Disorders of flavin adenine dinucleotide metabolism: MADD and related deficiencies

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Abstract

Multiple acyl-coenzyme A dehydrogenase deficiency (MADD), or glutaric aciduria type II (GAII), is a clinically heterogeneous disorder caused by mutations in electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO) – the two enzymes responsible for the re-oxidation of enzyme-bound flavin adenine dinucleotide ($FADH_2$) via electron transfer to the respiratory chain at the level of coenzyme Q10. Over the past decade, an increasing body of evidence has further coupled mutations in FAD metabolism (including intercellular riboflavin transport, FAD biosynthesis and FAD transport) to MADD-like phenotypes. In this review we provide a detailed description of the overarching and specific metabolic pathways involved in MADD. We examine the eight associated genes (*ETFA, ETFB, ETFDH, FLAD1, SLC25A32* and *SLC52A1-3*) and clinical phenotypes, and report ~423 causative mutations following a systematic literature review. Finally, we focus attention on the value and shortcomings of current diagnostic approaches, as well as current and future therapeutic options for MADD and its phenotypic disorders.

Keywords: Multiple acyl-CoA dehydrogenase deficiency, glutaric aciduria type II, ETF, ETFDH, FAD, riboflavin homeostasis

FAD metabolism and MADD facts:

- Flavoproteins are intricately involved in the mitochondrial metabolism and require oxidised FAD as cofactor.
- The re-oxidation of $FADH_2$ is mediated by the ETF-ETFQO system, while the synthesis and transport of FAD depends on the combined action of RFVT1-3, FADS and MFT.
- Genetic defects in *ETFA*, *ETFB* and *ETFDH* lead to a potentially fatal mitochondrial myopathy called Multiple acyl-CoA dehydrogenase deficiency (MADD).
- Disorders associated with mutations in *FLAD1, SLC25A32* and *SLC52A1-3* also frequently present with MADD-like phenotypes and -biochemistry.
- The effective diagnosis of MADD, resulting from >430 mutations, is challenging and involves a combination of biochemical investigations, enzyme/flux assays and next generation sequencing.
- Apart from MADD Types I and II and severe FADS dysfunction, the majority of MADD(-like) phenotypes are very amendable to treatment by riboflavin.

1. Introduction

Acyl-coenzyme A (CoA) dehydrogenases (ACDHs), a family of homologous mitochondrial flavoproteins that contain flavin adenine dinucleotide (FAD) as a non-covalently-bound prosthetic group, are key players in mitochondrial metabolism (Battaile et al., 2002; Henriques et al., 2010). Of the 11 ACDHs currently identified, a total of nine partake in: (i) mitochondrial fatty acid β-oxidation (FAO), (ii) branched chain amino acid catabolism (BCAA) and (iii) amino acid catabolism (Ghisla and Thorpe, 2004; Zhang et al., 2019). These include (i) acyl-CoA dehydrogenase 9 (ACAD9), short-, medium-, long-, and very-long-chain acyl-CoA dehydrogenase (SCAD, MCAD, LCAD and VLCAD), (ii) isovaleryl-CoA-, isobutyryl-CoA-, and short branched-chain acyl-CoA dehydrogenase (IVD, IBD and SBCAD) and (iii) glutaryl-CoA dehydrogenase (GCDH). To enable the sustained operation of these enzymes, their reduced FAD groups require continuous re-oxidation by the concerted action of electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO) (Ghisla and Thorpe, 2004). In addition, three other mitochondrial dehydrogenases rely on ETF and ETFQO as redox partners, including 2-hydroxyglutarate- (2HGDH), dimethylglycine- (DMGDH; involved in amino acid metabolism), and sarcosine dehydrogenase (SDH; involved in choline metabolism) (Toplak et al., 2019; Zhang et al., 2019).

The importance of the ETF-ETFQO system is exemplified by the existence of a variety of different inborn errors of metabolism, collectively called multiple acyl-CoA dehydrogenase deficiency (MADD) (Goodman and Frerman, 1984; Grünert, 2014). MADD, also known as glutaric aciduria type II (GAII), may be classified as a FAO disorder or lipid storage myopathy and is historically caused by mutations in the genes coding for ETF and ETFQO (Vasiljevski et al., 2018; Wanders et al., 2010). More recently, mutations in the genes coding for enzymes involved in the metabolism of riboflavin - the precursor of FAD - have also been shown to present as MADD-like phenotypes (Bosch et al., 2012; Green et al., 2010; Haack et al., 2012; Ho et al., 2011; Johnson et al., 2012, 2010; Olsen et al., 2016; Schiff et al., 2016; Taylor et al., 2014). In this review, we present the current state of knowledge on the metabolic pathways which are affected in MADD and the genes involved in the disorders of FAD metabolism from the perspective of both the ETF-ETFQO system as well as riboflavin transport and metabolism. We provide an overview of the phenotypes and diagnostic procedures used to identify these disorders and discuss available and future therapeutic avenues.

2. Pathways

In its fully oxidized state, FAD functions by accepting hydrogen ions and electrons from the substrates of mitochondrial flavoproteins. In order to maintain mitochondrial flavoproteome homeostasis, the resulting $FADH_2$ needs to be re-oxidized by the action of ETF, which is a heterodimer comprised of a 30 kDa ETFα and 28 kDa ETFβ subunit located in the mitochondrial matrix (Frerman and Goodman, 2001; Ruzicka and Beinert, 1975). This protein in turn, is re-oxidized by ETFQO, a 64 kDa monomer embedded in the inner mitochondrial membrane, which feeds the transferred electrons into the respiratory chain at the level of coenzyme Q10 (CoQ10) (Frerman, 1987). Together, ETF and ETFQO couple various metabolic pathways to the oxidative phosphorylation (OXPHOS) system for the production of adenosine triphosphate (ATP) (Fig. 1).

In humans, FAD and its precursor flavin mononucleotide (FMN) are produced from riboflavin, a water-soluble vitamin (B2) which itself cannot be synthesized in humans, but instead is obtained from dietary sources and in part from intestinal microflora (Fig. 1) (Barile et al., 2016). From the small (and less so, the large) intestine, free riboflavin is transported into the enterocyte via the action of riboflavin transporter 3 (RFVT3), and released into the blood by RFVT1 and 2. From here, riboflavin (bound to albumin/immunoglobulins) can be transferred to various tissues and transported into cells by RFVT1-3, which are expressed in a tissue-specific manner (see Barile et al., 2016 for review). Once inside the cytoplasm, riboflavin is phosphorylated to FMN by riboflavin kinase (RFK), and subsequently adenylated to FAD by

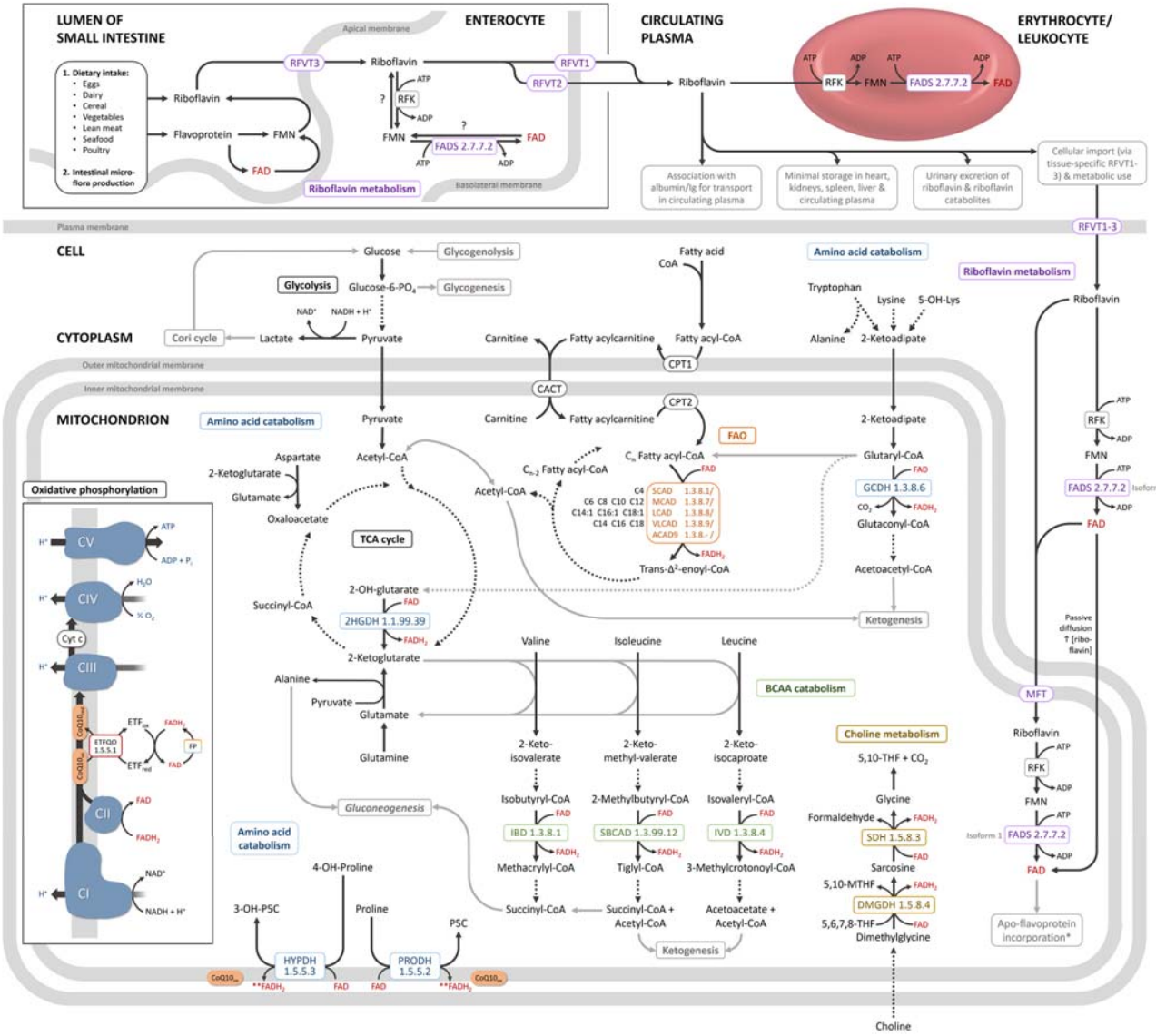
LUMEN OF SMALL INTESTINE
ENTEROCYTE
Apical membrane
1. Dietary intake:
• Eggs
• Dairy
• Cereal
• Vegetables
• Lean meat
• Seafood
• Poultry
2. Intestinal micro-flora production
Riboflavin
Flavoprotein
FMN
FAD
RFVT3
RFVT1
RFVT2
RFK
FADS 2.7.7.2
Riboflavin metabolism
Basolateral membrane
CIRCULATING PLASMA
ERYTHROCYTE/ LEUKOCYTE
Association with albumin/Ig for transport in circulating plasma
Minimal storage in heart, kidneys, spleen, liver & circulating plasma
Urinary excretion of riboflavin & riboflavin catabolites
Cellular import (via tissue-specific RFVT1-3) & metabolic use
Plasma membrane
CELL
CYTOPLASM
Glucose
Glycogenolysis
Glycolysis
Glucose-6-PO4
Glycogenesis
Cori cycle
Lactate
Pyruvate
Fatty acid
CoA
Fatty acyl-CoA
Carnitine
Fatty acylcarnitine
CPT1
CACT
CPT2
Amino acid catabolism
Tryptophan
Lysine
5-OH-Lys
Alanine
2-Ketoadipate
Outer mitochondrial membrane
Inner mitochondrial membrane
MITOCHONDRION
Acetyl-CoA
Aspartate
2-Ketoglutarate
Glutamate
Oxaloacetate
TCA cycle
FAO
Glutaryl-CoA
GCDH 1.3.8.6
Glutaconyl-CoA
Acetoacetyl-CoA
Ketogenesis
Trans-Δ²-enoyl-CoA
2-OH-glutarate
2HGDH 1.1.99.39
Succinyl-CoA
Oxidative phosphorylation
CV
CIV
CIII
CII
CI
Cyt c
ETFQO 1.5.5.1
FP
Valine
Isoleucine
Leucine
BCAA catabolism
Alanine
Pyruvate
Glutamate
Glutamine
2-Keto-isovalerate
2-Keto-methyl-valerate
2-Keto-isocaproate
Isobutyryl-CoA
2-Methylbutyryl-CoA
Isovaleryl-CoA
IBD 1.3.8.1
SBCAD 1.3.99.12
IVD 1.3.8.4
Methacrylyl-CoA
Tiglyl-CoA
3-Methylcrotonoyl-CoA
Succinyl-CoA
Succinyl-CoA + Acetyl-CoA
Acetoacetate + Acetyl-CoA
Gluconeogenesis
Amino acid catabolism
4-OH-Proline
Proline
3-OH-PSC
P5C
HYPDH 1.5.5.3
PRODH 1.5.5.2
Choline metabolism
5,10-THF + CO2
Glycine
Formaldehyde
SDH 1.5.8.3
Sarcosine
5,10-MTHF
DMGDH 1.5.8.4
5,6,7,8-THF
Dimethylglycine
Choline
Riboflavin metabolism
Passive diffusion ↑ [ribo-flavin]
MFT
Isoform 1
Isoform 2
Apo-flavoprotein incorporation*

Fig. 1. Riboflavin metabolism and flavoproteins affected in MADD(-like disorders) In the small intestine, flavoproteins are denatured, thereby releasing FMN and FAD which can be converted into riboflavin via non-specific hydrolases. Riboflavin is then absorbed into the blood and subsequently taken up by different blood cells (including erythrocytes and leukocytes) or bound to albumin/immunoglobulins to ensure transport through the circulation and tissue-uptake via cell-type specific RFVTs for cellular use or urinary excretion. Once inside the cell, riboflavin can be converted to the different enzymatically active cofactors (FMN and FAD) within the cytoplasm and mitochondrion. A deficiency of FAD, caused by a genetic defect in ETF, ETFQO, RFVT1−3, FADS or MFT may affect at least 14 flavoproteins, resulting in the accumulation of their associated metabolites. Dashed lines indicate omitted metabolic steps. Question marks refer to presently undefined hydrolases and mitochondrial import-export mechanisms. * Hypothesized to occur in the mitochondrion and cytoplasm via protein-protein interactions between FADS and apo-flavoproteins; ** Hypothesized to donate electrons directly to $CoQ10_{ox}$. Abbreviations: 2HGDH, 2-hydroxyglutarate dehydrogenase; ACAD9, acyl-CoA dehydrogenase 9; ADP and ATP, adenosine diphosphate and -triphosphate (respectively); CACT, carnitine-acylcarnitine translocase; CI−CV, mitochondrial respiratory chain complexes I−V; CoA, coenzyme A; CPT1 and CPT2, carnitine palmitoyltransferase 1 and 2; CoQ10, coenzyme Q10; Cyt c, cytochrome c; DMGDH, dimethylglycine dehydrogenase; ETF, electron transfer flavoprotein; ETFQO, ETF-ubiquinone oxidoreductase; FAD and $FADH_2$, flavin adenine dinucleotide (oxidised and reduced, respectively); FADS, FAD synthase; FAO, mitochondrial fatty acid β-oxidation; FMN, flavin mononucleotide; FP, flavoprotein; GCDH, glutaryl-CoA dehydrogenase; HYPDH, hydroxyproline dehydrogenase; IBD, isobutyryl-CoA dehydrogenase; Ig, immunoglobulin; IVD, isovaleryl-CoA dehydrogenase; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; MFT, mitochondrial FAD transporter; MTHF, methyl-tetrahydrofolate; NAD^+ and NADH, nicotinamide adenine dinucleotide (oxidised and reduced, respectively); OH, hydroxy; ox, oxidised; P5C, Δ^1-pyrroline-5-carboxylate; P_i, inorganic phosphate; PO_4, phosphate; PRODH, proline dehydrogenase; red, reduced; RFK, riboflavin kinase; RFVT1−3, riboflavin transporter 1−3; SBCAD, short branched-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; SDH, sarcosine dehydrogenase; TCA, tricarboxylic acid; THF, tetrahydrofolate; VLCAD, very-long-chain acyl-CoA dehydrogenase.

FAD synthase (FADS) (Henriques et al., 2010). A similar process takes place in the mitochondrion, following the import of riboflavin via the mitochondrial folate/FAD transporter (MFT). The enzymatically active cofactor (FAD) can then be transported to and from the mitochondrion (via MFT), and incorporated into apo-flavoproteins. Since humans have a very low storage capacity for riboflavin, any surplus will be excreted in urine, a factor which contributes to riboflavin's low toxicity, even at pharmacological doses.

Due to the central catalytic function of FAD in ETF, ETFQO, RFVT1-3, FADS and MFT, as well as its role in CoQ10 reduction, at least 14 flavoproteins [nine ACDHs, 2HGDH, DMGDH, SDH, proline dehydrogenase (PRODH) and hydroxyproline dehydrogenase (HYPDH)], distributed across a multitude of interconnected secondary metabolic pathways, may be affected in MADD(-like disorders) (Frerman and Goodman, 2001; Henriques et al., 2010). Under normal physiological conditions, ATP is produced via glycolysis and FAO, of which the latter is the main energy pathway in the heart, skeletal muscle and kidney. However, in MADD the ACDH-catalysed reactions within the FAO and BCAA catabolic pathways are deficient and metabolism shifts to glycolysis, followed by glycogenolysis in order to cope with the energy demand. Accordingly, pyruvate (and subsequently lactate) increases, while acetyl-CoA production declines and various fatty acyl-CoA species accumulate, resulting in the secondary accumulation of the corresponding acylglycine and -carnitine conjugates. The decrease in acetyl-CoA limits ketogenesis and results in the proteolytic degradation of muscle tissue to supply glucogenic and ketogenic substrates for energy production. The decreased availability of FAD further reduces the catabolism of BCAAs and other amino acids (lysine, 5-hydroxylysine, tryptophan, alanine, glutamate, aspartate sarcosine and 4-hydroxyproline), leading to the accumulation of their related intermediates. MADD caused by mutations in *ETFDH* may also be associated with secondary CoQ10 deficiency, which, together with the metabolic stress, will lead to a decreased activity of the respiratory chain complexes (Gempel et al., 2007). Consequently, depending on the severity of the genetic variant, MADD may give rise to varying degrees of disturbed energy homeostasis, as well as the accumulation of toxic metabolites and cellular starvation.

3. Clinical and Genetic features

Deficiencies of those flavoproteins that rely on ETF and ETFQO and their cofactor (FAD and thus riboflavin) lead to a number of disease phenotypes that are generally recognized as *metabolic myopathies* (Gaspar et al., 2019). These disorders have overlapping clinical features, and are biochemically characterized by a set of metabolite abnormalities typical for MADD, as described in more detail in the diagnosis section. The deficiencies and resulting

Table 1: Clinical phenotypes of genetic disorders associated with ETF, ETFQO and FAD metabolism

Deficiency	ETF and ETFQO			Riboflavin transporters			FAD synthase	Mitochondrial folate/FAD transporter
OMIM	#231680/608053/130410/#231675			607883/#615026	607882	613350/#211500/ #211530	610595/#255100	610815
Gene*	*ETFA*, *ETFB*, *ETFDH* for classification as glutaric aciduria II (GA2)A, GA2B, GA2C, respectively			*SLC52A1*	*SLC52A2*	*SLC52A3*	*FLAD1*	*SLC25A32*
Protein	ETFA, ETFB, ETFQO			RFVT1	RFVT2	RFVT3	FADS	MFT
Clinical phenotypes**	MADD Type I (MADD-S, RU-MADD)	MADD Type II (MADD-S, RU-MADD)	MADD Type III (MADD-M, RR-MADD)	Riboflavin deficiency	BVVLS-2	Fazio-Londe disease/BVVLS-1	Lipid storage myopathy	Riboflavin-responsive exercise intolerance
Inheritance	AR	AR	AR	AD, possibly haploinsufficiency, maternal	AR	AR	AR	AR
Onset	neonatal, severe	neonatal	mild and/or late-onset	neonatal/ pregnancy	all ages	all ages	all ages	early age
Clinical and biochemical features	• with congenital anomalies • multi-system involvement • hypotonia • cardiomyopathy • hepatomegaly • nonketotic hypoglycemia • metabolic acidosis • hyperammonemia (see text for detailed metabolic profile)	• without congenital anomalies	• high variability • recurrent episodes of lethargy, vomiting • muscle, cardiac, liver involvement • hypoglycemia • metabolic acidosis (see text for detailed metabolic profile)	• transient neonatal riboflavin deficiency • lethargy, hypotonia • poor peripheral circulation • hypothermia • lactic acidosis • MADD-type metabolites	• sensory ataxia • optic atrophy • early-onset muscle weakness of upper limbs and axial muscles • hearing loss • cranial nerve deficits • respiratory symptoms • feeding difficulties • MADD-type metabolites possible	• muscle weakness in upper limbs and axial muscles • hearing loss (BVVLS-1 only) • cranial nerve deficits • respiratory symptoms • feeding difficulties • sensory symptoms • MADD-type metabolites possible	• hypotonia • muscle weakness • swallowing and speech difficulties • respiratory symptoms • lipid storage myopathy • acylcarnitine abnormalities or ethylmalonic- and/or adipic aciduria	• recurrent exercise intolerance • acylcarnitine abnormalities
Effective Treatment***	diet (low fat and protein)	diet (low fat and protein)	riboflavin, carnitine, glycine, CoQ10	riboflavin	riboflavin	riboflavin	riboflavin	riboflavin
Prognosis (with treatment)	fatal	fatal	good	good	good	good	mostly fatal	good
References	Frerman and Goodman, 2001; Grünert, 2014; van Rijt *et al*., 2019			Chiong *et al*., 2007; Mosegaard *et al*., 2017	Bosch, 2020; Foley *et al*., 2014; Jaeger and Bosch, 2016	Bosch, 2020; Bosch *et al*., 2011; Jaeger and Bosch, 2016	Bosch, 2020; Olsen *et al*., 2016	Bosch, 2020; Schiff *et al*., 2016

* See Table S1 for mutations; ** Recognised by OMIM; *** Variability should be recognised; R, autosomal recessive; AD, autosomal dominant.

phenotypes associated with the genes involved in this metabolism are summarized in Table 1. Table S1 provides a list of 423 published pathogenic genetic variants (mutations) that have been associated with these phenotypes, as well as 159 additional potential pathogenic variants from the Ensembl Genome Browser (https://www.ensembl.org/). Table S2 provides an overview of the distribution of the variants in the gene structures. It should be noted that most of these 582 variants where not scrutinized using current classification guidelines to determine pathogenicity (Richards et al., 2015).

3.1. Multiple acyl-CoA dehydrogenase deficiency (MADD/GAII)

The first case of MADD/GAII was described over 40 years ago (Przyrembel et al., 1976). The disorder presents with a heterogeneous and multi-system phenotype which may be divided into three clinical classes including Types I and II – two ultimately fatal, neonatal-onset subtypes presenting with or without congenital features [MADD-severe (S)], and Type III – a mild and/or later-onset form [MADD-mild (M)]. As the disease severity correlates well with its response to high-dose riboflavin treatment, it has been suggested to classify these phenotypes as riboflavin-unresponsive (RU-MADD) or -responsive (RR-MADD) (Yıldız et al., 2019).

Together, MADD has a general prevalence of 1 to 9:1,000,000 and an estimated birth prevalence of 1:200,000 (https://www.orpha.net/; 26791), with wide variation reported between different countries and ethnicities. Historically, MADD is caused by autosomal recessive mutations in the *ETFA* (36), *ETFB* (19) and *ETFDH* (270) genes, which encode the alpha and beta subunits of ETF, and ETFQO, respectively (Table S1). The majority of these reported mutations are private and more or less equally distributed in both MADD Types I and II (Table S1), while Type III is most frequently caused by mutations in *ETFDH* (Grünert, 2014). In addition, MADD Types I and II display a strong genotype-phenotype correlation, with the former most often being associated with homozygous null mutations and the latter resulting from mutations often leading to amino acid substitutions which allow some level of residual enzyme activity. Conversely, while individuals with MADD Type III have at least one missense mutation and do not display a significantly reduced activity of the affected enzyme(s), the genotype-phenotype correlation is poor (Grünert, 2014).

3.2. Riboflavin transporter deficiencies

Although dietary deficiency of riboflavin has been well-documented (Powers, 2003), genetic disorders of the three riboflavin transporters (RFTV1-3) have only been recognized in the past two decades. Of these, the least frequently reported is a deficiency of RFVT1, which has only

been described in three cases of dominantly inherited, adult-onset or neonatal (transiently expressed) riboflavin deficiency (Chiong et al., 2007; Ho et al., 2011; Mosegaard et al., 2017). More frequently, two related autosomal recessive disorders of RFVT2 [Brown-Vialetto-Van Laere syndrome (BVVLS)-2] and RFVT3 (Fazio-Londe disease/BVVLS-1) have been described, with BVVLS-2 showing distinguishing features of ataxia and optic atrophy (Bosch et al., 2011; Cornelius et al., 2012; Foley et al., 2014; Ho et al., 2011; Olsen et al., 2016; Schiff et al., 2016). At present, two, 28 and 52 mutations, have been published for *SLC52A1, 2* and *3,* respectively (see Table S1).

3.3. FAD synthase and transporter deficiencies

Two additional, riboflavin-responsive phenotypes, resulting from dysfunction in the synthesis or transport of FAD, have been identified. A lipid storage myopathy with a variable age of onset and a mostly fatal outcome, is associated with FADS deficiency, resulting from mutations in *FLAD1,* of which 13 have been reported (Table S1). Furthermore, three mutations have hitherto been identified in *SLC25A32,* which cause a deficiency of the inner mitochondrial membrane carrier MFT and result in a milder, recurrent exercise intolerance phenotype. To date, however, no mutations have been identified in human *RFK* (FAD biosynthesis) (Ryder et al., 2019). Owing to the many unsolved cases of riboflavin deficiency, it is very likely that the number of affected genes will increase in the future as research advances (Olsen et al., 2016).

4. Diagnosis

As summarized in Fig. 1, dysfunction of ETF, ETFQO and the riboflavin metabolism, together with the associated secondary flavoprotein deficiencies, affects a myriad of metabolic pathways. These deficiencies are expressed as phenotypes that are recognized in a clinical setting through their varied neonatal- or late-onset MADD clinical presentations, and via newborn screening (NBS) programs in which they are included (Frerman and Goodman, 2001; Houten et al., 2016; Sahai et al., 2014).

4.1 Conventional diagnostic approach

In the event of a MADD-related clinical presentation (symptomatic patients) or abnormal NBS profile (asymptomatic patients), the differential diagnosis of MADD(-like disorders), as illustrated in Fig. 2, typically proceeds via broad-spectrum metabolite analyses. This may be

followed by functional (enzymatic/flux) assays, with a range of options for genetic analyses essential to confirm the diagnosis.

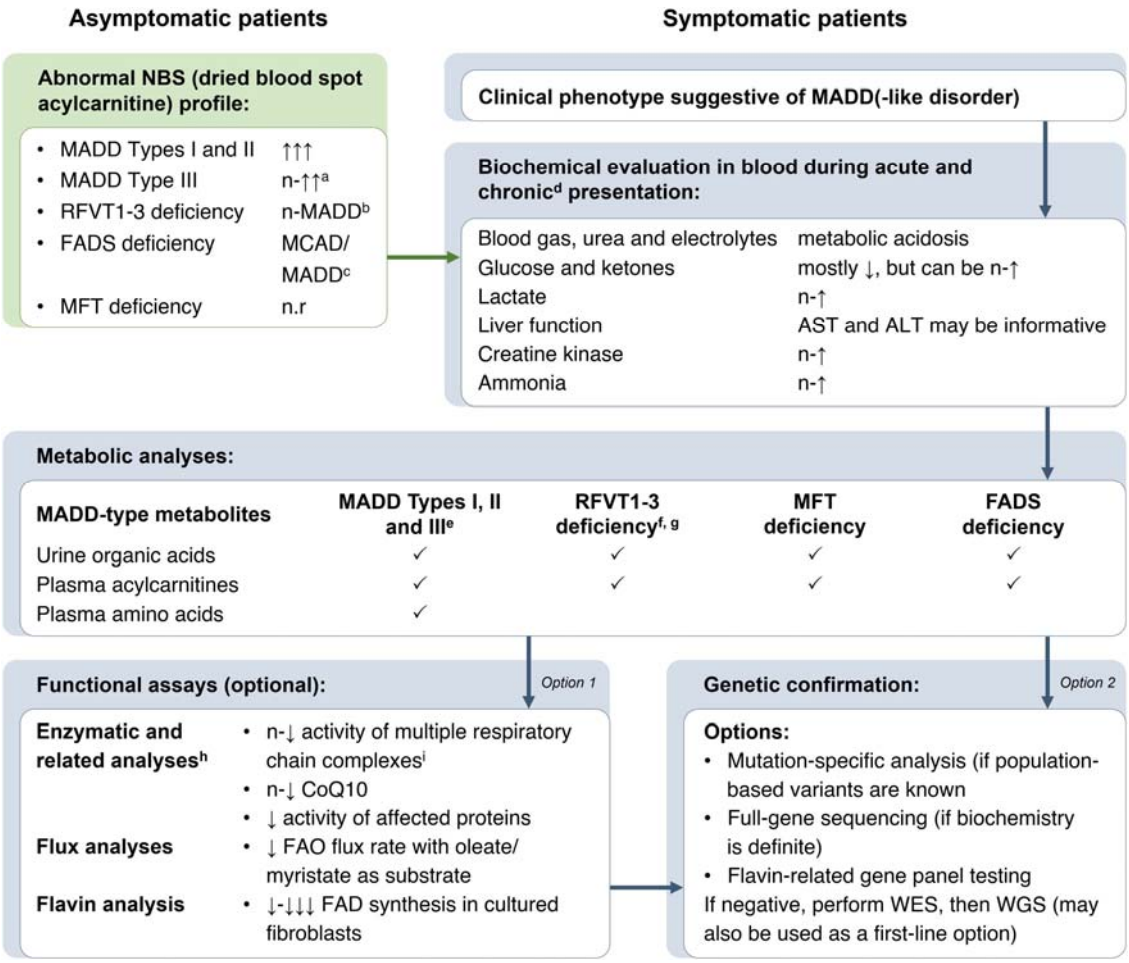

Fig. 2. **Overview of the differential diagnosis for MADD(-like disorders)** [a] Low birth weight and/or total parenteral nutrition may result in a MADD-like acylcarnitine profile (Shai et al., 2008); [b] NBS is not feasible for the diagnosis of RFVT2 and 3 deficiencies (due to suspected compensation by maternal riboflavin), whereas RFVT1 deficiency has displayed normal or abnormal (MADD) NBS profiles in the two reported cases (Bosch et al., 2011; Ho et al., 2011; Mosegaard et al., 2017); [c] NBS has only been reported in three cases (Muru et al., 2019; Ryder et al., 2019; Yamada et al., 2019); [d] Biochemical parameters may normalize and abnormal liver function is more to likely to occur later in the disease progression; [e] Metabolic analysis is most indicative during catabolic status/metabolic crisis, as the profile may be normal otherwise; [f] Some genotypes may have normal metabolic profiling; [g] Metabolite analysis can be indicative, but may be normal in some patients (molecular analysis of RFTV1-3 is required for definite diagnosis) (Jaeger and Bosch, 2016); [h] Should enzymatic assays be unavailable, diagnosis may proceed directly to genetic analyses or a combination of flux- and genetic assays; [i] Only CII activity has been analysed in MFT deficiency (Hellebrekers et al., 2017). ✓, can be indicative; ↓, ↓↓↓, slight and substantial decrease; ↑, ↑↑ and ↑↑↑, slight, moderate and substantial increase.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CoQ10, coenzyme Q10; FAD, flavin adenine dinucleotide; FADS, FAD synthase; FAO, mitochondrial fatty acid β-oxidation; MADD, multiple acyl-CoA dehydrogenase deficiency; MFT, mitochondrial folate/FAD transporter; n, normal; n.r, not reported; NBS, newborn screening; RFVT, riboflavin transporter; WES, whole exome sequencing; WGS, whole genome sequencing.

At present, metabolic investigations remain the cornerstone of diagnosis and involve the analysis of urinary organic acids (GC–MS) along with plasma amino acids (GC-/LC–MS/MS) and acylcarnitines (LC–MS/MS). Urinary organic acids suggestive of MADD include a combination of elevated glutaric-, ethylmalonic-, adipic-, suberic-, sebacic-, dodecanedioic-, 2-hydroxyglutaric-, 3-hydroxyisovaleric-, and 5-hydroxyhexanoic acid, in conjunction with C4 and C5 glycine conjugates. Moreover, elevated concentrations of proline, 4-hydroxyproline (neonatal-onset) and sarcosine (late-onset) are common biochemical features (Frerman and Goodman, 2001; Grünert, 2014). Acylcarnitine profiling, which is also used in NBS, is highly predictive and reflects the intra-mitochondrial accumulation of various acyl-CoAs as well as the corresponding acylcarnitines generated by carnitine palmitoyltransferase 2 (CPT2) and carnitine acetyltransferase (CrAT). A typical MADD-specific acylcarnitine profile would include C4, C5, C5-DC, C6, C8, C10, C12, C14:1, C16 and C18:1 acylcarnitine species (Grünert, 2014; Houten et al., 2016; Violante et al., 2013; Wanders et al., 2010). Important, however, is that abnormal metabolite profiles may, in some instances, only be observed during symptomatic events and may improve or even normalize when clinical symptoms are absent (Chaya et al., 2018). For metabolites to be most indicative, samples should be obtained during the catabolic state or metabolic crisis, or before the next feeding (for neonates), following minimal medication intake.

Although metabolic profiling can be highly predictive for a particular inborn error of metabolism, enzymatic and/or flux analyses have, in general, been shown to be paramount in pinpointing the enzyme defect with certainty and disclosing the level of the deficiency. However, in the case of MADD, direct measurement of ETFα, ETFβ and ETFQO activity has been notoriously difficult to perform routinely, mainly due to the requirement of purified ETF, which is not commercially available. As reviewed by Wanders et al. (2010), radio- and stable isotope-labelled FAO flux analyses, which are based on methods developed in the 1990s in fibroblasts and lymphocytes, have proven to be of great value to distinguish MADD from other FAO disorders (Manning et al., 1990; Niezen-Koning et al., 1992; Olpin et al., 1992). In addition, FAO flux analysis using oleate as substrate in patients' fibroblasts has been found to correlate with the clinical phenotype as determined using a recently proposed MADD-disease severity scoring system (MADD-DS3) (van Rijt et al., 2019). Considering the clinical and biochemical variability that makes MADD prone to misdiagnosis when using a differential diagnosis with

limited assays (Frerman and Goodman, 2001; Vasiljevski et al., 2018; Yıldız et al., 2018), the study of van Rijt et al. (2019) illustrated the extensive nature of the investigations required to obtain markers for early prediction of disease severity that would allow preventative and follow-up treatment.

4.2 Postgenomic era diagnostic approach

With the expanding ability, lowered cost and advantages of whole exome- (WES) and whole genome sequencing (WGS) to study complex inherited diseases, the application of these genomic tools have become an increasingly popular option to include earlier in the diagnostic process at several laboratories (Haack et al., 2012; Lieber et al., 2013; Neveling et al., 2013; Wortmann et al., 2017, 2015). Altogether, genetic approaches have resulted in the rapid expansion of gene and mutation identification, as summarized in Table S1. Of the eight genes and ~436 reported mutations, as well as other potential variants involved in MADD(-like disorders), five genes and the vast majority of mutations have been identified using next generation sequencing (NGS). With a limited number of genes involved in this group of disorders, gene panels have proven to be exceptionally useful, particularly when run in parallel with functional (flux) assays. Nevertheless, the value of biochemical and enzymatic/flux analyses, as well as the use of disease expression systems in unraveling the impact of genetic variants, diagnostic variability and the pathophysiology and treatment of MADD, should not be underestimated (Henriques et al., 2009; Tolomeo et al., 2020).

5. Treatment and Prognosis

As a first-line treatment, early oral supplementation of riboflavin at a high dose (100-400 mg/day) is advised for all MADD(-like) phenotypes (Ryder et al., 2019). This vitamin has been shown to dramatically improve the clinical and biochemical abnormalities associated with MADD Type III (especially in cases of *ETFDH* deficiency), RFVT1-3 defects, MFT deficiency and milder cases of FADS dysfunction without major side effects (Auranen et al., 2017; Jaeger and Bosch, 2016; Ryder et al., 2019; Schiff et al., 2016). Studies on *ETFDH* deficiency have attributed the molecular mechanism of riboflavin's response to the chaperone function of FAD, which promotes folding of the variant protein or otherwise stabilizes its folding intermediates or mature form (Cornelius et al., 2012; Henriques et al., 2009). However, this improvement, the extent of which depends on the nature of the variant, usually does not completely correct for the variant-induced structural defect(s). Other possible explanations for the beneficial effect of riboflavin include the increase of flavin content to compensate for the loss of FAD, and the induction of flavoprotein-transcription and -translation by FAD (Henriques et al., 2010; Nagao and Tanaka, 1992).

In MADD(-like disorders), riboflavin supplementation is often combined with a high-caloric diet reduced in fat and protein, the strict avoidance of fasting, and/or oral supplementation with glycine and L-carnitine to promote the export of acylglycine and -carnitine conjugates (Gordon, 2006). The latter is especially important since acyl-CoA esters cannot traverse the mitochondrial- or plasma membrane, but should be approached with caution as L-carnitine may worsen symptoms, at least in some cases (Gempel et al., 2007). Riboflavin may further be combined with CoQ10 to decrease the oxidative stress and treat MADD Type III with secondary CoQ10 deficiency, whereas bezafibrate has been shown to improve MADD in the absence of riboflavin, although its *in vivo* application requires further research (Cornelius et al., 2014; Gempel et al., 2007; Xi et al., 2014; Yamada et al., 2017; Yamaguchi et al., 2012). Moreover, the efficacy and safety of D,L-3-hydroxybutyric acid (100-2,600 mg/kg/day, administered orally or via nasogastric/gastrostomy tube) to treat MADD by bypassing the disturbed ketogenesis was recently demonstrated, with the authors reporting a longer survival rate (notably in MADD Type II) and clinical improvement in 70% of the patients (including MADD Type II, III and RFVT3 deficiency) (van Rijt et al., 2020).

Despite these treatment options, mortality remains high in severe MADD(-like) phenotypes (most notably MADD Types I and II) (van Rijt et al., 2020). More effective treatment strategies are thus still desperately needed and, consequently, future research in enzyme replacement-, gene- and gene expression therapies hold great promise in MADD as the phenotype can often be rescued by even a slight increase in the activity of the variant protein (Ørngreen and Vissing, 2017). Likewise, disease expression models are of immeasurable value in the study of therapeutic options (Tolomeo et al., 2020)..

6. Conclusion

FAD and the flavoproteins it supports, participate in a number of catabolic pathways which all converge on the mitochondrion. Deficiencies of FAD's synthesis, transport and dedicated redox machinery are thus expected to have a severe impact on a wide range of metabolic functions. In this article, we have provided a succinct overview of these processes and the clinical phenotypes resulting from genetic deficiencies of the FAD metabolism, which, at present, include eight genes with at least 436 reported mutations that lead to a range of different MADD(-like) phenotypes. *ETFDH* remains the gene with the most (64 %) reported mutations followed by *SLC52A3* (12 %), while the other genes each exhibit less than 9 % of the reported mutations. With the exception of MADD Types I and II, an important feature of these disorders is the possibility of effective treatment. At present, these disorders are included in few NBS programmes and an effective early metabolic diagnosis relies

predominantly on disease expression (catabolic state) (Austin and Dawkins, 2017; Baynam et al., 2020). Together, this warrants the development of novel diagnostic approaches – notably with the focus on finding more successful and direct avenues for diagnostics in a more inclusive global population. Over the past decade genomic approaches have, alongside biochemical investigations, proven their value in the diagnosis of MADD(-like disorders). Considering the limited number of genes involved, the earlier inclusion of mutational analysis in diagnosis, and the development of targeted gene therapy should therefore be considered in earnest.

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