An Update on the Genetics, Clinical Presentation and Pathomechanisms of Human Riboflavin Transporter Deficiency

Benjamin O'Callaghan[1], Annet M Bosch[2], Henry Houlden[1*]

[1]MRC Centre for Neuromuscular Diseases, Department of Neuromuscular Diseases, UCL Queen Square Institute of Neurology and National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, UK

[2]Amsterdam UMC, University of Amsterdam, Pediatric Metabolic Diseases, Emma Children's Hospital, Meibergdreef 9, Amsterdam, Netherlands.

*Corresponding: h.houlden@ucl.ac.uk Tel: 020 7837 3611

Manuscript word count: 4114

Summary Word Count: 174

Tables: 1 Figs: 1

SUMMARY: Riboflavin Transporter Deficiency (RTD) is a rare neurological condition that encompasses the Brown-Vialetto-Van Laere and Fazio-Londe syndromes since the discovery of pathogenic mutations in the *SLC52A2* and *SLC52A3* genes that encode human riboflavin transporters RFVT2 and RFVT3. Patients present with a deteriorating progression of peripheral and cranial neuropathy that causes muscle weakness, vision loss, deafness, sensory ataxia and respiratory compromise which when left untreated can be fatal. Considerable progress in the clinical and genetic diagnosis of RTDs has been made in recent years and has permitted the successful lifesaving treatment of many patients with high dose riboflavin supplementation.

In this review we first outline the importance of riboflavin and its efficient transmembrane transport in human physiology. Reports on 109 patients with a genetically confirmed diagnosis of RTD are then summarised in order to highlight commonly presenting clinical features and possible differences between patients with pathogenic *SLC52A2* (RTD2) or *SLC52A3* (RTD3) mutations. Finally, we focus attention on recent work with different models of RTD that have revealed possible pathomechanisms contributing to neurodegeneration in patients.

Take Home Message: Here we outline the genetics, clinical features, and underlying pathomechanisms of human riboflavin transporter deficiencies (RTDs). Lifesaving treatment with oral riboflavin should be started as soon as a RTD is suspected and continued until the diagnosis has been confirmed or excluded by genetic evaluation.

COMPLIANCE WITH ETHICS GUIDELINES

Author Contributions: Ben O'Callaghan drafted the article. Annet Bosch and Henry Houlden conceived and revised the content.

Guarantor: Ben O'Callaghan serves as guarantor for the article.

Corresponding Author: Henry Houlden

Conflict of Interest: Ben O'Callaghan, Annet Bosch and Henry Houlden declare that they have no conflict of interest.

Funding: Ben O'Callaghan is supported by a PhD studentship from the MRC Centre for Neuromuscular Diseases.

Ethics Approval: This article does not contain any studies with human or animal subjects performed by any of the authors, and does not require ethics approval.

Keywords: *SLC52A2*, *SLC52A3*, RFVT, riboflavin, RTD

INTRODUCTION

Riboflavin belongs to the metabolic B class of vitamins (Vitamin B2) and is the sole precursor for the biologically active cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). During evolution, humans and other higher animals have lost the ability to synthesise riboflavin and instead rely on dietary sources. Emphasising the importance of riboflavin in human physiology and furthermore its efficient absorption and homeostasis are the riboflavin transporter deficiencies (RTDs) (ORPHA 97229 https://www.orpha.net/; OMIM 211500, 211530 and 614707) caused by recessive, biallelic mutations in the genes encoding human riboflavin transporters (RFVTs).

Essential Role of Riboflavin in Human Physiology

Following cellular absorption, riboflavin is rapidly converted into activated flavin cofactors: FMN through riboflavin kinase (RFK: EC 2.7.1.26) mediated phosphorylation of riboflavin, and subsequently FAD by flavin adenine dinucleotide synthetase 1 (FLAD1: EC 2.7.7.2) mediated adenylation of FMN. FMN and FAD are incorporated into 90 different proteins collectively termed the "flavoproteome" (Lienhart et al. 2013), the large majority of which are oxidoreductases localised to the mitochondria that catalyse electron transfer during various redox metabolic reactions including: oxidative decarboxylation of amino acids and glucose, and β-oxidation of fatty acids. Of particular note are a collection of flavoproteins that are crucial for mitochondrial oxidative phosphorylation (OXPHOS) function including: electron-transferring flavoprotein (ETF) and electron-transferring flavoprotein-dehydrogenase (ETFDH: EC 1.5.5.1), which together transfer electrons from various reduced flavin groups to Complex III via Coenzyme Q10; and constituent subunits of Complexes I (NADH Ubiquinone Oxidoreductase Core Subunit V1, NDUFV1: EC 1.6.99.3) and II (Succinate Dehydrogenase Subunit A, SDHA: EC 1.3.5.1).

Central to the successful incorporation of flavin cofactors into mitochondrial flavoproteins is the transport of FAD from the cytosol, into the mitochondrial matrix by the mitochondrial FAD transporter (MFT encoded by *SLC25A32*). Biallelic mutations in *SLC25A32* have been associated with riboflavin-responsive exercise intolerance (Schiff et al. 2016) and more recently a severe neuromuscular phenotype (Hellebrekers et al. 2017), highlighting the subcellular importance of flavin availability within mitochondria in particular. For further discussion on the mitochondrial FAD transporter, readers are referred to an accompanying review in this issue that addresses disorders of riboflavin metabolism (Balasubramaniam et al. 2019).

Other important roles of flavoproteins include: the activation of other B class vitamins, redox homeostasis, transcriptional regulation through enzymatic chromatin modifications, caspase independent apoptosis and cytoskeletal reorganisation (Lienhart et al. 2013; Barile et al. 2016).

Considering the importance of flavins in metabolically active cells it is unsurprising that inadequate supply of riboflavin has been implicated in diseases of energy demanding tissues, particularly the nervous system.

Human Riboflavin Transporters

In order to maintain a sufficient supply of flavins to cells throughout the body, humans and other higher animals have established an effective carrier-mediated system to transport riboflavin across plasma membranes. Three human RFVT homologues have been identified: RFVT1-3 encoded by genes *SLC52A1-3* respectively (note RFVT2 and RFVT3 were designated RFT3 and RFT2 respectively in previous nomenclature) (Yonezawa et al. 2008; Yamamoto et al. 2009; Yao et al. 2010; Yonezawa and Inui 2013). RFVT1 and RFVT2 display 87 % amino acid sequence identity, whereas RFVT3 only exhibits 44 % and 45 %

amino acid sequence identity with RFVT1 and RFVT2 respectively (ClustalW: http://www.clustal.org/omega/).

Transmembrane Topology

Some confusion surrounding the transmembrane (TM) topology of RFVTs is present in the literature. Based on initial *in silico* predictions, RFVT1 and RFVT2 were predicted to have 10 TM domains (Yonezawa et al. 2008; Yao et al. 2010) whereas RFVT3 was predicted to have 11 TM domains (Yonezawa and Inui 2013). *In silico* predictions made using other membrane topology algorithms predict all three RFVTs to have 11 TM domains however (Yamamoto et al. 2009; Udhayabanu et al. 2016; Colon-Moran et al. 2017), and this is supported by immunostaining of hemagglutinin (HA) tagged RFVT1 constructs that indicate an intracellular N-terminus and extracellular C-terminus (Mattiuzzo et al. 2007). Knowing the correct RFVT topology might be important for correlating disease causing mutation sites with differences in phenotypical presentations and/or responsiveness to therapeutic interventions.

Tissue Distribution

mRNA expression of the three different RFVT genes in human tissues has been assessed (Yao et al. 2010) and is largely in accordance with more recent gene expression data from the GTEx V7 dataset (https://gtexportal.org/). *SLC52A1* is mainly expressed in the placenta and intestine. *SLC52A2* is rather ubiquitously expressed but is particularly abundant in nervous tissues. *SLC52A3* is most highly expressed in testis but also intestine and prostate. These different but overlapping expression profiles might explain the vulnerability of certain tissues to mutations in one or more of the *SLC52A* genes.

RIBOFLAVIN TRANSPORTER DEFICIENCIES (RTDs)

Brown-Vialetto-Van Laere (BVVL) and Fazio-Londe (FL) are two phenotypically continuous syndromes presenting with a progressive sensorimotor and cranial neuropathy. Both share a core phenotype of: bulbar palsy (e.g. dysphagia, dysphonia, tongue atrophy), axial and distal muscle weakness, optic atrophy, sensory ataxia and respiratory compromise due to diaphragm paralysis (Bosch et al. 2011; Horvath 2012; Manole and Houlden 2015; Jaeger and Bosch 2016). Sensorineural deafness is present in BVVL only. Since 2010 biallelic mutations in the human riboflavin transporter genes *SLC52A3* (previously C20orf54) and *SLC52A2* have been demonstrated to be the cause of the BVVL and FL syndromes which were renamed to Riboflavin Transporter Deficiencies (RTDs) (Green et al. 2010; Johnson et al. 2010, 2012, Bosch et al. 2011, 2012; Foley et al. 2014; Manole and Houlden 2015). RTD2 and RTD3 refer to disorders caused by *SLC52A2* and *SLC52A3* mutations respectively *(Tables S2 and S3).*

Transient Riboflavin Deficiency

Although pathogenic mutations in *SLC52A1* have not been described in patients with a typical RTD phenotype, there have been two reports of transient riboflavin deficiency occurring in the newborn children of mothers harbouring one heterozygous *SLC52A1* mutation (OMIM 615026), in one case in combination with a riboflavin deficiency due to deficient maternal intake *(Table S1)* (Ho et al. 2011; Mosegaard et al. 2017). In both cases the children but not the mothers showed clinical symptoms of riboflavin deficiency after birth that had subsided by two years of age. Whilst *SLC52A1* is expressed in both the human small intestine and placenta, the transient nature of the clinical presentation suggests that these cases were caused by placental haploinsufficiency, and associated impairment in the transport of riboflavin from the mother to the fetus.

Genetically Diagnosed Cases of Riboflavin Transporter Deficiency

An article in this journal three years ago (Jaeger and Bosch 2016) summarised reports of 70 genetically confirmed RTD patients that had been published at that time (Green et al. 2010; Johnson et al. 2010, 2012; Bosch et al. 2011; Anand et al. 2012; Koy et al. 2012; Dezfouli et al. 2012; Haack et al. 2012; Ciccolella et al. 2012, 2013; Spagnoli et al. 2014; Foley et al. 2014; Bandettini Di Poggio et al. 2014; Srour et al. 2014; Cosgrove et al. 2015; Horoz et al. 2016; Menezes et al. 2016a; Davis et al. 2016).

There have since been a further 10 publications reporting on 23 newly diagnosed RTD2 cases (Petrovski et al. 2015; Menezes et al. 2016b; Guissart et al. 2016; Allison et al. 2017; Manole et al. 2017; Woodcock et al. 2017; Çıralı et al. 2017; Babanejad et al. 2018; Nimmo et al. 2018; Set et al. 2018), and 12 reporting on 27 newly diagnosed RTD3 cases (van der Kooi et al. 2016; Manole et al. 2017; Thulasi et al. 2017; Bashford et al. 2017; Chaya et al. 2017; Woodcock et al. 2017; Hossain et al. 2017; Kurkina et al. 2017; Khadilkar et al. 2017; Nimmo et al. 2018; Camargos et al. 2018; Gowda et al. 2018). A patient harbouring a heterozygous pathogenic mutation in *SLC52A3* and heterozygous *SLC52A2* variant of unknown significance has been described (Allison et al. 2017), which will be considered as a RTD3 case here. The possibility that both heterozygous mutations within the two different riboflavin genes are synergistically disrupting the same metabolic pathway to a pathogenic level cannot be excluded however. Finally, a patient with homozygous mutations in both *SLC52A2* and *SLC52A3* (Udhayabanu et al. 2016) has also been described (RTD2/3). In total, various degrees of information are available on 109 patients (52 RTD2, 56 RTD3 and 1 RTD2/3) with 71 different *SLC52A* mutations (24 *SLC52A2*, 47 *SLC52A3*) *(Table 1)*.

SLC52A2 and *SLC52A3* Pathogenic Variants

Pathogenic variants in *SLC52A2* and *SLC52A3* are distributed throughout all coding exons (Ex2-5) and include nonsense and missense mutations affecting RFVT amino acid residues

constituting: transmembrane domains, intracellular loops, extracellular loops and C-terminus *(Tables S2 and S3)*. Single nucleotide substitutions within intron-exon boundaries have also been identified in *SLC52A2* and *SLC52A3* that likely cause splicing defects (Bosch et al. 2011; Manole et al. 2017; Çıralı et al. 2017). Single/double nucleotide insertions/deletions causing frameshift mutations have been identified in *SLC52A3* (Green et al. 2010; Bandettini di Poggio et al. 2013; Manole et al. 2017), in addition to a more recently described in-frame insertion of 60 nucleotides (20 amino acid peptide) (Camargos et al. 2018).

Using heterologous expression systems the impact of different pathogenic *SLC52A2/3* mutations on RFVT2/3 function has been assessed *in vitro* (Nabokina et al. 2012; Haack et al. 2012; Foley et al. 2014; Subramanian et al. 2015; Petrovski et al. 2015; Udhayabanu et al. 2016). In most cases the disease causing mutation reduces RFVT cell surface expression which when assessed appears to be due to retainment in the endoplasmic reticulum (ER), indicative of protein misfolding and/or trafficking defect. In some instances riboflavin transport is impaired but with an apparently normal cell surface expression. Of the 15 mutant RFVTs assessed only one (*SLC52A3* Genbank NM_033409.3 c.1048T>A; RFVT3 p.Leu350Met) has been shown to be functionally normal (Nabokina et al. 2012). Evidence for a reduction in mRNA stability has also been shown for a *SLC52A2* single nucleotide substitution (Ciccolella et al. 2013). Finally, impaired riboflavin uptake has been described in fibroblasts from patients harbouring compound heterozygous *SLC52A2* mutations (Ciccolella et al. 2013; Manole et al. 2017).

Clinical Differentiation of RTD2 and RTD3

Disease Onset

The large majority of patients with either RTD2 or RTD3 present early in life but until now only in RTD3 has a late onset (as late as the third decade) been reported (Bashford et al. 2017; Camargos et al. 2018). Late onset RTD (>10y) might therefore be more suggestive of a

SLC52A3 mutation. Hearing loss and muscle weakness are among the most common presenting symptoms at onset of both RTD2 and RTD3. Abnormal gait and/or ataxia is often a presenting feature of RTD2 but rarely RTD3. By contrast RTD3 commonly presents with bulbar symptoms, whereas in RTD2 these are generally observed later in the disease course. Other symptoms regularly described upon RTD onset include hypotonia, facial weakness and respiratory dysfunction due to diaphragmatic paralysis as well as muscle weakness.

Common Symptoms

Whilst hearing loss as a consequence of cranial nerve VIII degeneration is a presenting symptom of many patients, others develop sensorineural hearing loss later in the disease course, and this remains the most commonly observed clinical feature of RTD2 and RTD3. Bulbar symptoms such as dysphagia and dysarthria are present in most patients and a large number display feeding difficulties as a result of dysphagia that in many instances necessitates a nasogastric tube or gastrostomy feeding device. Artificial respiratory devices are also often required, with respiratory symptoms due to neurogenic diaphragm paralysis being very common. Weakness and hypotonia of both limb and axial muscles was prevalent and commonly associated with neurogenic muscular atrophy, particularly of distal muscles. Facial weakness caused by cranial nerve VII (facial nerve) degeneration was common in RTD3 but rarely seen in RTD2. Abnormal gait and/or ataxia remains a distinguishing feature of RTD2, with RTD3 patients rarely showing signs later during the disease course. *SLC52A2* mutations have recently been associated with spinocerebellar ataxia with blindness and deafness type 2 (SCABD2) (Guissart et al. 2016; Babanejad et al. 2018). Finally, vision loss caused by cranial nerve II (optic nerve) atrophy was observed in numerous RTD3 cases but appears to be a much more prevalent feature of RTD2.

Neurodiagnostic Tests

Neurophysiological studies are suggestive of peripheral neuropathy in the large majority of RTD patients tested but normal results are also observed, particularly in RTD3. Motor and sensory nerve conduction studies are indicative of an axonal rather than demyelinating neuropathic phenotype, with signs of anterior horn dysfunction and chronic denervation in most RTD cases. Slightly slowed sensorimotor conduction velocities suggestive of demyelination have been described in a minority of RTD2 cases (Guissart et al. 2016; Allison et al. 2017), and a single RTD3 patient (Bandettini di Poggio et al. 2013; Bandettini Di Poggio et al. 2014) however.

In the large majority of RTD cases brain magnetic resonance imaging (MRI) is unremarkable. Abnormal MRI observations rarely described in RTD2 brain include: mild atrophy of the cerebellar vermis (Guissart et al. 2016), optic nerve abnormalities (Woodcock et al. 2017; Set et al. 2018) and thinning/shortening of the corpus callosum (Srour et al. 2014; Set et al. 2018). Cerebellar abnormalities described in RTD3 brain MRI include: hyperintense T2-weighted signals within cerebellar peduncles (Koy et al. 2012; Bandettini Di Poggio et al. 2014), and volume loss of peduncles and vermis over an 8 year period (Bandettini Di Poggio et al. 2014). Intense T2-weighted signals have also been noted in cortical, subcortical (basal ganglia and internal capsule) and brainstem (vestibular nuclei and central tegmental tract) regions of some RTD3 patients (Koy et al. 2012; Spagnoli et al. 2014; Hossain et al. 2017; Nimmo et al. 2018). Spinal MRI has been conducted much less frequently, but abnormal T2-weighted intensities have been described in ventral nerve roots and dorsal regions of the spinal cord (Koy et al. 2012; Spagnoli et al. 2014; Davis et al. 2016; Woodcock et al. 2017; Khadilkar et al. 2017) in accordance with the sensorimotor phenotype of RTD.

Neuropathology

Assessment of sural nerve biopsies from RTD2 (Haack et al. 2012; Foley et al. 2014; Srour et al. 2014) and RTD3 (Johnson et al. 2010; Chaya et al. 2017) patients show evidence for axonal neuropathy and degeneration which preferentially affects large calibre myelinated axons (Foley et al. 2014; Srour et al. 2014; Chaya et al. 2017), in accordance with the sensory impairments observed in these patients.

Recent neuropathological observations described in the central nervous system of two RTD3 patients are also reflective of the RTD clinical phenotype (Manole et al. 2017). In line with the bulbar symptoms that are commonly observed, nuclei and tracts of cranial nerves IX, X and XII showed marked neuronal loss and gliosis. Loss of neurons was also observed in the nuclei of cranial nerves III and IV in accordance with eye movement impairments observed in these patients. The nuclei of cranial nerve VIII and tracts of cranial nerve II showed evidence of degeneration, underscoring the clinical presentation of sensorineural deafness and vision loss respectively. Gliosis and neuronal loss was also evident in midbrain (medial lemniscus, central tegmental tract) brainstem (pons, medulla), cerebellum (white matter structures including cerebellar peduncles, cerebellar nuclei) and spinal cord (anterior horn, spinothalamic tracts, spinocerebellar tracts), fitting with MRI observations that have been made in some RTD3 patients *(see above)*. Of particular interest was the presence of symmetrical lesions in the brainstem of both patients that showed demyelination and macrophage infiltration but with relative sparing of the neurons. The authors highlighted the similarities of these lesions to neuropathological observations made in mitochondrial disease patients.

Biochemical Tests

An increase in plasma acylcarnitines is indicative of an impairment in the metabolism of fatty acids by mitochondrial β-oxidation and is a characteristic observation of the multiple acyl-

CoA dehydrogenation defect (MADD) syndromes caused by mutations in ETF (encoded by *ETFA* and *ETFB*) or ETFDH (encoded by *ETFDH*) flavoproteins (OMIM 231680). Identification of a MADD-like acylcarnitine profile in BVVL patients without *ETFA, ETFB* or *ETFDH* mutations led to a hypothesis of impaired riboflavin absorption and was key to the initial identification of BVVL as a RTD (Bosch et al. 2011). However, nearly half of the RTD cases described since show normal acylcarnitine profiles on diagnosis and thus it cannot be used to exclude a RTD diagnosis.

Urine organic acid analysis has been reported less frequently, and in half of RTD cases results are normal. Ethylmalonic aciduria suggestive of impairments in fatty-acid, methionine and/or isoleucine oxidation is the most common abnormality noted (4/10 RTD2, 5/12 RTD3). Flavoproteins constitute important steps in the metabolic pathways responsible for branched-chain, lysine and tryptophan amino acid catabolism (Barile et al. 2016) and elevations in acylglycines associated with impairments of such pathways have also been described.

Assessment of plasma flavin status necessitates mass spectrometry analysis and is not routinely done in the clinical setting. In the small number of patients assessed, plasma flavin levels are generally within the normal range but low levels have been reported in both RTD2 (Srour et al. 2014) and RTD3 (Bosch et al. 2011). Following high dose riboflavin treatment, increases in plasma flavin levels are observed in both RTD2 and RTD3 cases (Bosch et al. 2011; Haack et al. 2012; Foley et al. 2014), highlighting the partial redundancy of RFVT homologues in intestinal absorption. Nevertheless, with many patients presenting with normal flavins at diagnosis, plasma flavin status cannot be used as a tool to exclude a RTD diagnosis.

Measurements of the erythrocyte glutathione reductase activity coefficient (EGRAC) are representative of flavin status and more routinely done in the clinic. An abnormal EGRAC measurement without acylcarnitine abnormalities has been reported in a single RTD3 case, which normalised following riboflavin supplementation (Chaya et al. 2017).

Genetic Diagnostic Strategy

Whilst there does appear to be differences in the commonest clinical signs linked with RTD2 or RTD3, there is no observation that can definitively distinguish between the two. It is therefore recommended that genetic analysis of *SLC52A2* and *SLC52A3* is performed simultaneously rather than sequentially in suspected RTD cases (Manole and Houlden 2015). Even though mutations in *SLC52A1* are yet to be associated with a typical RTD phenotype, it remains a viable candidate that should also be considered. Whole or focused exome analysis using next generation sequencing (NGS) technology might then be performed, with a filtering strategy targeting genes associated with: similar clinical phenotypes (e.g. amyotrophic lateral sclerosis, OMIM 105400; Joubert syndrome, OMIM 213300; Nathalie syndrome, OMIM 255990; Madras motor neuron disease, ORPHA 137867; MADD, OMIM 231680), riboflavin metabolism, the flavoproteome and/or mitochondrial metabolism.

High Dose Riboflavin Therapy

Identification of causative *SLC52A* mutations in these debilitating disorders has not only advanced their genetic diagnoses but also highlighted high dose oral riboflavin supplementation as an effective therapeutic intervention. Excess riboflavin is excreted in the urine and toxicity has not been reported, making riboflavin therapy a safe intervention. Over 70 % of patients demonstrate improvements in muscle strength, motor abilities, respiratory function and/or cranial nerve deficits, with some patients no longer requiring ventilatory support. No deaths have been reported in riboflavin treated patients, whilst over half of untreated patients reported have died (Jaeger and Bosch 2016).

Effective doses which have been used vary between 10-80 mg/kg/day, whilst doses below 10 mg/kg/day are reported to be ineffective (personal communications). Doses as high as 80 mg/kg body weight per day (Chaya et al. 2017; Forman et al. 2018) have been tolerated with

minimal side effects, although gastrointestinal side effects are rarely noted (Bosch et al. 2011; Foley et al. 2014; Woodcock et al. 2017; Nimmo et al. 2018).

Responses to high dose riboflavin are similarly observed in the majority of RTD2 and RTD3 cases (i.e. genotype is not predictive of treatment response). Clinical improvement following riboflavin treatment is observed for the majority of RTD patients (19/30 RTD2, 20/23 RTD3) with the remaining patients showing stabilisation of the current disease state (10/30 RTD2, 1/23). In only two RTD3 patients has no beneficial response to riboflavin supplementation been reported. In one of these cases treatment was not started until 29 years after disease onset (Davis et al. 2016), at which point irreversible neurodegenerative changes will have occurred. In the second non-responsive case, treatment was discontinued after 1 week (Koy et al. 2012) which might have preceded a latent response, as clinical improvement is frequently not observed for months following the beginning of treatment. For example patient 2 reported by (Nimmo et al. 2018) was started on 80 mg/kg/day riboflavin at 8 months of age but his ventilator dependency had not improved by 10 months of age and for this reason a tracheostomy was performed. However, with continued riboflavin treatment clinical improvement was observed, and by the age of 14 months he was able to maintain spontaneous respiration.

Generally the most positive responses are reported in patients that receive riboflavin supplementation shortly after disease onset (Foley et al. 2014). Of note, a newly born sibling of an RTD3 patient harbouring the same pathogenic mutations has been administered riboflavin since birth and remains asymptomatic after 1 year (Horoz et al. 2016), whilst patient 2 from the first report of RTD3 who was symptomatic and treated from 3 months of age (Bosch et al. 2011) is now still asymptomatic at 8 years of age.

For these reasons it is recommended that riboflavin is administered immediately upon suspected RTD in order to prevent irreversible neurological changes, and continued until an

alternate unrelated cause of disease has been identified. Esterified derivatives of riboflavin are less reliant on RFVTs for cellular absorption and might therefore represent a strategy for future RTD therapeutics with improved bioavailability (Manole et al. 2017).

RECENT INSIGHTS INTO RTD PATHOMECHANISMS

Whilst there has been great advancement in RTD diagnosis and treatment, much less progress has been made in determining the pathomechanisms that lead to cranial and peripheral nerve degeneration. Flavins are important to the function of cells throughout the whole body, yet neurons appear to be especially vulnerable to riboflavin depletion. Recent work has started to unravel possible downstream consequences of RFVT dysfunction that might lead to neurodegeneration *(Figure 1)*.

In a study by (Rizzo et al. 2017), human induced pluripotent stem cell (hIPSC) lines were established from a RTD2 and RTD3 patient and differentiated into motor neurons. RTD motor neurons displayed an increase in neurofilament heavy chain (NFH) expression and its aggregation in inclusions, something previously characterised as an early event leading to motor neuron degeneration in amyotrophic lateral sclerosis (ALS) (Chen et al. 2014). An associated reduction in axonal length was also observed, however in a more recent study by (Manole et al. 2017) no such cytoskeletal abnormalities were described in the motor axons of *Drosophila* with knockdown of the *Drosophila* homologue of *SLC52A* (*drift*).

The phenotypic overlap of RTD with primary mitochondrial diseases and important role of flavins in mitochondrial function, might point towards mitochondrial dysfunction as an important pathomechanism contributing to neurodegeneration in RTD. In accordance, mitochondria within neurons of *drift* knockdown *Drosophila* are structurally abnormal, show reduced activity of OXPHOS complexes I and II and more depolarised mitochondrial membrane potential (Manole et al. 2017). Such abnormalities are also seen in RTD2

fibroblasts (Manole et al. 2017) and RTD muscle biopsies (Foley et al. 2014; Chaya et al. 2017; Nimmo et al. 2018). OXPHOS activity is normal in hIPSC-derived RTD motor neurons, but impairments in mitochondrial fusion and autophagy (mitophagy) were seen (Rizzo et al. 2017), both of which are important for maintaining a healthy mitochondrial network in post mitotic cells.

Neurons are among the most energy demanding cells of the body making them particularly sensitive to impairments in cellular metabolic processes. Increases in the release of mitochondrial derived reactive oxygen species (ROS) have also been implicated as pathomechanisms contributing to neuronal death. Mitochondrial dysfunction and concurrent impairments in their clearance might therefore be contributing to the specific vulnerability of neurons in RTD patients, and represent an additional pathomechanism that is shared with many other neurodegenerative conditions including primary mitochondrial diseases and ALS (Golpich et al. 2017).

CONCLUSION

The RTDs are an excellent example of how the genetic diagnosis of an inborn error of metabolism can translate an effective rational based therapy back in to the clinic. Although clinical improvements upon riboflavin supplementation are observed in many patients, some cases only show a stabilisation of the current disease state indicating quick intervention with riboflavin supplementation is important to avoid irreversible damage from occurring. Therefore, start of oral riboflavin supplementation upon suspicion of RTD diagnosis without awaiting test results is of utmost importance and lifesaving. Positive clinical responses to riboflavin supplementation might occur with some latency and for this reason riboflavin therapy should be continued in all suspected or genetically diagnosed RTD cases, even if no apparent clinical improvement has initially occurred. In the foreseeable future newborn

screening of *SLC52A1-3* might ensure riboflavin therapy is administered prior to the presentation of symptoms. Whilst biochemical screening parameters might in some instances be suggestive of RTD, diagnosis can only be made by genetic analysis. Genetic analysis of *SLC52A1-3* should therefore be the basis for such newborn screening tests. Understanding the pathomechanisms contributing to irreversible neuronal damage caused by riboflavin depletion might reveal additional targets for novel therapeutic intervention in patients which receive a delayed diagnosis.

REFERENCES

Allison T, Roncero I, Forsyth R, et al (2017) Brown-Vialetto-Van Laere Syndrome as a Mimic of Neuroimmune Disorders: 3 Cases from the Clinic and Review of the Literature. J Child Neurol 32:528–532. doi: 10.1177/0883073816689517

Anand G, Hasan N, Jayapal S, et al (2012) Early use of high-dose riboflavin in a case of Brown-Vialetto-Van Laere syndrome. Dev Med Child Neurol 54:187–9. doi: 10.1111/j.1469-8749.2011.04142.x

Babanejad M, Adeli OA, Nikzat N, et al (2018) SLC52A2 mutations cause SCABD2 phenotype: A second report. Int J Pediatr Otorhinolaryngol 104:195–199. doi: 10.1016/J.IJPORL.2017.11.014

Balasubramaniam, S. , Christodoulou, J. and Rahman, S. Disorders of Riboflavin Metabolism. J Inherit Metab Dis.. Accepted Author Manuscript. 2019. doi:10.0.3.234/jimd.12058

Bandettini di Poggio M, Gagliardi S, Pardini M, et al (2013) A novel compound heterozygous mutation of *C20orf54* gene associated with Brown-Vialetto-Van Laere syndrome in an Italian family. Eur J Neurol 20:e94–e95. doi: 10.1111/ene.12163

Bandettini Di Poggio M, Monti Bragadin M, Reni L, et al (2014) Brown-Vialetto-Van Laere syndrome: Clinical and neuroradiological findings of a genetically proven patient. Amyotroph Lateral Scler Front Degener 15:141–144. doi: 10.3109/21678421.2013.837931

Barile M, Giancaspero TA, Leone P, et al (2016) Riboflavin transport and metabolism in humans. J Inherit Metab Dis 39:545–557. doi: 10.1007/s10545-016-9950-0

Bashford JA, Chowdhury FA, Shaw CE (2017) Remarkable motor recovery after riboflavin therapy in adult-onset Brown-Vialetto-Van Laere syndrome. Pract Neurol 17:53–56. doi: 10.1136/practneurol-2016-001488

Bosch AM, Abeling NGGM, Ijlst L, et al (2011) Brown-Vialetto-Van Laere and Fazio Londe syndrome is associated with a riboflavin transporter defect mimicking mild MADD: a new inborn error of metabolism with potential treatment. J Inherit Metab Dis 34:159–64. doi: 10.1007/s10545-010-9242-z

Bosch AM, Stroek K, Abeling NG, et al (2012) The Brown-Vialetto-Van Laere and Fazio Londe syndrome revisited: natural history, genetics, treatment and future perspectives. Orphanet J Rare Dis 7:83. doi: 10.1186/1750-1172-7-83

Camargos S, Guerreiro R, Bras J, Mageste LS (2018) Late-onset and acute presentation of Brown-Vialetto-Van Laere syndrome in a Brazilian family. Neurol Genet 4:e215. doi: 10.1212/NXG.0000000000000215

Chaya S, Zampoli M, Gray D, et al (2017) The First Case of Riboflavin Transporter Deficiency in sub-Saharan Africa. Semin Pediatr Neurol. doi: 10.1016/J.SPEN.2017.03.002

Chen H, Qian K, Du Z, et al (2014) Modeling ALS with iPSCs Reveals that Mutant SOD1 Misregulates Neurofilament Balance in Motor Neurons. Cell Stem Cell 14:796–809. doi:

444 10.1016/J.STEM.2014.02.004

445 Ciccolella M, Catteruccia M, Benedetti S, et al (2012) Brown–Vialetto–van Laere and Fazio–
446 Londe overlap syndromes: A clinical, biochemical and genetic study. Neuromuscul
447 Disord 22:1075–1082. doi: 10.1016/J.NMD.2012.05.007

448 Ciccolella M, Corti S, Catteruccia M, et al (2013) Riboflavin transporter 3 involvement in
449 infantile Brown-Vialetto-Van Laere disease: two novel mutations. J Med Genet 50:104–
450 7. doi: 10.1136/jmedgenet-2012-101204

451 Çıralı C, Ergin H, Özdemir ÖM, et al (2017) P385 Hypotonic infant with riboflavin
452 transporter deficiency due to slc52a2 mutations. In: Posters. BMJ Publishing Group Ltd
453 and Royal College of Paediatrics and Child Health, p A181.3-A182

454 Colon-Moran W, Argaw T, Wilson CA (2017) Three cysteine residues of SLC52A1, a
455 receptor for the porcine endogenous retrovirus-A (PERV-A), play a critical role in cell
456 surface expression and infectivity. Virology 507:140–150. doi:
457 10.1016/J.VIROL.2017.04.019

458 Cosgrove J, Datta S, Busby M (2015) Adult onset Brown–Vialetto–Van Laere syndrome with
459 opsoclonus and a novel heterozygous mutation: A case report. Clin Neurol Neurosurg
460 128:1–3. doi: 10.1016/J.CLINEURO.2014.10.016

461 Davis A, Josifova D, Lloyd-Owen S, et al (2016) Brown-Vialetto-Van Laere syndrome: a 28-
462 year follow-up. J Neurol Neurosurg Psychiatry 87:681–2. doi: 10.1136/jnnp-2014-
463 310088

464 Dezfouli MA, Yadegari S, Nafissi S, Elahi E (2012) Four novel C20orf54 mutations
465 identified in Brown-Vialetto-Van Laere syndrome patients. J Hum Genet 57:613–7. doi:
466 10.1038/jhg.2012.70

467 Foley AR, Menezes MP, Pandraud A, et al (2014) Treatable childhood neuronopathy caused

by mutations in riboflavin transporter RFVT2. Brain 137:44–56. doi: 10.1093/brain/awt315

Forman EB, Foley AR, King MD (2018) Dramatic improvement of a rare syndrome with high dose riboflavin treatment. Pediatr Neurol. doi: 10.1016/J.PEDIATRNEUROL.2018.05.005

Golpich M, Amini E, Mohamed Z, et al (2017) Mitochondrial Dysfunction and Biogenesis in Neurodegenerative diseases: Pathogenesis and Treatment. CNS Neurosci Ther 23:5–22. doi: 10.1111/cns.12655

Gowda VK, Udhayabanu T, Varalakshmi P, et al (2018) Fazio-Londe syndrome in siblings from India with different phenotypes. Brain Dev. doi: 10.1016/J.BRAINDEV.2018.02.010

Green P, Wiseman M, Crow YJ, et al (2010) Brown-Vialetto-Van Laere syndrome, a ponto-bulbar palsy with deafness, is caused by mutations in c20orf54. Am J Hum Genet 86:485–9. doi: 10.1016/j.ajhg.2010.02.006

Guissart C, Drouot N, Oncel I, et al (2016) Genes for spinocerebellar ataxia with blindness and deafness (SCABD/SCAR3, MIM# 271250 and SCABD2). Eur J Hum Genet 24:1154–1159. doi: 10.1038/ejhg.2015.259

Haack TB, Makowski C, Yao Y, et al (2012) Impaired riboflavin transport due to missense mutations in SLC52A2 causes Brown-Vialetto-Van Laere syndrome. J Inherit Metab Dis 35:943–8. doi: 10.1007/s10545-012-9513-y

Hellebrekers DMEI, Sallevelt SCEH, Theunissen TEJ, et al (2017) Novel SLC25A32 mutation in a patient with a severe neuromuscular phenotype. Eur J Hum Genet 25:886–888. doi: 10.1038/ejhg.2017.62

Ho G, Yonezawa A, Masuda S, et al (2011) Maternal riboflavin deficiency, resulting in

transient neonatal-onset glutaric aciduria Type 2, is caused by a microdeletion in the riboflavin transporter gene GPR172B. Hum Mutat 32:E1976–E1984. doi: 10.1002/humu.21399

Horoz OO, Mungan NO, Yildizdas D, et al (2016) Brown-Vialetto-Van Laere syndrome: two siblings with a new mutation and dramatic therapeutic effect of high-dose riboflavin. J Pediatr Endocrinol Metab 29:227–231. doi: 10.1515/jpem-2015-0198

Horvath R (2012) Update on clinical aspects and treatment of selected vitamin-responsive disorders II (riboflavin and CoQ10). J Inherit Metab Dis 35:679–687. doi: 10.1007/s10545-011-9434-1

Hossain MA, Obaid A, Rifai M, et al (2017) Early onset of Fazio-Londe syndrome: the first case report from the Arabian Peninsula. Hum Genome Var 4:17018. doi: 10.1038/hgv.2017.18

Jaeger B, Bosch AM (2016) Clinical presentation and outcome of riboflavin transporter deficiency: mini review after five years of experience. J Inherit Metab Dis 39:559–564. doi: 10.1007/s10545-016-9924-2

Johnson JO, Gibbs JR, Megarbane A, et al (2012) Exome sequencing reveals riboflavin transporter mutations as a cause of motor neuron disease. Brain 135:2875–82. doi: 10.1093/brain/aws161

Johnson JO, Gibbs JR, Van Maldergem L, et al (2010) Exome sequencing in Brown-Vialetto-van Laere syndrome. Am J Hum Genet 87:567-9; author reply 569-70. doi: 10.1016/j.ajhg.2010.05.021

Khadilkar S V., Faldu HD, Udani V, et al (2017) Reversible posterior column dysfunction in Brown-Vialetto-Von Laere syndrome. Muscle Nerve 56:E28–E31. doi: 10.1002/mus.25694

516 Koy A, Pillekamp F, Hoehn T, et al (2012) Brown-Vialetto-Van Laere Syndrome: A
517 Riboflavin-Unresponsive Patient With a Novel Mutation in the C20orf54 Gene. Pediatr
518 Neurol 46:407–409. doi: 10.1016/J.PEDIATRNEUROL.2012.03.008

519 Kurkina MV, Baydakova GV, Kokh EE, et al (2017) Case report of patients with Fazio-
520 Londe syndrome. Eur J Paediatr Neurol 21:e131–e132. doi:
521 10.1016/j.ejpn.2017.04.1025

522 Lienhart W-D, Gudipati V, Macheroux P (2013) The human flavoproteome. Arch Biochem
523 Biophys 535:150–162. doi: 10.1016/j.abb.2013.02.015

524 Manole A, Houlden H (2015) Riboflavin Transporter Deficiency Neuronopathy. University
525 of Washington, Seattle

526 Manole A, Jaunmuktane Z, Hargreaves I, et al (2017) Clinical, pathological and functional
527 characterization of riboflavin-responsive neuropathy. Brain 140:2820–2837. doi:
528 10.1093/brain/awx231

529 Mattiuzzo G, Matouskova M, Takeuchi Y (2007) Differential resistance to cell entry by
530 porcine endogenous retrovirus subgroup A in rodent species. Retrovirology 4:93. doi:
531 10.1186/1742-4690-4-93

532 Menezes MP, Farrar MA, Webster R, et al (2016a) Pathophysiology of motor dysfunction in
533 a childhood motor neuron disease caused by mutations in the riboflavin transporter. Clin
534 Neurophysiol 127:911–918. doi: 10.1016/j.clinph.2015.05.012

535 Menezes MP, O'Brien K, Hill M, et al (2016b) Auditory neuropathy in Brown-Vialetto-Van
536 Laere syndrome due to riboflavin transporter RFVT2 deficiency. Dev Med Child Neurol
537 58:848–854. doi: 10.1111/dmcn.13084

538 Mosegaard S, Bruun GH, Flyvbjerg KF, et al (2017) An intronic variation in SLC52A1
539 causes exon skipping and transient riboflavin-responsive multiple acyl-CoA

dehydrogenation deficiency. Mol Genet Metab 122:182–188. doi: 10.1016/J.YMGME.2017.10.014

Nabokina SM, Subramanian VS, Said HM (2012) Effect of clinical mutations on functionality of the human riboflavin transporter-2 (hRFT-2). Mol Genet Metab 105:652–657. doi: 10.1016/J.YMGME.2011.12.021

Nimmo GAM, Ejaz R, Cordeiro D, et al (2018) Riboflavin transporter deficiency mimicking mitochondrial myopathy caused by complex II deficiency. Am J Med Genet Part A 176:399–403. doi: 10.1002/ajmg.a.38530

Petrovski S, Shashi V, Petrou S, et al (2015) Exome sequencing results in successful riboflavin treatment of a rapidly progressive neurological condition. Cold Spring Harb Mol case Stud 1:a000257. doi: 10.1101/mcs.a000257

Rizzo F, Ramirez A, Compagnucci C, et al (2017) Genome-wide RNA-seq of iPSC-derived motor neurons indicates selective cytoskeletal perturbation in Brown-Vialetto disease that is partially rescued by riboflavin. Sci Rep 7:1–13. doi: 10.1038/srep46271

Schiff M, Veauville-Merllié A, Su CH, et al (2016) *SLC25A32* Mutations and Riboflavin-Responsive Exercise Intolerance. N Engl J Med 374:795–797. doi: 10.1056/NEJMc1513610

Set KK, Weber ARB, Serajee FJ, Huq AM (2018) Clinical Reasoning: Siblings with progressive weakness, hypotonia, nystagmus, and hearing loss. Neurology 90:e625–e631. doi: 10.1212/WNL.0000000000004973

Spagnoli C, Pitt MC, Rahman S, De Sousa C (2014) Brown-Vialetto-van Laere syndrome: A riboflavin responsive neuronopathy of infancy with singular features. Eur J Paediatr Neurol 18:231–234. doi: 10.1016/j.ejpn.2013.09.006

Srour M, Putorti ML, Schwartzentruber J, et al (2014) Mutations in riboflavin transporter

present with severe sensory loss and deafness in childhood. Muscle Nerve 50:775–779. doi: 10.1002/mus.24224

Subramanian VS, Kapadia R, Ghosal A, Said HM (2015) Identification of residues/sequences in the human riboflavin transporter-2 that is important for function and cell biology. Nutr Metab (Lond) 12:13. doi: 10.1186/s12986-015-0008-3

Thulasi V, Veerapandiyan A, Pletcher BA, et al (2017) A Case of Brown-Vialetto-Van Laere Syndrome Due To a Novel Mutation in SLC52A3 Gene: Clinical Course and Response to Riboflavin. Child Neurol open 4:2329048X17725610. doi: 10.1177/2329048X17725610

Udhayabanu T, Subramanian VS, Teafatiller T, et al (2016) SLC52A2 [p.P141T] and SLC52A3 [p.N21S] causing Brown-Vialetto-Van Laere Syndrome in an Indian patient: First genetically proven case with mutations in two riboflavin transporters. Clin Chim Acta 462:210–214. doi: 10.1016/J.CCA.2016.09.022

van der Kooi A, Jaeger B, van Spaendonck K, Bosch A (2016) Riboflavin transporter deficiency diagnosed 30 years after onset of symptoms. Neuromuscul Disord 26:S201. doi: 10.1016/j.nmd.2016.06.416

Woodcock IR, Menezes MP, Coleman L, et al (2017) Genetic, Radiologic, and Clinical Variability in Brown-Vialetto-van Laere Syndrome. Semin Pediatr Neurol. doi: 10.1016/J.SPEN.2017.03.001

Yamamoto S, Inoue K, Ohta K -y., et al (2009) Identification and Functional Characterization of Rat Riboflavin Transporter 2. J Biochem 145:437–443. doi: 10.1093/jb/mvn181

Yao Y, Yonezawa A, Yoshimatsu H, et al (2010) Identification and Comparative Functional Characterization of a New Human Riboflavin Transporter hRFT3 Expressed in the Brain. J Nutr 140:1220–1226. doi: 10.3945/jn.110.122911

588 Yonezawa A, Inui K (2013) Novel riboflavin transporter family RFVT/SLC52: Identification,
589 nomenclature, functional characterization and genetic diseases of RFVT/SLC52. Mol
590 Aspects Med 34:693–701. doi: 10.1016/J.MAM.2012.07.014

591 Yonezawa A, Masuda S, Katsura T, Inui K (2008) Identification and functional
592 characterization of a novel human and rat riboflavin transporter, RFT1. Am J Physiol
593 Physiol 295:C632–C641. doi: 10.1152/ajpcell.00019.2008

594

595

596

597

598

599

600

601

602

603

604

605

606

607

608

609

610

611 **Table 1: Clinical Features of RTD2 and RTD3 patients described in published**
612 **literature. *Numbers in brackets represent number of patients showing symptom at**
613 **disease onset (see text for details).**

	RTD2 (n=52)	**RTD3 (n=56)**	**RTD2/3 (n=1)**	**Total RTD (n=109)**
Age of Onset	Mean 2.9yr SD 2.3yr Range 0-10yr	Mean 7.8yr SD 8.6yr Range 0.2-35yr	9yr	Mean 5.3yr SD 6.6yr Range 0-35yr
Gender	Males 22/52 *42 %* Females 30/52 *58 %*	Males 24/56 *43 %* Females 30/56 *54 %*	Males 1/1 *100 %* Females 0/1 *0 %*	Males 47/109 *43 %* Females 60/109 *55 %*
Bulbar Symptoms	26/52 (*1) *50 %*	34/56 (*15) *61 %*	1/1 (*0) *100 %*	61/109 (*16) *56%*
Optic Atrophy	37/52 (*7) *71 %*	13/56 (*2) *23 %*	0/1 (*0) *0 %*	50/109 (*9) *46 %*
Hearing Loss	47/52 (*21) *90 %*	47/56 (*20) *84 %*	1/1 (*0) *100 %*	95/109 (*41) *87 %*
Muscle Weakness /Hypotonia	43/52 (*8) *83 %*	47/56 (*12) *84 %*	1/1 (*0) *100 %*	91/109 (*20) *83 %*
Facial Weakness	3/52 (*0) *6 %*	26/56 (*7) *46 %*	1/1 (*0) *100 %*	30/109 (*7) *28 %*
Gait Abnormality / Ataxia	32/52 (*22) *62 %*	7/56 (*1) *13 %*	0/1 (*0) *0 %*	39/109 (*23) *36 %*
Nystagmus	12/52 (*6) *23 %*	4/56 (*2) *7 %*	1/1 (*0) *100 %*	17/109 (*8) *16 %*
Feeding Difficulties	13/52 (*0) *25 %*	28/56 (*7) *50 %*	0/1 (*0) *0 %*	41/109 (*7) *38 %*
Respiratory Symptoms	26/52 (*5) *50 %*	41/56 (*12) *73 %*	1/1 (*1) *100 %*	68/109 (*17) *62 %*
Peripheral Neuropathy (EMG/NCS)	41/42 *98 %*	29/37 *78 %*	Not Performed	70/79 *89 %*
Abnormal Cranial MRI	5/29 *17 %*	5/21 *24 %*	0/1 *0 %*	10/51 *20 %*
Abnormal Spinal MRI	1/4 *25 %*	5/8 *63 %*	Not Performed	6/12 *50 %*
Plasma Acylcarnitine Abnormalities	20/30 *67 %*	9/16 *56 %*	Not Performed	29/46 *63 %*
Plasma Flavin Abnormalities	2/17 *12 %*	3/7 *43 %*	1/1 *100 %*	6/25 *24 %*
Urine Organic Acid Abnormalities	4/10 *40 %*	8/13 *62 %*	Not Performed	12/23 *52 %*

Patients Administered Riboflavin Therapy	30/52 *58 %*	23/56 *41 %*	1/1 *100 %*	54/109 *50 %*

EMG: Electromyography, NCS: Nerve Conduction Study

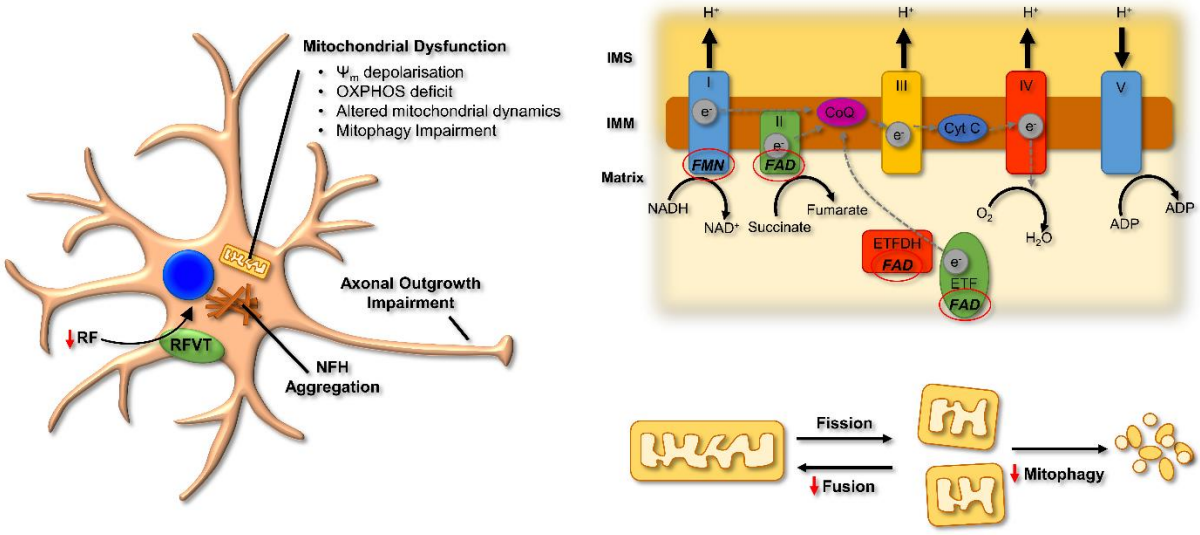

Figure 1: Cellular Pathomechanisms of Riboflavin Transporter Deficiency

RFVT dysfunction alters a number of cellular processes which have been implicated in the specific vulnerability of neural cells in other neurodegenerative conditions. Of particular note are deficits in mitochondrial oxidative phosphorylation caused by a reduced availability of necessary flavin cofactors (red circles), and impairments in the dynamic pathways responsible for maintaining a healthy mitochondrial network. RF, riboflavin; RFVT, riboflavin transporter; NFH, neurofilament heavy chain; Ψ_m, mitochondrial membrane potential; IMS, intermembrane space; IMM, inner mitochondrial membrane; CoQ, coenzyme Q10; Cyt C, cytochrome C; ETF, electron transferring flavoprotein; ETFDH, electron transferring flavoprotein dehydrogenase.

630

Table S1: Pathogenic SLC52A1 Variants (Genbank NM_071986.3)

DNA Nucleotide Changed	Mutation Type	Intron/Exon	Mutation Site	Publications	Functional Studies / Other Details
Microdeletion spanning Ex2-Ex3				Ho et al., 2011	Heterozygous deletion identified in the mother of a child that presented with riboflavin deficiency as a new-born.
c.1134+11G> A	Splicing loss >> Ex4 skipping			Mosegaard et al., 2017	Heterozygous mutation identified in a mother and new-born child with transient riboflavin deficiency. Mutation introduces binding site for splice inhibiting hnRNPA1 and skipping of Ex4.

Table S2: Pathogenic SLC52A2 Variants (Genbank NM_024531.4)

DNA Nucleotide Changed	Mutation Type	Intron/Exon	Mutation Site	Publications	Functional Studies / Other Details
c.-110–1G> A	5' Ex2 Splice Site	In1-2		Çıralı et al. 2017	Not Performed
c.92G>C	p.Trp31Ser	Ex2	TM1	Foley et al. 2014	Riboflavin uptake impaired but cell surface expression maintained.
c.155C>T	p.Ser52Phe	Ex3	TM2	Ciccolella et al., 2013	Reduced SLC52A2 mRNA expression shown in heterozygous carriers fibroblasts.
c.231G>A	p.Glu77Lys	Ex3	Int. TM2-TM3	Manole et al., 2017	Not Performed

c.297G>C	p.Trp99Cys	Ex3	TM3	Çıralı et al. 2017	Not Performed
c.368T>C	p.Leu123Pro	Ex3	TM4	Haak et al., 2012; Subramanian et al., 2015	Impaired riboflavin uptake and reduction in total protein. Reduction in cell surface expression with majority retained intracellularly colocalised with ER markers.
c.383C>T	p.Ser128Leu	Ex3	TM4	Manole et al., 2017	Not Performed
c.401C>T	p.Pro134Leu	Ex3	TM4	Guissart et al., 2016	
c.421C>A	p.Pro141Thr	Ex3	Int. TM4-TM5	Udhayabanu et al., 2016	Patient homozygous for SLC52A2 variant but also harboured homozygous SLC52A3 c.62A>G (p.N21S). Riboflavin uptake impaired but cell surface expression was maintained.
c.505C>T	p.Arg169Cys	Ex3	TM5	Allison et. al., 2017; Woodcock et al., 2017	Not Performed
c.700C>T	p.Gln234*	Ex3	Int. TM6-TM7	Foley et al. 2014	Impaired riboflavin uptake and absent cell surface expression.
c.808C>T	p.Gln270*	Ex3	Int. TM6-TM7	Petrovski et al., 2015	Absent cell surface expression.
c.851C>A	p.Ala284Asp	Ex3	TM7	Foley et al. 2014	Impaired riboflavin uptake and absent cell surface expression.
c.865C>T	p.Ala288Val	Ex3	TM7	Manole et al., 2017	
c.914A>G	p.Tyr305Cys	Ex3	Ext. TM7-TM8	Foley et al. 2014	Impaired riboflavin uptake and almost absent cell surface expression.
c.916G>A	p.Gly306Arg	Ex3	Ext. TM7-	Johnson et al., 2012; Foley	Not Performed

			TM8	et al. 2014; Srour et al., 2014; Menezes et al., 2016a; Menezes et al., 2016b	
c.917G>A	p.Gly306Glu	Ex3	Ext. TM7-TM8	Nimmo et al., 2018	Not Performed
c.935T>C	p.Leu312Pro	Ex3	TM8	Foley et al. 2014; Allison et al., 2017; Manole et al., 2017	Impaired riboflavin uptake and reduced cell surface expression.
c.973T>G	p.Cys325Gly	Ex3	TM8	Babanejad et al., 2018	Not Performed
c.1016T>C	p.Leu339Pro	Ex4	TM9	Haak et al., 2012; Foley et al., 2014; Subramanian et al., 2015; Menezes et al., 2016a; Menezes et al., 2016b; Manole et al., 2017	Impaired riboflavin uptake and absent cell surface expression. Retained intracellularly colocalised with ER markers.
c.1088C>T	p.Pro363Leu	Ex4	Ext. TM9-TM10	Manole et al., 2017	Not Performed
c.1255G>A	p.Gly419Ser	Ex5	TM11	Ciccolella et al., 2013	Not Performed
c.1258G>A	p.Ala429Thr	Ex5	Ext. C-term	Foley et al., 2014	Not Performed
c.1327T>C	p.Cys443Arg	Ex5	Ext. C-term	Manole et al., 2017; Set et al., 2018	Not Performed

Table S3: Pathogenic SLC52A3 Variants (Genbank NM_033409.3)

DNA Nucleotide Changed	Mutation Type	Intron/Exon	Mutation Site	Publications	Functional Studies / Other Details
c.44G>T	p.Gly15Val	Ex2	TM1	Horoz et al., 2015	Not Performed
c.49T>C	p.Trp17Arg	Ex2	TM1	Bosch et al., 2011; Nabokina et al., 2012	Riboflavin uptake impaired but cell surface expression unaffected.
c.62A>G	p.Asn21Ser	Ex2	TM1	Dezfouli et al., 2012; Udhayabanu et al., 2016; Gowda et al., 2018	Riboflavin uptake impaired and protein retained intracellularly colocalised with ER markers.
c.71G>A	p.Trp24*	Ex2	TM1	Hossain et al., 2017	Not Performed
c.82C>A	p.Pro28Thr	Ex2	Ext. TM1-TM2	Johnson et al., 2010; Nabokina et al., 2012	Riboflavin uptake impaired and

					protein retained intracellularly.
c.106G>A	p.Glu36Lys	Ex2	Ext. TM1-TM2	Green et al., 2010; Nabokina et al., 2012; Manole et al., 2017; Allison et al., 2017	Riboflavin uptake impaired and protein retained intracellularly colocalised with ER markers.
c.160G>A	p.Gly54Arg	Ex2	TM2	Johnson et al., 2012	Not Performed
c.173T>A	p.Val58Asp	Ex2	TM2	Ciccolella et al., 2012	Not Performed
c.193C>T	p.Arg65Trp	Ex2	Int. TM2-TM3	Davis et al., 2016	Not Performed
c.211G>A	p.Glu71Lys	Ex2	Int. TM2-TM3	Johnson et al., 2010; Nabokina et al., 2012	Riboflavin uptake impaired and protein retained intracellularly.

c.211G>T	p.Glu71*	Ex2	Int. TM2-TM3	Green et al., 2010	Not Performed
c.224T>C	p.Ile75Thr	Ex2	TM3	Johnson et al., 2012	Not Performed
c.354G>A	p.Val118Met	Ex2	TM4	Manole et al., 2017	Not Performed
c.374C>A	p.Thr125Asn	Ex2	TM4	Chaya et al., 2017; Manole et al., 2017	Not Performed
c.383C>T	p.Pro128Leu	Ex2	TM4	Cosgrove et al., 2015	Not Performed
c.394C>T	p.Arg132Trp	Ex2	Int. TM4-TM5	Green et al., 2010; Nabokina et al., 2012	Riboflavin uptake impaired and protein retained intracellularly.
c.403A>G	p.Thr135Ala	Ex2	TM5	Manole et al., 2017	Not Performed
c.497G>C	p.Cys166Ser	Ex2	Ext. TM5-TM6	Kurkina et al., 2017	Not Performed
c.634C>T	p.Arg212Cys	Ex3	Ext. TM5-TM6	Manole et al., 2017	Not Performed
c.639C>G	p.Tyr213*	Ex3	Ext. TM5-	Green et al., 2010;	Not

			TM6		Performed
c.659C>A	p.Pro220His	Ex3	TM6	Dezfouli et al., 2012	Not Performed
c.670T>C	p.Phe224Leu	Ex3	TM6	Green et al., 2010	Not Performed
c.935C>T	p.Ala312Val	Ex3	TM7	Dezfouli et al., 2012; Khadilkar et al., 2017	Not Performed
c.955C>T	p.Pro319Ser	Ex3	Ext. TM7-TM8	Ciccolella et al., 2012	Not Performed
c.989G>T	p.Gly330Val	Ex3	Ext. TM7-TM8	Koy et al., 2012	Not Performed
c.1048T>A	p.Leu350Met	Ex3	TM8	Green et al., 2010; Nabokina et al., 2012	Riboflavin uptake unaffected.
c.1074G>A	5' Ex4 Splice Site	Ex4		Manole et al., 2017	Not Performed
c.1081C>G	p.L361V	Ex4	Int. TM8-TM9	Bandettini Di Poggio et al., 2013; Bandettini Di Poggio et al., 2014	Present on same allele as c.1127A>G (p.Tyr376Cys) variant.
c.1124G>A	p.Gly375Asp	Ex4	TM9	Dezfouli et al., 2012	Not Performed

c.1127A>G	p.Tyr376Cys	Ex4	TM9	Bandettini Di Poggio et al., 2013; Bandettini Di Poggio et al., 2014	Present on same allele as c.1081C>G (p.L361V) variant.
c.1128C>G	p.Tyr376*	Ex4	Int. TM6-TM7	Van der Kooi et al., 2016	Not Performed
c.1128-1129_insT	p.Tyr376Leufs*129	Ex4	Int. TM6-TM7	Manole et al., 2017	Not Performed
c.1156T>C	p.Cys386Arg	Ex4	Ext. TM9-TM10	Thulasi et al., 2017	Not Performed
c.1198-2A>C	5' Ex5 Splice Site	In4-5		Bosch et al., 2011	Not Performed
c.1203insT	p.Ser402Phefs*103	Ex5	TM10	Bandettini Di Poggio et al., 2013; Bandettini Di Poggio et al., 2014	Not Performed
c.1222G>C	p.Gly408Arg	Ex5	TM10	Kurkina et al., 2017	Not Performed
c.1223G>A	p.Gly408Asp	Ex5	TM10	Nimmo et al., 2018	Not Performed
c.1232_1233insCTAC GCTTCCCTCCCGGCC CCGCAGGTGGCCTCGTG	p.Ser411_Tyr412insTyrAla SerLeuProAlaProGlnValAla SerTrpValLeuPheSerGlyCy	Ex5	TM10	Camargos et al., 2018	Not Performed

GGTGCTTTTCAGCGGCTGCCTCAG	s LeuSer				
c.1237T>C	p.Val413Ala	Ex5	TM10	Green et al., 2010; Bashford et al., 2017; Manole et al., 2017	Not Performed
c.1238T>C	p.Val413Ala	Ex5	TM10	Ciccolella et al., 2012; Davis et al., 2016	Not Performed
c.1292G>A	p.Trp431*	Ex5	TM11	Cosgrove et al., 2015	Not Performed
c.1294G>A	p.Trp431*	Ex5	TM11	Manole et al., 2017	Not Performed
c.1296C>A	p.Cys432*	Ex5	TM11	Ciccolella et al., 2012	Not Performed
c.1316G>A	p.Gly439Asp	Ex5	TM11	Woodcock et al., 2017	Not Performed
c.1325_1326delTG	p.Leu442Argfs*35	Ex5	TM11	Green et al., 2010	Not Performed
c.1371C>G	p.Phe457Leu	Ex5	Ext. C-term	Green et al., 2010	Not Performed
c.1381G>T	p.Asp461Tyr	Ex5	Ext. C-term	Bashford et al., 2017	Not Performed