutarate, branched chain amino acids and alpha ketoacids	YES - established for myopathic form ^[146]

Ethylmalonic encephalopathy	<i>ETHE1</i>	602473	Encephalopathy, Vasculopathy (petechial purpura, orthostatic acrocyanosis, chronic hemorrhagic diarrhoea)	Increase in ethylmalonic, C4 and C5 acylcarnitines. Complex IV deficiency	Limited data ($n = 4$) ^[152]
Combined oxidative phosphorylation deficiency 6	<i>AIFM1</i>	300816	Encephalomyopathy	Combined respiratory chain deficiencies	Limited data ($n = 2$) for ataxia ^[160] and neurological condition ($n = 1$, gross, fine motor and communication) ^[155]
Cowchock syndrome	<i>AIFM1</i>	310490	X-linked Charcot-Marie-Tooth disease (CMTX4) with axonal sensorimotor neuropathy, deafness and cognitive Impairment		
Deafness, X-linked 5	<i>AIFM1</i>	300614	Auditory neuropathy with peripheral neuropathy		
Spondyloepimetaphyseal dysplasia, X-linked, with hypomyelinating leukodystrophy	<i>AIFM1</i>	300232	Spondyloepimetaphyseal dysplasia with hypomyelination		

Haploinsufficiency of the *SLC52A1* (OMIM#615026) due to maternal microdeletion and heterozygous intronic variant has been reported to cause a transient riboflavin responsive neonatal multiple acyl-CoA dehydrogenase deficiency that resolved with oral supplementation of riboflavin^[27-29].

Disorders of Flavocoenzyme Transport

Mitochondrial FAD transporter deficiency (OMIM #616839)

SLC25A32 encoding the mitochondrial FAD transporter has been described in two patients with biallelic mutations to date^[30,31]. The first, a 14-year-old girl presented with riboflavin-responsive recurrent exercise intolerance and biochemical findings of MADD^[30]. The second, a 51-year-old Dutch patient with a severe neuromuscular phenotype had initially presented aged three years with muscle weakness post- influenza infection. He subsequently developed impaired motor skills, progressive exercise intolerance in childhood, early-onset ataxia, myoclonus, dysarthria, and dysphagia^[31]. Muscle biopsy in both patients demonstrated ragged-red fibers, lipid storage, and decreased staining for succinate dehydrogenase (SDH, FAD-dependent mitochondrial respiratory chain Complex II) and COX (mitochondrial respiratory chain Complex IV). Complex II deficiency was revealed in cultured skin fibroblasts and muscle from the first and second patients, respectively^[30,31]. Dramatic improvements in exercise tolerance and endurance and biochemical abnormalities were reported in both patients following oral riboflavin supplementation^[30,31].

SLC25A32 has recently been revealed to be a novel regulator of cancer cell proliferation and mitochondrial FAD metabolism. *SLC25A32* knock-down in sensitive tumor cells resulted in inhibition of the FAD-dependent Complex II, increased succinate levels, and reduced oxygen consumption rate^[32]. These findings corroborate evidence of decreased Complex II protein levels and OXPHOS activity, which is a marker for mitochondrial FAD in muscle of patients with severe neuromuscular phenotype and novel variants in *SLC25A32*^[30,31]. Reduction of mitochondrial FAD concentrations by inhibition of *SLC25A32* is anti-proliferative in a subset of tumor cell lines and has potential clinical applications as a novel cancer target by increasing oxidative stress and reducing tumor growth^[32].

Disorders of Flavocoenzyme Metabolism

Flavin adenine dinucleotide synthase (FAD) synthase deficiency (OMIM # 255100)

The *FLAD1* gene encodes FAD synthase (EC 2.7.7.2), which catalyzes the adenylation of FMN into the redox cofactor FAD. Human FADS has previously been shown to be a bifunctional enzyme with both FAD synthase and hydrolase activity^[33]. The enzyme contains an N-terminal molybdopterin binding (MPTb) domain, which has FAD hydrolase activity, and a C-terminal 3'-phosphoadenosine-5'-phosphosulfate

(PAPS) reductase domain, which is sufficient to catalyze FAD synthesis and has thus been renamed the FADS domain^[33,34]. Multiple FADS isoforms generated due to alternative splicing of the encoding *FLAD1* gene result in distribution within different subcellular localizations^[34-36]. Two isoforms, the more abundant cytosolic (FADS2) and the mitochondrial (FADS1), have been described in detail^[33]. Human FADS1, FADS2, and FADS3 possess both catalytic domains and are bifunctional, while FADS4 and FADS5 present the sole mMPTb domain and are monofunctional enzymes^[37]. A novel cytosolic FADS6 isoform recently characterized contains only the PAPS domain with considerable FAD synthesis activity, which allows survival of affected patients with biallelic *FLAD1* frameshift variants^[37]. The FADS6 isoform has approximately 70% of the FAD-synthesizing capacity of human FADS2. Its catalytic efficiency (kcat/Km) is however higher due to a lower Km for FMN and ATP compared to FADS2^[38].

The recently described clinical spectrum for FAD synthase deficiency has ranged from neonatal-onset, a lethal disease with metabolic myopathy, cardiomyopathy, swallowing, speech difficulties, and respiratory insufficiency, to later-onset, a potentially treatable neuromuscular disorder with lipid storage myopathy, MADD-like metabolic aberrations, and combined respiratory-chain deficiency^[34]. To date, sixteen patients have been reported with age of onset ranging from birth to 44 years^[34,39-45]. Most patients (92%) have however presented in infancy, with five out of thirteen dying within the first twelve months of life. The first patient described by Taylor *et al.*^[39], a Turkish male infant of consanguineous parents, presented at age four months with respiratory insufficiency, lipid myopathy, decreased activities of mitochondrial respiratory chain Complexes I and IV, and early death at eight months of age. A homozygous 4-bp deletion (c.397_400delTTCT) in the *FLAD1* gene was identified, resulting in a frameshift and premature termination (Phe134CysfsTer8). Functional studies were not performed; however, in silico predictions of the deleterious effect of the mutations supported a causal association.

A further 15 patients from 13 unrelated families affected by MADD and/or multiple respiratory-chain deficiency and lipid storage myopathy were diagnosed with *FLAD1* deficiency. Apart from one patient, all were identified by whole-exome sequencing or through candidate gene panels^[3]. Characteristic clinical manifestations included early infantile onset hypotonia, swallowing, speech difficulties, respiratory insufficiencies, and a fatal course (seven died within the first year of life and one at age 16 years). Cardiorespiratory collapse was the cause of death for a neonate who presented at 32 h of age and died at three days. One patient died of multiorgan failure at seven months due to recurrent episodes of sudden cardiac arrest necessitating a pacemaker. Another infant with cardiomyopathy and recurrent supraventricular tachycardias who required implantable cardioverter defibrillator placement showed a dramatic response to riboflavin supplementation and was still alive at 22 years. Interestingly, the acute metabolic decompensations observed in the neonatal forms of MADD caused by *ETFA*, *ETFB*, and *ETFDH* defects, including hypoglycemia, metabolic acidosis, and hyperammonemia have not been reported in early-onset FADS deficiency^[41]. The adult onset presentations at 20 and 44 years included exercise intolerance and progressive muscle weakness, with gait difficulties, bilateral foot drop, and arm weakness in the older patient. These individuals were alive at 44 and 56 years, respectively^[34,40].

Metabolic findings in FADS deficiency are typical of riboflavin deficiency or MADD, with increased acylcarnitines (C4-C14 even chain acylcarnitines; C5 and C5-DC; and C10:1, C14:1, C16:1, C18:1, and C18:2) and urinary organic acids (increased urinary excretion of ethylmalonic, adipic, suberic, glutaric and methylsuccinic acids, hexanoylglycine, and tiglylglycine)^[3]. Characteristic muscle pathology with pronounced lipid storage and global decrease of COX and/or SDH histochemical staining has been reported^[39]. Multiple respiratory chain enzyme deficiencies involving Complexes I-III were described in skeletal muscle biopsies in seven of eight cases tested^[34,39,41].

A possible genotype-phenotype correlation has been proposed with homozygous carriers of frameshift variants affecting the MPTb domain displaying a more severe clinical course than that observed in

individuals harboring single amino acid changes in the FADS domain that are potentially more responsive to riboflavin therapy^[34].

Riboflavin supplementation resulted in clinical improvements in nine of ten patients treated^[34,39-42,44,45]. All five children who presented in infancy and treated with riboflavin were alive, two at 8 years, one at 22 years, one at 15 months^[44], and one at 2 years 5 months^[45]. Treatment with riboflavin in two patients carrying homozygous c.401_404delTTCT mutations in *FLAD1* at age three months resulted in milder improvements in spontaneous activity, muscle tone, vomiting, and alertness; however, it failed to prevent disease progression and demise by six and five months, respectively^[41]. The same variant had previously been reported in an untreated Turkish infant with multiple respiratory chain complex deficiencies who died at six months of age^[34]. It has been proposed that the partial response to riboflavin supplementation and residual FADS activity described in patients with biallelic frameshift mutations in exon 2 of *FLAD1* may be attributed to the existence of a FADS isoform that lacks exon 2, but has an intact and functional FADS domain^[34]. However, the doses of riboflavin used in these patients may have been inadequate in ensuring long-term survival^[41].

A trial of riboflavin therapy should be offered to all FADS-deficient individuals while awaiting mutational analysis. The benefits of early diagnosis and treatment is exemplified by the fatal outcome of the untreated brother of an older sister who responded to riboflavin^[34].

Riboflavin kinase deficiency (OMIM # 613010)

RFK (EC 2.7.1.26) is a ubiquitous rate-limiting enzyme that catalyzes the first step in flavocoenzyme biosynthesis by phosphorylating riboflavin to form FMN, an obligatory step in riboflavin metabolism. FMN is subsequently adenylated by FAD synthetase to generate FAD^[46]. Embryonic lethality demonstrated before Day 7.5 of gestation in complete knockout mice was potentially attributable to secondary mitochondrial dysfunction from the effect on flavocoenzyme deficiencies on the electron transport chain, which is critically involved in energy generation^[47]. To date, there have been no reported human phenotypes of RFK deficiency. It is possible that defects in RFK may be incompatible with life due to its critical role noted above. Hypomorphic *RFK* mutations may result in clinical phenotypes that are not that dissimilar to patients with *FLAD1* mutations^[3].

SECONDARY FLAVOPROTEOME DEFECTS ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION

Primary Mitochondrial Disorders

Acyl-CoA dehydrogenase-9 deficiency (ACAD9) (OMIM #611126)

Acyl-CoA dehydrogenase 9 (ACAD9), a mitochondrial protein, was initially demonstrated to catalyze the initial rate-limiting step in the beta-oxidation of long-chain fatty acids^[48]. It was subsequently proposed to be a novel assembly factor crucial for oxidative phosphorylation Complex I biogenesis, independent of its role in fatty acid oxidation^[49]. More recently, human ACAD9 was showed to be a bifunctional enzyme involving primarily oxidative phosphorylation Complex I biogenesis, with an additional moonlighting function in fatty acid oxidation (FAO)^[50].

Major clinical presentations of ACAD9 deficiency include biventricular hypertrophic cardiomyopathy (85%), muscle weakness (75%), exercise intolerance (72%) and lactic acidosis^[51]. Two subgroups of ACAD9 deficient patients have been described, including early-onset, often lethal cardiac involvement presenting in infancy. Those surviving from this group performed more poorly than later-onset presentations. Interestingly, developmental delay and severe intellectual disability were only seen with early disease onset^[51]. ACAD9 is the only long-chain ACAD enzyme expressed in the brain and is especially abundant during fetal life^[49,52]. Hence, it has been proposed that the neurological symptoms present in the most

severely affected ACAD9-deficient patients could be attributed to a deficiency in brain FAO. To date, no patients with two *ACAD9* null mutations have been described, suggesting that the complete absence of the protein is lethal. Embryonic lethality was also proposed for the failure to generate homozygous knockout *ACAD9* mice^[49].

Riboflavin supplementation resulted in alleviation of symptoms in 65% patients, and, most notably, improved survival was observed when commenced within the first year of life^[51]. Riboflavin responsiveness may be related to its essential function as a precursor of FAD cofactor for ACAD enzyme activity and stability. It also increases ACAD9 protein levels and rescues Complex I assembly, while also functioning as a chemical chaperone by improving folding of *ACAD9* mutant proteins^[49]. Schiff *et al.*^[49] (2015) suggested that similar specific interventions used for VLCAD defects including avoidance of fasting, medium-chain triglycerides, or triheptanoin anaplerotic therapy could be beneficial in terms of long-term outcomes in patients.

FAD-dependent OXidoREDuctase deficiency (FOXRED1) (OMIM #618241)

FOXRED1 has been proposed to be a dual function protein. It plays a key role as an assembly factor for Complex I biogenesis, and, secondly, due to its oxidoreductase activity, it is hypothesized to participate in glycine metabolism which modulates glutathione biosynthesis, an antioxidant protecting the cells from ROS^[53-55]. Co-immunoprecipitation experiments suggest that FOXRED1 facilitates Complex I assembly by associating with the 370-kDa subcomplex and two other FAD-dependent Complex I assembly factors, ACAD9 and probably AIFM1^[55].

FOXRED1 mutations have been recognized as a cause of Complex I deficiency. To date, eight patients from six families have been described with variable clinical spectrum and severity^[55-60]. Age of onset of clinical manifestations ranged from birth to early infancy with one prenatal onset of oligohydramnios, severe intrauterine growth retardation, and periventricular cysts^[60]. The clinical manifestations included Leigh syndrome and infantile-onset encephalomyopathy with epilepsy, mild to severe psychomotor retardation, and hypotonia. Non-neurological features included congenital lactic acidosis, cardiomyopathy, hepatomegaly, kyphoscoliosis, optic atrophy, roving eye movements, strabismus, and distal renal tubular acidosis^[55-58,60]. Brain neuroimaging (MRI) findings ranged from normal to delayed myelination and features consistent with Leigh syndrome^[55-57]. Enzymatic studies supported Complex I deficiency in all patients. Additionally, a milder decrease in Complex II was observed in one patient^[59]. The majority of mutations identified were missense. Riboflavin supplementation was reported in only two patients^[57,60], of whom one had prenatal onset and died at three months of age^[60]. Outcome was available in 6/8 patients: 4/6 are alive with median age of 17 years (age range: 10-22 years) and another died at eight years. In contrast to patients with other nuclear encoded Complex I defects who typically have a severe clinical presentation and associated early death, it has been suggested that pathogenic variants in the *FOXRED1* gene result in partial loss of function and are probably hypomorphic due to the longer survival of patients^[61].

COQ6 deficiency (OMIM #614650)

Human Coenzyme Q10 monooxygenase 6 (COQ6) is a flavoprotein involved in the biosynthesis of Coenzyme Q10 which operates as a redox carrier by transferring electrons from respiratory chain Complexes I and II to Complex III. Coenzyme Q10 is also a potent antioxidant^[62] and a cofactor of many mitochondrial dehydrogenases. It is required for pyrimidine nucleoside biosynthesis and has been implicated in the inhibition of apoptosis by preventing the collapse of the inner mitochondrial membrane^[63]. Studies using human cell line lacking functional COQ6 showed impaired COQ synthesis, severe ATP deficiency, and increased production of reactive oxygen species^[64].

In 2011, Heeringa *et al.*^[65] reported a novel cause for steroid-resistant nephrotic syndrome associated with sensorineural hearing loss due to variants in the *COQ6* gene. Twenty-three patients in 16 families with similar clinical manifestations and two mutations in the *COQ6* gene have been identified to date^[65-71]. Extra-renal manifestations such as ataxia, seizures, muscle weakness, white matter changes, and optic atrophy were less common^[65,68]. The age of onset of the renal disease varied from 2 months to 6.4 years but was most common in infancy and early childhood. The course was progressive with median age of onset of end stage renal disease between 13 months and 19 months^[65,68]. The most common histopathologic finding was focal segmental glomerulosclerosis. Abnormal mitochondrial proliferation in the podocytes was also noted on electron microscopy^[68].

Coenzyme Q10 supplementation alone at 5-30 mg/kg/day was reported in a few patients. Significant improvement in proteinuria with normal renal function was noted on follow-up of these patients^[66,70,72-74]. However, hearing loss had not improved after Coenzyme Q10 supplementation^[66,72]. Hence, early supplementation of Coenzyme Q10 seemed to help avoid development of massive proteinuria and consequently chronic renal failure^[72].

GFER deficiency (OMIM #613076)

Human Growth Factor ERV-1-like (*GFER*) (also known as augments of liver regeneration 1, ALR1) is one of the sulfhydryl oxidases with a FAD-binding domain, which together with disulfide carrier Mia40 forms part of the disulfide relay system (DRS) and constitutes the mitochondrial import and assembly (MIA) pathway. The MIA pathway plays an important role in the biogenesis of many mitochondrial intramembrane space (IMS) proteins^[75]. *GFER* has two distinct isoforms: the long isoform (205 amino acids, 23 kD), which is mainly located in the IMS of the mitochondria, and the shorter isoform (125 amino acids, 15 kD), which is present in the nucleus. Mia40 functions as an import receptor of cysteine-rich substrates in the IMS and promotes oxidative folding of these proteins. It is re-oxidized by *GFER* in a disulfide-transfer reaction with electrons transferred to cytochrome c, thus connecting the DRS to the electron-transport chain of the mitochondria and establishing its role in Complex IV activity^[76].

In 2009, Di Fonzo *et al.*^[77] reported homozygous missense mutations (c.581 G > A p.R194H) in the *GFER* gene in three siblings from a consanguineous Moroccan family. Subsequent functional studies using analogous mutation (R182H) in yeast ERV1 showed altered mitochondrial morphology, mitochondrial instability, reduced cytochrome c oxidase activity, weaker FAD binding, decreased thermal protein stability, and altered protein folding^[77,78]. Daithanka *et al.*^[79] (2010) also characterized the human *GFER*/ALR protein and demonstrated that the R194H led to marked loss of protein stability by an increased rate of FAD dissociation and enhanced proteolysis susceptibility with only minimal effect on enzymatic activity.

There are eight patients in four families reported with mutations in the *GFER* gene^[77,80,81], to date. The age range of the patients was 3 to 21 years with one deceased at 21 years of age. The predominant features were neuromuscular including hypotonia and psychomotor retardation of variable severity, muscle hypotrophy, progressive muscle weakness requiring ventilatory support, and, additionally, cataracts and lactic acidosis. Other clinical features included movement disorder, dysautonomia, cachexia and orthopedic problems^[81], hearing loss^[77], and adrenal insufficiency^[80]. MRI of the brain showed normal findings (4/6), cerebellar and moderate cortical atrophy (1/6), and thin corpus callosum (1/6). Histopathologic muscle findings included abnormal mitochondrial morphology, ragged red fibers, COX negative, and atrophic fibers. Enzymatic testing revealed isolated Complex IV deficiency or combined deficiencies of Complexes I-IV. The missense mutation (c.581 G > A; p.R194H) was identified in six out of the eight patients.

Riboflavin supplementation was not reported in these patients. Experiments have indicated that the FAD cofactor is detached from Erv1 R182H during the catalytic reaction and inactivated the protein^[78]. The

functional defect was shown to recover by adding extra FAD^[78]. Hence, riboflavin supplementation in patients with *GFER* mutations may potentially have clinical benefit, but this is speculative.

FDXR deficiency (OMIM #617717)

FDXR (Ferredoxin Reductase) has one FAD/NAD(P)-binding domain and two NAD(P)-binding domains. It is the sole ferredoxin reductase in humans and has important roles in iron-sulfur (Fe-S) cluster biogenesis and heme synthesis^[82]. Mitochondrial membrane associated FDXR reduces the ferredoxins, FDX1 and FDX2, and transfers electrons from NADPH to the mitochondrial cytochrome P450 system, thus initiating the mitochondrial electron transport chain reaction^[83]. Studies using patient derived fibroblasts showed loss of FDXR function led to decreased enzyme activity, reduced functions of Complexes I-III, significant increase in reactive oxygen species production, and mitochondrial iron overload^[84,85].

There are 27 patients in 19 families with mutations in the *FDXR* gene reported in the literature^[84-86]. A cohort of eight patients presented predominantly with auditory neuropathy and optic atrophy with onset in childhood or adolescence^[84]. There were no other clinical features except for mild language delay in one and retinitis pigmentosa, ophthalmoplegia, and lower limbs hypoalgesia in another patient^[84]. However, clinical variability was present in another cohort of patients who presented with neurological features of early onset, i.e., infancy to childhood^[85]. Apart from visual problems, i.e., retinitis pigmentosa and/or optic atrophy, and hearing loss, this cohort of 13 unrelated patients also had other clinical features such as hypotonia, global developmental delay, axonal sensorimotor polyneuropathy, regression after febrile illness, ataxia, failure to thrive, progressive microcephaly, and encephalopathy^[85]. Respiratory chain enzyme activities, when reported, showed normal activities or combined deficiencies. Post-mortem findings of one FDXR patient showed neuronal loss and vacuoles in the cerebral cortex and increased biomarkers for gliosis, astrocyte activation, and neurodegeneration^[86]. These findings were also observed in a homozygous p. R389Q mouse model, suggesting that inflammation could be a major component of the pathology in the neurodegeneration of *FDXR* mutations^[86].

NDUFV1 deficiency (OMIM # 618225)

NADH: ubiquinone oxidoreductase flavoprotein 1 (NDUFV1) is a 51-kDa highly conserved nuclear encoded subunit comprising the electron input (N) functional module of Complex I^[87]. It contains NADH-, FMN-, and Fe-S-binding sites^[88].

Mutations in the *NDUFV1* gene causing Complex I deficiency have been identified in 41 patients in 33 families^[56,88-110]. The median age of onset of clinical manifestations was 8.0 months (range: birth to 6 years). The most common clinical features were motor delay or neurodevelopmental regression usually precipitated by an illness, hypotonia, dystonia, seizures, spasticity, and cognitive impairment. Oculomotor impairment including ptosis, strabismus, and ophthalmoplegia was also frequently observed. Typical Leigh syndrome^[88,107] and late-onset Leigh syndrome have been reported^[95,103]. The clinical course was variable with some patients having slower progression of neurological manifestations^[90,91,103]. Brain MRI and MRS findings included features of typical Leigh syndrome, white matter changes, basal ganglia changes, elevated lactate peak, infantile striatal necrosis^[97], and cavitating leukoencephalopathy^[109]. Outcome was available in 34 patients; ten died at a median age of six months (range: three days to three years). The median age among surviving patients was seven years (range: 1-15 years) and severity of neurocognitive outcome varied from mild learning disability^[91,96] to significant impairment^[101,103]. Riboflavin supplementation was only reported in seven patients^[94,97,98,101] and was given with other supplements such as thiamine, Coenzyme Q10, creatine, and alpha lipoic.

NDUFV2 deficiency (OMIM #618229)

Human NADH dehydrogenase ubiquinone flavoprotein 2 (NDUFV2), a 24-kDa binuclear [2Fe-2S] cluster containing protein, is highly conserved and one of the nuclear encoded subunits of Complex I which is

involved in electron transfer. It is also one of the subunits comprising the electron input (N) functional module of Complex I^[87].

Phenotypic variability is observed in patients with Complex I deficiency due to mutations in the *NDUFV2* gene. Bénit *et al.*^[111] 2003 reported three affected siblings in a consanguineous family presenting with early onset hypertrophic cardiomyopathy, truncal hypotonia, feeding difficulties, growth retardation, and early death within the first year of life. Another patient also presented with hypertrophic cardiomyopathy and encephalopathy^[112]. However, a separate family with three affected siblings presented with Leigh syndrome without cardiac involvement^[113]. These patients reported by Cameron *et al.*^[113] had seizures by 10 months of age, progressive clinical course, and two died at 19 months and 10 years. One of these patients was started on riboflavin plus other supplements. One sibling is still alive at 32 years despite a vegetative state by three years of age. All these patients were homozygous for a 4-bp deletion in intron 2 (IVS2 + 5_ + 8delGTAA) of the *NDUFV2* gene^[113]. Studies using human disease cell model showed that this deletion caused a significant reduction in the mitochondrial targeting ability of the NDUFV2 protein^[114].

In a cohort of 37 children with cavitating leukoencephalopathies, three (3/37, 8.1%) were compound heterozygotes for pathogenic mutations in the *NDUFV2* gene^[109]. The age of onset of symptoms was 4-46 months with stable/improved clinical course and frontal predominant and deep white matter patterns on brain MRI^[109].

Succinic dehydrogenase subunit A deficiency (OMIM #252011)

Succinic dehydrogenase subunit A (SDHA), a flavoprotein with FAD as cofactor, is a nuclear encoded and catalytic subunit of Complex II (succinate dehydrogenase) which oxidizes succinate to fumarate in the Krebs' cycle and transfers electrons to ubiquinone in the mitochondrial electron transport chain^[115].

Mutations in *SDHA* gene result in both a mitochondrial disease causing Complex II deficiency and tumor susceptibility. A large proportion (47%) of germline mutations in *SDHA* has been reported in gastrointestinal stromal tumors^[116]. Other tumors such as paraganglioma/pheochromocytoma, pituitary adenoma, and renal carcinoma have also demonstrated *SDHA* germline mutations^[117-121].

Complex II deficiency due to mutations in *SDHA* presents with variable clinical presentations and age of onset from prenatal to adulthood. The first report of a nuclear gene mutation causing a mitochondrial respiratory chain deficiency in humans was identified in two sisters presenting with developmental regression at 10 months of age, pyramidal tract signs, leukodystrophy with early demise, Complex II deficiency, and homozygous mutations (R554W) in the *SDHA* gene^[122,123]. Further reports of patients with *SDHA* mutations have since been described^[124-131]. Leigh or Leigh-like syndrome was a common phenotype. Slower progression with survival up to 11 years of age has been observed^[126,127,130], and, in one patient, there was no apparent cognitive impairment at 10 years of age^[130]. Isolated cardiomyopathy presenting from 32 weeks in utero to 10 years of age was associated with high mortality in two large Bedouin families with homozygous G555E mutations in the *SDHA* gene^[132].

Adult presentation has been reported in two sisters (62 and 56 years) with normal cognition, late-onset (mid-40s), and slowly progressive cerebellar ataxia, visual impairment, optic atrophy, proximal weakness, and partial Complex II deficiency in skeletal muscle^[133]. Riboflavin supplementation did not prevent neurological progression in these two patients. These patients were found to be heterozygous for a missense mutation (R408C) in a highly conserved region of the mature *SDHA* protein, suggesting an autosomal dominant inheritance pattern^[134]. Another family with three affected members who presented with optic atrophy, dilated cardiomyopathy, ataxia, and isolated Complex II deficiency in fibroblast were also found to be heterozygous for a R451C missense mutation. This family serves as the second report supporting an autosomal dominant inheritance^[135].

Secondary mitochondrial dysfunction

Multiple Acyl-CoA Dehydrogenase Deficiency (OMIM# 231680)

Multiple acyl-coenzyme A dehydrogenase deficiency, also known as glutaric aciduria Type II (GAII), is an autosomal recessive disorder that affects the oxidation of fatty acids, BCAA, lysine, tryptophan, and choline. MADD is caused by deficiency of one of the two electron-transfer flavoproteins which transfer electrons from acyl-CoA dehydrogenases to Coenzyme Q in the respiratory chain: Electron Transfer Flavoprotein (ETF), encoded by *ETF A* and *ETF B* genes, and electron-transfer flavoprotein dehydrogenase (ETF DH), encoded by *ETF DH*^[136]. Metabolic defects resulting from impaired beta-oxidation include decreased ATP biosynthesis, excessive lipid accumulation in various organs, and insufficient gluconeogenesis^[137].

The clinical phenotype is variable and has been classified into neonatal onset forms, with the most severely affected patients presenting with congenital anomalies (Type I) or without anomalies (Type II), and mild and/or later onset (Type III)^[138]. Severely affected patients present in the first few days of life with non-ketotic hypoglycemia, hyperammonemia, and metabolic acidosis accompanied by hypotonia, encephalopathy, hepatomegaly, cardiomyopathy, and poor prognosis. An odor of sweaty feet similar to that in isovaleric acidemia may be observed^[139]. Some patients have congenital anomalies (including large cystic kidneys, hypospadias, and neuronal migration defects that can be prenatally detected by fetal MRI and facial dysmorphism (low set ears, high forehead, and midfacial hypoplasia)^[139]. The most frequent clinical presentation is the milder myopathic Type III form, which manifests with fluctuating proximal and axial myopathy with exercise intolerance and occasionally respiratory insufficiency^[138] or rhabdomyolysis, although often with hepatomegaly, encephalopathy, and episodic lethargy, as well as vomiting and hypoglycemia often triggered by metabolic stress, episodes of which have been lethal in 5% of patients^[140]. Although most decompensations occur in childhood, severe metabolic crises have also been reported in adulthood^[138]. The majority of the 350 cases of late-onset MADD described in the literature carry mutations in the *ETF DH* gene (93%), while mutations in the *ETF A* (5%) and *ETF B* (2%) genes are less frequent^[138].

Laboratory parameters include increased creatine kinase and lactate levels, with low carnitine. Diagnostic confirmation is made by the increase of short-, medium-, and long-chain acyl-carnitines on acylcarnitine analysis and characteristic urinary organic acid pattern comprising elevated levels of glutaric, ethylmalonic, 3-hydroxyisovaleric, 2-hydroxyglutaric, 5-hydroxyhexanoic, and relevant ketonuria, particularly glycine conjugates of C4 and C5 acids^[137,138]. Muscle biopsies usually reveal lipid storage myopathy and secondary mitochondrial dysfunction with decreased Complexes I and II + III, attributable to deficiency of Coenzyme Q, which has been associated with increased ROS generation due to electron leak from misfolded variant ETF DH proteins and impaired Q10 binding affinity^[138].

A clear genotype-phenotype correlation has been reported with the heterogenous subtypes of MADD. Type I disease is usually associated with homozygosity for null mutations. Even minor amounts of residual ETF/ETF DH activity suffices to prevent embryonic development of congenital anomalies observed in Type II disease, whereas higher residual activity is found in the late-onset form/ Type III disease. The genotype-phenotype correlation within Type III patients is however poor, as exogenous stressors including febrile infections may modulate the residual activity^[138].

Riboflavin supplementation has successfully ameliorated clinical symptoms and metabolic abnormalities in almost all patients (98%) with late-onset MADD, the majority of whom have *ETF DH* mutations (93%)^[138]. High levels of FAD and FMN due to riboflavin supplementation promote folding and stability of flavoproteins, particularly at fever simulating temperatures (40 °C), permitting certain mutant flavoenzymes to reach a folding efficiency and stability that attenuates the enzyme deficiency^[7]. Combined treatment of riboflavin and Coenzyme Q10 has been advocated for use in riboflavin-responsive MADD based on studies

which demonstrated improved mitochondrial dynamics with decreased ROS production and restoration of CoQ10 levels in patient fibroblasts co-treated with CoQ10 and riboflavin^[141].

Dihydrolipoamide dehydrogenase (E3) deficiency (OMIM# 246900)

The *DLD* gene encodes dihydrolipoamide dehydrogenase (also known as E3), a common flavoprotein shared by three mitochondrial α -ketoacid dehydrogenase multi-enzyme complexes: pyruvate dehydrogenase complex (PDHC), α -ketoglutarate dehydrogenase complex, and branched-chain α -keto acid dehydrogenase complex. It catalyzes the third stage of the reaction by re-oxidizing the reduced lipoyl moiety of E2 and generating lipoic acid and NADH^[142]. E3 is also a component of a fourth mitochondrial multienzyme complex, the glycine cleavage system, where it functions as the L protein, a housekeeping enzyme that, to date, has not appeared to impair the function of the system in vivo when associated with pathogenic variants of *DLD*^[143]. The metabolic derangements incorporate PDHC and TCA cycle defects: increased blood lactate and pyruvate, elevated plasma alanine, BCAA, presence of allo-isoleucine in plasma, and increased urinary lactic, pyruvic, 2-ketoglutaric, and branched-chain 2-hydroxy- and 2-ketoacid^[143,144].

The phenotypic spectrum of DLD deficiency includes: (1) classical E3 deficiency, which includes early-onset encephalopathy with progressive hypotonia, failure to thrive, hypoglycemia, ketoacidosis, and encephalopathy or Leigh-like encephalopathy; (2) a primarily hepatic presentation with Reye-like syndrome, wherein patients typically have normal intellect without residual neurologic deficits between acute metabolic episodes; and (3) a milder riboflavin responsive myopathic phenotype with exertional fatigue, intermittent elevation of blood lactate, ketoacidosis, creatine kinase, and mitochondrial proliferation^[143,145,146]. Riboflavin supplementation led to complete resolution of muscle weakness, improvement of metabolic abnormalities, partial restoration of the DLD protein, resolution of mitochondrial proliferation in muscle, and reduced ROS production in fibroblasts, supporting the evidence of a chaperone-like effect of riboflavin in promoting DLD protein stability and folding^[146].

*Ethylmalonic aciduria secondary to *ETHE1* dysfunction (OMIM# 602473)*

Ethylmalonic encephalopathy (EE) is a rare, devastating, invariably fatal neurodegenerative disease caused by mutations in the *ETHE1* gene, which encodes a mitochondrial sulfur dioxygenase critical in hydrogen sulfide (H₂S) detoxification^[147]. The characteristic clinical features are hydrogen sulfide mediated and include vasculopathy due to diffuse microvasculature injury responsible for multiple necrotic brain lesions, which lead to early-onset psychomotor regression, seizures, and global neurological impairment that subsequently evolve to severe psychomotor delay with spastic tetraparesis. Vascular lesions include diffuse and spontaneous relapsing petechial purpura, orthostatic acrocyanosis, hemorrhagic suffusions of mesothelial surfaces, and intestinal mucosa associated with chronic hemorrhagic diarrhea^[148].

Biochemical abnormalities resulting from accumulation of hydrogen sulfide and its derivative thiosulfate in crucial tissues including liver, brain, and colonic mucosa leading to inhibition of both short-chain acyl-CoA dehydrogenase with consequent elevation of ethylmalonate, C4- and C5-acylcarnitines predominantly in muscle and brain, and cytochrome c oxidase deficiency, blocking mitochondrial respiration and increasing lactic acid^[147,148]. Urinary thiosulfate is also markedly elevated^[149].

Current treatment modalities include the off-label use of common drugs such as N-acetylcysteine and metronidazole to lower the production and promote detoxification of toxic H₂S. This combined exposure has produced some encouraging results with amelioration of some neurological abnormalities and marked attenuation or disappearance of the vascular lesions and diarrhea^[150], suggesting disease modification from the invariably fatal clinical course of EE^[151]. Therapeutic trial with riboflavin and/or Coenzyme Q10 resulted in decreased C4- and C5-acylcarnitines in one patient and mild improvements in chronic mucoid diarrhea and motor and cognitive functions in three patients^[152].

AIFM1 deficiency (OMIM#300816, OMIM#310490, OMIM#300614, OMIM#300232)

The X-linked *AIFM1* gene encodes the apoptosis-inducing factor (AIF), a mitochondrial flavoprotein with a dual role, first as a FAD-dependent NADH oxidoreductase and second as a caspase-independent death effector which regulates apoptosis^[153,154]. *AIFM1* mutations have been associated with wide spectrum of clinical phenotypes with X-linked recessive inheritance including a severe, early-onset mitochondrial encephalomyopathy with combined oxidative phosphorylation deficiency^[155], prenatal ventriculomegaly^[156], the Cowchock syndrome, an X-linked Charcot-Marie-Tooth disease (CMTX4) with axonal sensorimotor neuropathy, deafness and cognitive impairment^[157], auditory neuropathy spectrum disorder^[158], spondyloepimetaphyseal dysplasia with mental retardation^[159], and, more recently, cerebellar ataxia partially responsive to riboflavin^[160].

The *AIFM1* protein is highly expressed in hairy cells and spiral ganglion within the inner ear, consistent with its crucial role in maintaining normal auditory function^[158]. Hence, the predominant phenotypes described with *AIFM1* mutations are either mitochondrial encephalomyopathies or sensorineural hearing loss and auditory neuropathy as transmission from the inner ear to the auditory nerve and brain stem is distorted^[160]. Cerebellar ataxia, with or without vermian atrophy, was described only recently in relation to *AIFM1* mutations, and has further widened the symptomatology^[160].

Supplementation with riboflavin was reported to temporarily slow the disease progression in a patient with the severe encephalomyopathy^[155]. A clear improvement of ataxia by 44% and 20% was observed in two patients, respectively, as measured by the ICARS score, an objective assessment that quantifies the magnitude of improvement in progressive ataxias, with a score of 5%-20% considered as substantial in providing a clinical impact. Prompt diagnosis through early recognition of constellation of symptoms including ataxia with sensorineural hearing loss especially auditory neuropathy would be important since the symptoms may be partially amenable to riboflavin treatment^[160].

CONCLUSION

Defects of riboflavin metabolism present with a heterogeneous clinical spectrum and variable severity, ranging from severe, early-onset neurodegenerative disorders to adult-onset myopathy. Riboflavin has been used as a potential therapeutic agent in the “mitochondrial cocktail”, particularly in Complex I- and II-related primary mitochondrial disease to ameliorate oxidative stress. There is growing evidence of the beneficial role of riboflavin in treating defects of riboflavin metabolism. Riboflavin supplementation has dramatically led to clinical improvements in riboflavin transporter deficiencies (RTD2 and RTD3), which encompasses BVVL and FL syndromes and mitochondrial FAD transporter deficiency. It has also been proven efficacious in FAD synthase deficiency, late-onset MADD due to *ETFDH* mutations and deficiencies in *ACAD9*, dihydrolipoamide dehydrogenase (E3), and *AIFM1*. In view of the promising therapeutic effects of riboflavin in these disorders, early recognition and treatment including trial of riboflavin supplementation is imperative.

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Authors' contributions

Made equal contributions to writing and critically revising the manuscript: Balasubramaniam S, Yaplitto-Lee J

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Both authors declared that there are no conflicts of interest.

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