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REVIEW ARTICLE

Understanding the role of OXPHOS dysfunction in the pathogenesis of ECHS1 deficiency

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Mitochondria provide the main source of energy for eukaryotic cells, oxidizing fatty acids and sugars to generate ATP. Mitochondrial fatty acid β-oxidation (FAO) and oxidative phosphorylation (OXPHOS) are two key pathways involved in this process. Disruption of FAO can cause human disease, with patients commonly presenting with liver failure, hypoketotic glycaemia and rhabdomyolysis. However, patients with deficiencies in the FAO enzyme short-chain enoyl-CoA hydratase 1 (ECHS1) are typically diagnosed with Leigh syndrome, a lethal form of subacute necrotizing encephalomyelopathy that is normally associated with OXPHOS dysfunction. Furthermore, some ECHS1-deficient patients also exhibit secondary OXPHOS defects. This sequela of FAO disorders has long been thought to be caused by the accumulation of inhibitory fatty acid intermediates. However, new evidence suggests that the mechanisms involved are more complex, and that disruption of OXPHOS protein complex biogenesis and/or stability is also involved. In this review, we examine the clinical, biochemical and genetic features of all ECHS1-deficient patients described to date. In particular, we consider the secondary OXPHOS defects associated with ECHS1 deficiency and discuss their possible contribution to disease pathogenesis.

Keywords: ECHS1 deficiency; enoyl-CoA hydratase short-chain 1; fatty acid oxidation; mitochondrial disease; oxidative phosphorylation; OXPHOS

Abbreviations

$\Delta\Psi_m$, mitochondrial membrane potential; ACAD9, acyl-CoA dehydrogenase family member 9; BN-PAGE, blue native polyacrylamide gel electrophoresis; CACT, carnitine-acylcarnitine translocase; CPT I, carnitine O-palmitoyltransferase I; CPT II, carnitine O-palmitoyltransferase II; ECHS1, enoyl-CoA hydratase short-chain 1; ECHS1D, ECHS1 deficiency; ECI1, enoyl-CoA delta isomerase 1; Ecsit, evolutionarily conserved signalling intermediate in Toll pathway, mitochondrial; ETC, electron transport chain; ETF, electron transfer flavoprotein; FAO, fatty acid oxidation; FOXRED1, FAD-dependent oxidoreductase domain-containing protein 1; HADH, hydroxyacyl-CoA dehydrogenase; KAT, 3-ketoacyl-CoA thiolase; LCEH, long-chain 2,3-enoyl-CoA hydratase; LCHAD, long-chain L-3 hydroxyacyl-CoA dehydrogenase; LCKAT, long-chain 3-ketoacyl-CoA thiolase; LLS, Leigh-like syndrome; LS, Leigh syndrome; MCAD, medium-chain acyl-CoA dehydrogenase; mtDNA, mitochondrial DNA; MTP, mitochondrial trifunctional protein; NDUFAF1, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 1 (complex I intermediate-associated protein 30, mitochondrial); NDUFAF3, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 3 (C3orf60); NDUFAF5, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 5 (C20orf7); NDUFAF6, NADH dehydrogenase (ubiquinone) complex I, assembly factor 6 (C8orf38); OXPHOS, oxidative phosphorylation; PCC, propionyl-CoA carboxylase; PDC, pyruvate dehydrogenase complex; PED, paroxysmal exercise-induced dyskinesia (PED); SBCAD, short/branched-chain specific acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; VLCAD, very-long-chain acyl-CoA dehydrogenase.

Short-chain enoyl-coA hydratase (ECHS1) is a key enzyme involved in mitochondrial fatty acid β-oxidation (FAO). Since its initial identification in 2014, 46 patients have been described with ECHS1 deficiency (ECHS1D) [1–21]. Almost all of these patients have been diagnosed with Leigh syndrome (LS), a lethal form of subacute necrotizing encephalomyelopathy. Intriguingly, this severe disorder is typically associated with deficiencies in oxidative phosphorylation (OXPHOS) and has not been described in any other FAO diseases [22]. In addition, some patients with ECHS1D also exhibit defects in OXPHOS function, which appear to be secondary to the primary deficiency in ECHS1 enzymatic activity. The unusual clinical presentation of ECHS1D, in combination with the identification of secondary OXPHOS defects in some patients, suggests that the disruption of OXPHOS may play a role in the pathogenesis of ECHS1D. However, the mechanisms that are involved in this disease process are currently unknown.

Biochemical interactions between the FAO and OXPHOS pathways have long been recognized, but more recently physical interactions between the components of these two pathways have also been described [23]. Furthermore, other FAO deficiencies (apart from ECHS1D) also exhibit secondary OXPHOS defects, including reduced steady-state levels of mature OXPHOS complexes [24]. These findings suggest that certain FAO proteins, including ECHS1, may not only be involved in FAO but also in maintaining OXPHOS function, stability and/or biogenesis. As such, secondary defects in OXPHOS may be contributing to pathogenesis in FAO diseases, and understanding the mechanisms involved is critical if we are to develop new, effective therapies that address this OXPHOS dysfunction.

In this review, the clinical and biochemical features of all ECHS1D patients described to date will be discussed. Correlations between *ECHS1* genotype and the spectrum of LS clinical presentations have also been explored, with particular examination of the nine ECHS1D patients with reported defects in OXPHOS. The potential contribution of these secondary OXPHOS defects to disease pathogenesis is discussed, as well as their importance when considering the development of new treatments for FAO disorders, including ECHS1D.

Mitochondrial metabolism

Mitochondria are organelles responsible for powering the entire cell, oxidizing fatty acids, amino acids and the products of glucose (pyruvate and NADH), to generate adenosine triphosphate (ATP) [25]. ATP production occurs *via* oxidative phosphorylation (OXPHOS), a process performed by the electron transport chain (ETC), which consists of five membrane-bound complexes: complex I (NADH: ubiquinone oxidoreductase), complex II (succinate: ubiquinone oxidoreductase), complex III (ubiquinol: ferricytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase) and complex V (F_1F_0-ATP synthetase) [26] (Fig. 1). These complexes are comprised of many protein subunits that are encoded by the mitochondria's own genome, as well as the nuclear genome [26,27].

Oxidative phosphorylation, in combination with glycolysis and the tricarboxylic acid (TCA) cycle, generates approximately 30–38 molecules of ATP per single glucose molecule [28]. Glycolysis produces pyruvate and NADH, which are imported into the mitochondria. Pyruvate is oxidized into acetyl-CoA and enters the TCA cycle within the mitochondrial matrix, where it is further oxidized into a series of products to generate NADH and $FADH_2$. For each round of the TCA cycle, one pyruvate molecule can produce one $FADH_2$, one GTP and three NADH molecules. NADH and $FADH_2$ act as reducing equivalents, donating their electrons to OXPHOS complexes I and II, respectively (Fig. 1). The electrons are shuttled from these OXPHOS complexes *via* ubiquinone (Q) (Fig. 1) to complex III, then to cytochrome *c*. Cytochrome *c* is finally oxidized by complex IV with the subsequent reduction of O_2 to H_2O. This electron transfer through the OXPHOS complexes facilitates the pumping of protons from the mitochondrial matrix to the intermembrane space by complexes I, III and IV, generating a mitochondrial membrane potential ($\Delta\Psi_m$). $\Delta\Psi_m$ then drives complex V to phosphorylate ADP to form ATP [26].

While the OXPHOS complexes are pictured individually in Fig. 1 for simplicity, they also exist in 'supercomplexes' of more than one of each OXPHOS complex. These OXPHOS supercomplexes are metabolically active structures that have been observed in the following forms: CI_1CIII_2, $CI_1CIII_2CIV_1$ (often termed the 'respirasome'), $CIII_2CIV_1$ and $CI_2CIII_2CIV_{1-2}$ (termed the 'respiratory megacomplex') [29]. While the existence of mitochondrial supercomplexes is now accepted, the reasons behind why they form are still debated [30]. It is widely thought that the respirasome supercomplex enhances electron transfer between the OXPHOS complexes by channelling substrates; however, both structural information and biophysical information challenge this view [30]. Other current theories as to the existence of OXPHOS supercomplexes include the reduction of reactive oxygen species (ROS) production [31], the regulation of OXPHOS activity and assembly of OXPHOS complexes [32] and the prevention of

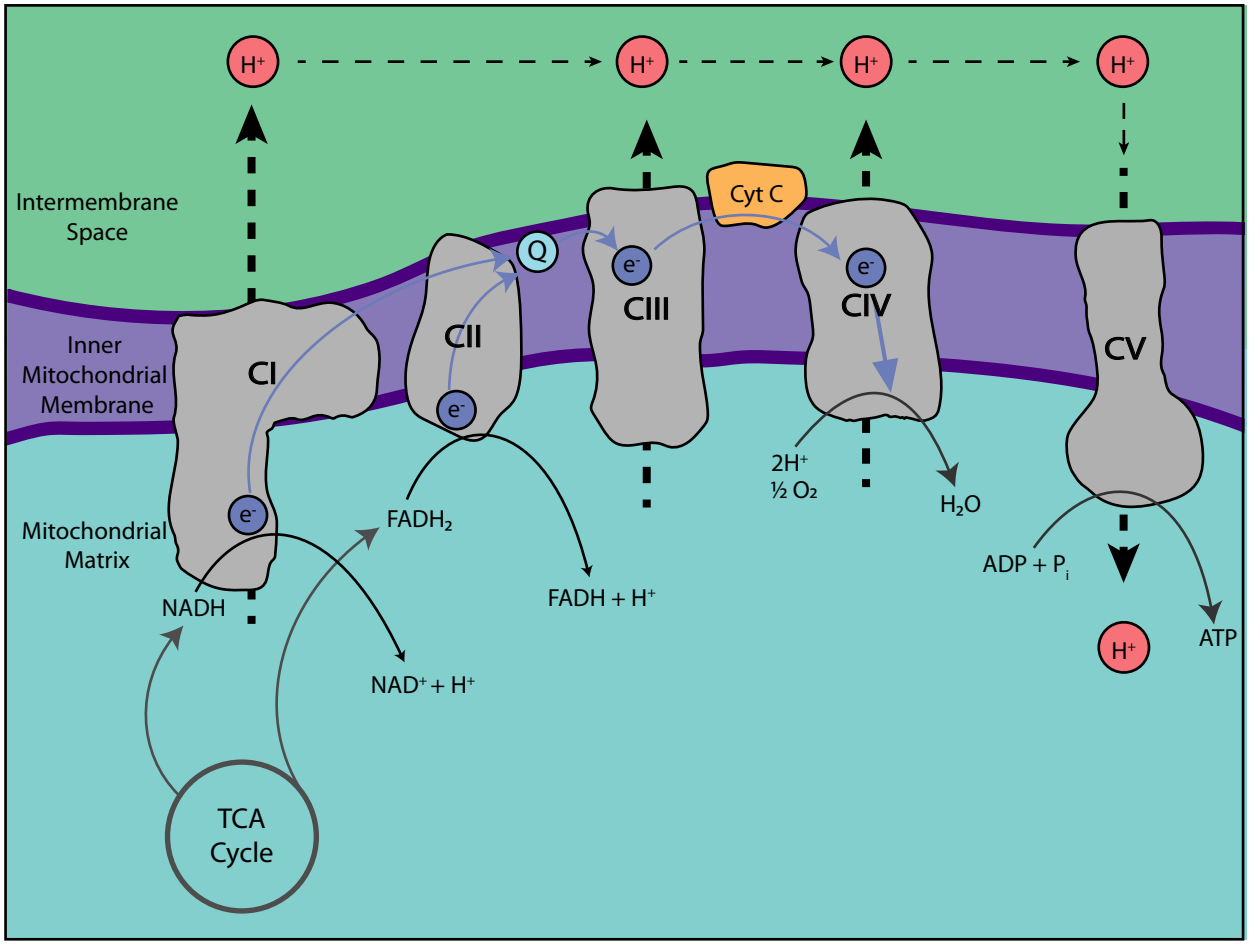

Fig. 1. Mitochondrial oxidative phosphorylation. Complex I oxidizes NADH to NAD^+ while complex II oxidizes $FADH_2$ to FADH, with the resulting electrons being transferred through the OXPHOS complexes *via* ubiquinone (shown as Q, also known as CoQ_{10}) to complex III and then cytochrome *c* (Cyt *c*), which is finally oxidized by complex IV to reduce O_2 to H_2O. This electron transfer through the OXPHOS complexes facilitates the pumping of protons (H^+) from the mitochondrial matrix to the intermembrane space by complexes I, III and IV, generating a mitochondrial membrane potential ($\Delta\Psi_m$). Complex V (ATP synthase) uses the $\Delta\Psi_m$ to power the condensation of ADP + P_i to generate ATP.

protein aggregation at the mitochondrial inner membrane [31]. An alternative theory is that the main purpose of supercomplexes is to allow for tighter packing of the OXPHOS complexes into the inner membrane [30]. Interestingly, enzymes involved in mitochondrial fatty acid β-oxidation can also be associated with OXPHOS supercomplexes [23], suggesting they provide both an enzymatic and structural link between the OXPHOS and FAO pathways (as will be discussed below).

Mitochondrial fatty acid β-oxidation

Fatty acid oxidation involves the metabolism of dietary fatty acids to produce energy. Fatty acids are the preferred substrate for catabolic metabolism in the liver, skeletal muscle and heart [33], providing 60–70% of the ATP required for regular heart function [34]. During periods of fasting or high metabolic intensity (such as endurance exercise), the liver metabolizes fatty acids to produce ketone bodies as an alternative energy source for the brain when glucose availability is limited [35,36]. FAO is also essential in newborns, where it drives nonshivering thermogenesis in brown adipose tissue to maintain body temperature [37–39].

Fatty acids are transported through the bloodstream to their target cells *via* serum albumin or lipoproteins. Upon arrival at their destination cells, short- and medium-chain fatty acids (C4–C12) can freely diffuse across the plasma membrane. However, longer fatty acids and saturated fatty acids require transport proteins, such as FAT/CD36, to facilitate their import

into the cell [40]. Upon entry into the cytosol, fatty acids are activated by acyl-CoA synthetases, converting them to fatty acyl-CoA esters. This activated form can now be metabolized *via* FAO, or form the preliminary substrate for phospholipid, triacylglycerol or cholesterol synthesis [41].

There are multiple types of FAO: α-FAO, β-FAO and ω-FAO, each occurring at different cellular locations with different substrate specificity. α-FAO occurs in peroxisomes, preparing fatty acids to enter β-FAO by removing methyl groups that would otherwise block oxidation of the β-carbon [42]. β-FAO occurs in both peroxisomes and mitochondria, with peroxisomal β-oxidation shortening very-long-chain fatty acids (> 22 carbon atoms in length) for subsequent β-oxidation within mitochondria (see below). ω-FAO is a minor catabolic pathway with broad substrate specificity, oxidizing the ω carbon (or ω-1 carbon) furthest away from the carboxyl group [43]. Interestingly, peroxisomal ω-oxidation of very-long-chain fatty acids has been suggested as a potential rescue pathway when β-FAO is disrupted [42,44].

The majority of β-FAO occurs within the matrix of the mitochondria, breaking down fatty acids < 22 carbons in length. Fatty acyl-CoAs are imported into the mitochondria *via* the carnitine shuttle system, consisting of carnitine *O*-palmitoyltransferase I and II (CPT I and CPT II, respectively) and carnitine-acylcarnitine translocase (CACT) (Fig. 2). CPT I is the rate-limiting enzyme for mitochondrial β-FAO as it controls the import of fatty acyl-CoA molecules into the mitochondria [45]. CPT I catalyses the addition of a carnitine group to a fatty acyl-CoA ester, forming an acylcarnitine, which can be imported by CACT. Upon entry, the carnitine group is removed by CPT II, allowing the re-established fatty acyl-CoA ester to enter the β-FAO spiral, with the carnitine exported back across the inner mitochondrial membrane *via* CACT.

β-FAO of the fatty acyl-CoA ester proceeds through a series of four enzymatic reactions: dehydrogenation, hydration, a second dehydrogenation and finally thiolysis. Fatty acid chain-length specific enzymes are involved at each of the four steps, beginning with dehydrogenation by very-long-chain (C24–12), medium-chain (C12–C6) or short-chain (C6–C4) acyl-CoA dehydrogenases (VLCAD, MCAD and SCAD, respectively). For very-long-chain and long-chain (C14–24) fatty acyl-CoA esters, the second, third and fourth steps of β-FAO are performed by the multi-domain mitochondrial trifunctional protein (MTP), which consists of long-chain 2,3-enoyl-CoA hydratase (LCEH), long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and long-chain 3-ketoacyl-CoA thiolase (LCKAT) (Fig. 2).

Medium- (C12–C6) and short-chain (C6–C4) fatty acyl-CoA esters are metabolized *via* a different set of enzymes that perform hydration (step 2; short-chain enoyl-CoA hydratase, ECHS1), dehydrogenation (step 3; hydroxyacyl-CoA dehydrogenase, HADH) and thiolysis (step 4; 3-ketoacyl-CoA thiolase, KAT) reactions.

The end product of the four enzymatic reactions of β-FAO is one acetyl-CoA molecule and a fatty acyl-CoA ester that is two carbons shorter in length. The resulting acetyl-CoA can enter the citric acid cycle to produce NADH and $FADH_2$, which are used by the ETC complexes to generate ATP. The shortened fatty acyl-CoA ester is metabolized again (and again) by β-FAO until only two acetyl-CoA molecules remain. As such, the β-FAO pathway is often described as an oxidation 'spiral' [46].

In addition, β-FAO also generates $FADH_2$ and NADH during its two dehydrogenation reactions. In the first dehydrogenation reaction, electron transfer flavoprotein (ETF) and ETF dehydrogenase transfer electrons from $FADH_2$ to ubiquinone, which is subsequently oxidized by OXPHOS complex III. Conversely, the second dehydrogenation reaction requires oxidized NAD^+ as a cofactor, with its reduction to NADH completed during hydration of the fatty acyl-CoA ester.

If the fatty acyl-CoA ester has an odd number of carbons, the last molecule cannot be released as acetyl-CoA, and instead, a three-carbon-long molecule, propanoyl-CoA, is the last product of the β-FAO spiral. When this occurs, propanoyl-CoA is carboxylated by propanoyl-CoA carboxylase to form (*S*)-methylmalonyl-CoA. (*S*)-methylmalonyl-CoA is then isomerized to form (R)-methylmalonyl-CoA by methylmalonyl-CoA epimerase. Finally, (R)-methylmalonyl-CoA is converted to succinyl-CoA by methylmalonyl-CoA mutase, which requires Vitamin B12 as a cofactor. Succinyl-CoA can then enter the TCA cycle to generate NADH and $FADH_2$ as reducing equivalents [47].

Short-chain enoyl-CoA hydratase

Short-chain enoyl-CoA hydratase (also called crotonase; EC 42.1.17) catalyses the second step of β-FAO and is expressed in most tissues, including brain, heart, kidney, liver, skeletal muscle and skin [48,49]. ECHS1 activity was first observed in bovine heart and liver, with the first *ECHS1* cDNA clones isolated in 1993 [49]. The *ECHS1* gene is found on chromosome 10q26.2-q26.3 and encodes eight exons, with the 5′ and 3′ UTRs contained within exons I and VIII, respectively [50]. *ECHS1* mRNA is transcribed

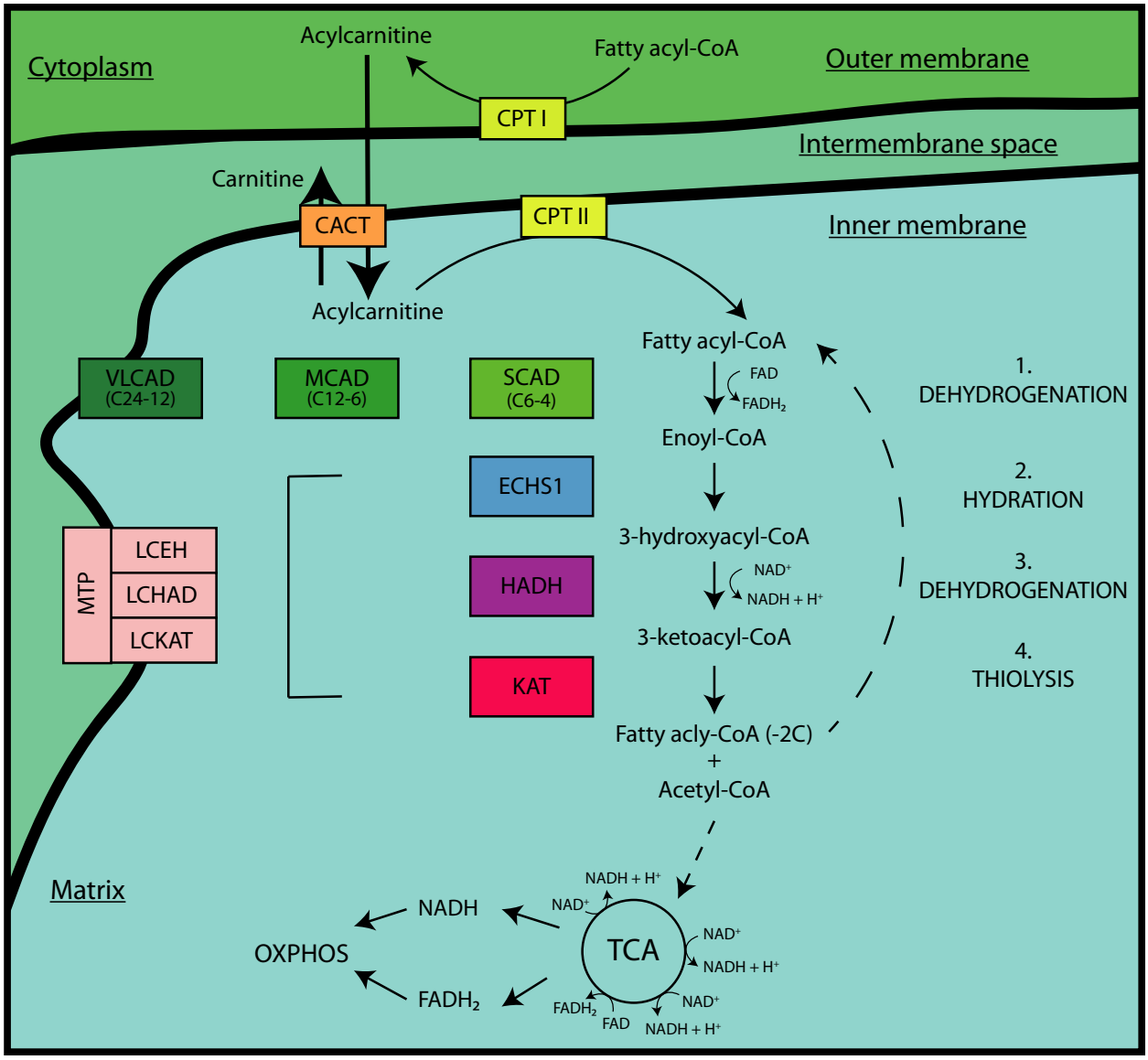

Fig. 2. Fatty acid β-oxidation. Fatty acyl-CoA esters are imported into the mitochondrial matrix as acylcarnitines by the carnitine shuttle system, which consists of CPT I, CACT and CPT II. Once inside the mitochondrial matrix, the carnitine is removed from the fatty acyl-CoA ester by CPT II. Four reactions occur in each round of β-FAO, catalysed by a group of enzymes with differing specificities based on the length of the carbon chain of the fatty acyl-CoA. The first reaction is dehydrogenation of fatty acyl-CoA esters by very-long-chain (VLCAD), medium-chain (MCAD) and short-chain (SCAD) acyl-CoA dehydrogenases to form enoyl-CoA and $FADH_2$. The second reaction is hydration of enoyl-CoA by the MTP, or ECHS1, to form 3-hydroxyacyl-CoA. The third reaction is dehydrogenation of 3-hydroxyacyl-CoA by MTP or HADH to form 3-ketoacyl-CoA and NADH. Finally, thiolysis of 3-ketoacyl-CoA by MTP or 3-ketoacyl-CoA thiolase (KAT) yields a fatty acyl-CoA (shortened by two carbon atoms) and acetyl-CoA. Acetyl-CoA enters the TCA cycle to generate more NADH and $FADH_2$, which is then utilized by OXPHOS for ATP production, while the shortened acyl-CoA molecule re-enters β-FAO until only two acetyl-CoA molecules remain.

as a single 1.4 kb transcript, which encodes a 290 amino acid precursor protein containing a 27-amino acid N-terminal mitochondrial targeting sequence that is cleaved upon entry into the mitochondria [51]. The resulting 28.3 kDa mature protein forms an active 188 kDa homohexamer 'dimer of trimers' [52,53].

ECHS1 has multiple functions in both amino acid and fatty acid metabolism. ECHS1 has affinity for intermediates in the isoleucine, leucine and valine pathways, with varying degrees of activity (Fig. 3). ECHS1 has very low affinity for tiglyl-CoA (isoleucine pathway), with increasing affinities for methacrylyl-CoA (valine pathway), 3-methylcrotonyl-CoA (leucine

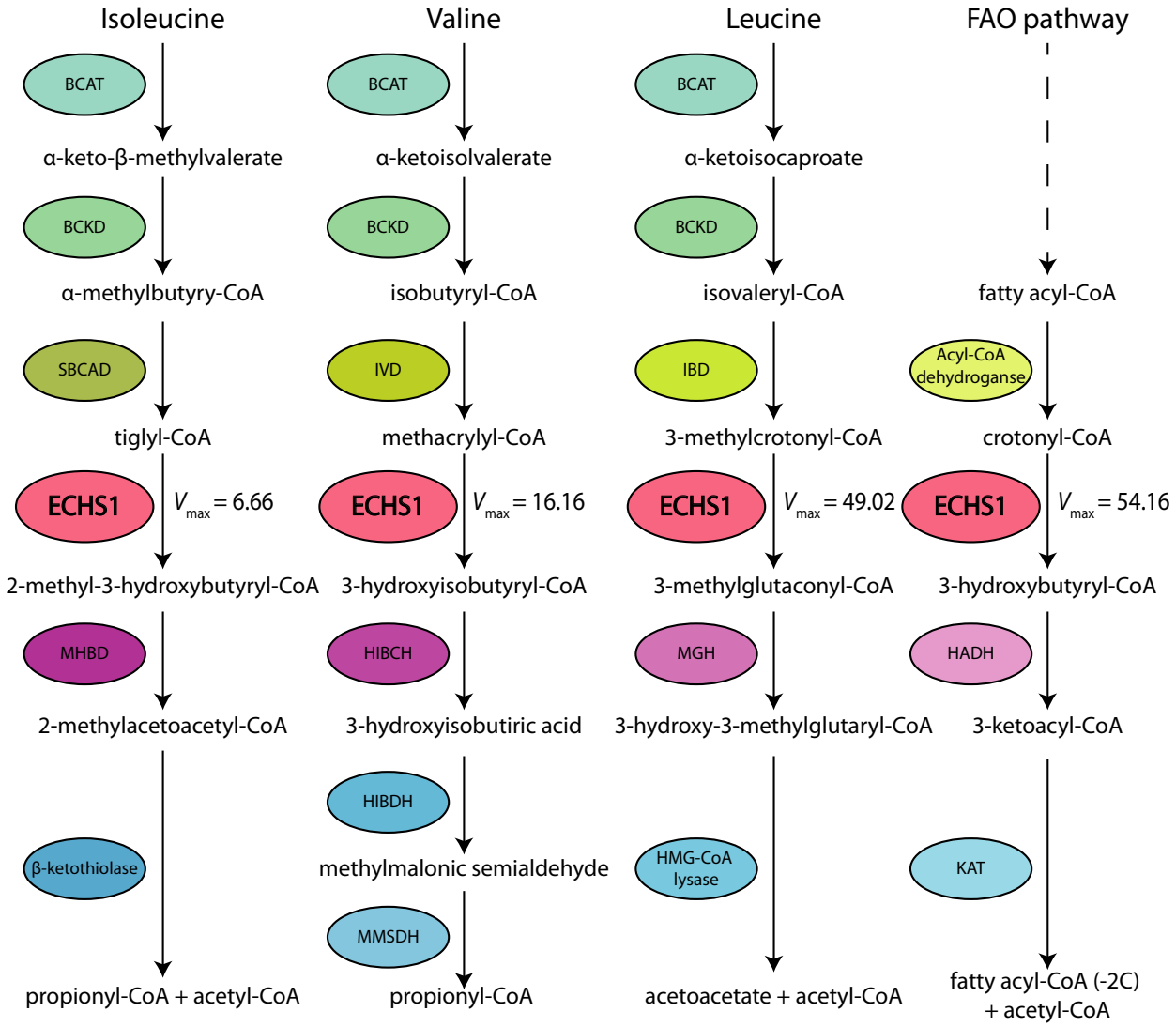

Fig. 3. ECHS1 catalyses reactions involved in amino acid metabolism, in addition to its role in FAO. ECHS1 has multiple substrates, including intermediates of the valine, leucine and isoleucine metabolic pathways. However, ECHS1 has the strongest binding affinity (V_{max}) for crotonyl-CoA in mitochondrial β-FAO. BCAT, branched-chain aminotransferase; BCKD, branched-chain α-keto acid dehydrogenase complex; SBCAD, short/branched-chain acyl-CoA dehydrogenase; IVD, isovaleryl-CoA dehydrogenase; IBD, isobutyryl-CoA dehydrogenase; MHBD, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase; HIBCH, 3-hydroxyisobutyryl-CoA hydrolase; MGH, 3-methylglutaconyl-CoA hydratase; HIBDH, 3-hydroxyisobutyrate dehydrogenase; MMSDH, methylmalonate semialdehyde dehydrogenase; KAT, 3-ketoacyl-CoA thiolase.

pathway) and crotonyl-CoA (β-FAO) [9,21]. ECHS1 does have a higher V_{max} for 3-methylcrotonyl-CoA than methacrylyl-CoA; however, it has been suggested that ECHS1 is nonessential for leucine metabolism [6]. As only a small amount of tiglyl-CoA is hydrated by ECHS1, it is also suggested that ECHS1 is not essential for isoleucine metabolism [21].

In β-FAO, ECHS1 catalyses the hydration of trans-Δ^2-enoyl-CoA thioesters to 3-L-hydroxyacyl-CoA thioesters [54]. ECHS1 has strongest substrate affinity for the 4-carbon crotonyl-CoA but can bind enoyl-CoA chains up to ten carbon atoms in length [52,55].

Mitochondrial β-FAO disorders

Mitochondrial disease affects approximately 1 in 4300 people and causes significant morbidity and mortality [56]. Patients commonly suffer from debilitating multi-systemic metabolic disorders, including brain, heart and skeletal muscle dysfunction [27]. Mitochondrial

disease is commonly associated with OXPHOS deficiencies; however, mitochondrial β-FAO deficiencies can also cause human disease [23]. The first β-FAO deficiencies were described in 1973 in CPT I [57], with the first β-FAO pathogenic mutations identified in *ACADM* (MCAD) in 1990 [58–60].

β-FAO disorders affect as many as 1 in 10 000 people in certain populations [61]. These diseases are typically inherited in an autosomal recessive manner, with at least 21 disease-causing β-FAO genes identified. Common symptoms of β-FAO disease include liver defects, hypoglycaemia and cardiac abnormalities such as dilated hypertrophic cardiomyopathy and arrhythmias [62] (Table 1). Milder forms of β-FAO disease can present with symptoms such as exercise-induced myopathy and rhabdomyolysis (the breakdown of muscle fibres) [63]. Disease presentation is not persistent, with some patients remaining asymptomatic until an episode of metabolic crisis, such as fasting, exercise or a fat-rich diet [64–66]. In infants, β-FAO is the primary pathway for generating ATP as metabolic rates are high, and stored glycogen levels are low [67,68]. As such, deficiencies in β-FAO are often more severe in younger patients, causing build-up of excess metabolites and fatty acid intermediates that result in toxicity and neonatal death in some cases [69,70]. β-FAO deficiencies have also been linked to sudden infant death syndrome [71], with deficiencies in MCAD and LCHAD attributed to 1–3% of sudden infant deaths [72]. As many patients die during their first episode of metabolic crisis, early detection of β-FAO deficiency is critical. Consequently, newborn screening (NBS) for β-FAO deficiencies using mass spectroscopy has been implemented in numerous

Table 1. FAO genes associated with diseases and their common symptoms. SBCAD, short/branched-chain specific acyl-CoA dehydrogenase; PCC, propionyl-CoA carboxylase.

Enzyme	Gene	Clinical phenotype	Primary tissue(s) effected	OMIM
CPT I	*CPT1A*	Metabolic acidosis, hypoglycaemia, liver dysfunction, coma, seizures	Liver	600528
	CPT1C			608846
CPT II	*CPT2*	Rhabdomyolysis, late-onset myopathies	Skeletal muscle	255110
CACT	*SLC25A20*	Neurologic abnormalities, cardiomyopathy, arrhythmias, skeletal muscle damage, liver dysfunction	Brain, liver, heart, skeletal muscle	613698
Organic cation/ carnitine translocase	*SLC22A5*	Hypoketotic hypoglycaemia, skeletal myopathy, cardiomyopathy	Kidney, skeletal muscle, heart, pancreas	603377
VLCAD	*ACADVL*	Hypoketotic hypoglycaemia, cardiomyopathy, myoglobinuria, rhabdomyolysis	Liver, skeletal muscle	201475
MCAD	*ACADM*	Hypoglycaemia coma, impaired ketogenesis, low plasma and tissue carnitine levels	Skeletal muscle	201450
SCAD	*ACADS*	Acidosis, neurological impairment, myopathy, developmental delay	Skeletal muscle	201470
SBCAD	*ACADSB*	Asymptomatic, impaired isoleucine dehydration observed	Asymptomatic	600301
HADH	*HADH*	Recurrent myoglobinuria, hypoketotic hypoglycaemic encephalopathy, hypertrophic/dilated cardiomyopathy	Skeletal muscle	231530
MTP	*HADHA*	Neonatal dilated cardiomyopathy or progressive neuromyopathy, hypoglycaemia	Brain, skeletal muscle	600890
	HADHB	Hypoketotic hypoglycaemia, episodic myoglobinuria	Liver	143450
ACAD9	*ACAD9*	Cardiorespiratory depression, hypertrophic cardiomyopathy, encephalopathy and severe lactic acidosis	Brain, skeletal muscle	611126
ETF	*ETFA*	Nonketotic hypoglycaemia, metabolic acidosis, large amounts of fatty acid and amino acid-derived metabolites excreted	Multi-systemic	608053
	ETFB			130410
	ETFDH			231675
ECHS1	*ECHS1*	Delayed psychomotor development, neurodegeneration, increased lactic acid, brain lesions in the basal ganglia	Brain, liver, skeletal muscle	616277
PCC	*PCCA*	Propionic academia, episodic vomiting, lethargy and ketosis, neutropenia, periodic thrombocytopenia, hypogammaglobulinemia	Liver, skeletal muscle	232000
	PCCB			232050
Methylmalonyl-CoA epimerase	*MCCE*	Severe metabolic acidosis, dehydration, tachypnoea, retarded motor development, spasticity	Skeletal muscle, brain	251120
Methylmalonyl-CoA mutase	*MUT*	Developmental retardation, chronic metabolic acidosis, lethargy, failure to thrive, recurrent vomiting, dehydration, respiratory distress, hypotonia	Brain	251000

countries, reducing β-FAO-related mortalities [73]. Screening for MCAD deficiency is common across many countries [74], including the Netherlands, where it has a relatively high prevalence of 1 in 8300 [75]. NBS can also include CPT I, CPT II, CACT, VLCAD, LCHAD, TFP and MCKAT [76,77], with some countries, including the USA and Japan, also screening for SCAD and M/SCHAD [74,77].

Treatment options for β-FAO disease remain limited, and no cure is presently available. Dietary interventions aimed at regulating fatty acid levels have been trialled, but have had limited success [78]. For defects such as VLCAD deficiency, where the metabolism of longer chain fatty acids is disrupted, supplementation with medium-chain fatty acids is thought to be a potential treatment option [69]. Clinical trials of triheptanoin, an artificially produced triglyceride consisting of medium-chain fatty acids, have been shown to improve cardiac structure and function, both at rest and during exercise, in patients with LCHAD, VLCAD and CPT II deficiencies [79]. Maintaining constant high blood glucose levels has also been trialled in some patients to reduce the effects of fasting; however, this does not address the underlying aetiology [78].

Emerging links between β-FAO and OXPHOS in pathophysiology

It is clear that the β-FAO and OXPHOS pathways are tightly linked biochemically. However, there is increasing evidence that physical interactions between the components of the β-FAO and OXPHOS pathways also exist, and that these physical interactions are important for protein activity and stability in both pathways. The first interactions between β-FAO and OXPHOS proteins were shown to involve hydroxyacyl-CoA dehydrogenase (HADH) and OXPHOS complex I [80]. Similarly, the electron transfer flavoprotein (ETF) was purified in a protein complex with OXPHOS complex III, where it can donate electrons directly to this OXPHOS complex [81].

More recently, several β-FAO enzymes have been shown to comigrate with OXPHOS supercomplexes by native gel electrophoresis, including VLCAD, LCAD, MCAD, ETF and MTP [23]. Furthermore, isolated fractions containing the OXPHOS supercomplex can directly oxidize palmitoyl-CoA and octanoyl-CoA, suggesting the OXPHOS supercomplex harbours β-FAO activity [23]. Taken together, these findings suggest that a metabolically active superstructure comprised of both β-FAO and OXPHOS complexes (potentially in the form of an OXPHOS supercomplex) exists within the mitochondria.

Interestingly, patients with primary β-FAO deficiencies can also exhibit significant secondary OXPHOS enzyme defects. As early as 1996, it was noted that patients with LCHAD deficiency also exhibit defects in OXPHOS complex I activity [82–85]. Historically, it has been thought that these secondary OXPHOS defects are due to the accumulation of fatty acyl-CoA intermediates that inhibit normal OXPHOS function. However, the identification of physical β-FAO-OXPHOS interactions (as described above) suggests that more complex mechanisms are involved.

Recently, secondary OXPHOS defects were described in patients with MCAD deficiency [24]. MCAD deficiency is one of the most common β-FAO disorders, presenting with hypoketotic hypoglycaemia, vomiting and lethargy [62]. Fibroblasts from MCAD-deficient patients exhibit reduced carbohydrate oxidation, as well as decreased steady-state levels of OXPHOS complexes I, III, IV and the OXPHOS supercomplex [24]. In addition, assembly of OXPHOS subunits into *de novo* OXPHOS complexes was also disrupted in cells lacking MCAD expression [24]. These findings highlight that the loss of MCAD is associated with a disruption of both OXPHOS complex assembly and stability, which subsequently contributes to defects in OXPHOS function.

Interestingly, the normalization of blood glucose levels can be used to alleviate symptoms following episodes of encephalopathy in MCAD-deficient patients [78]. However, this form of treatment is not always successful, potentially due to the disruption of carbohydrate metabolism caused by the secondary OXPHOS defects in these patients. Furthermore, increased oxidative stress, due to the disruption of OXPHOS, may also be contributing to MCAD deficiency disease pathogenesis. Increased ROS production has been reported in patients with MCAD deficiency [86,87], while cells lacking MCAD expression exhibit elevated ROS generation associated with increased sensitivity to OXPHOS complex III inhibition [24].

Further links between β-FAO and OXPHOS have been uncovered through studies examining the biogenesis of OXPHOS complex I. Complex I is the largest of the OXPHOS complexes, assembled from 45 subunits that are encoded by both nuclear and mitochondrial DNA [88,89]. Proper assembly of complex I requires the assistance of multiple assembly factors, including acyl-CoA dehydrogenase 9 (ACAD9) [90,91]. ACAD9 was initially identified as an acyl-CoA dehydrogenase with activity for C16:0 and C18:0 saturated fatty acids, with activity for C16:1, C18:1, C18:2 and C22:6 unsaturated fatty acids also described [92–94]. However, ACAD9 was subsequently shown to interact

with the *bona fide* complex I assembly factors NDUFAF1 and Ecsit, with *ACAD9* knockdown resulting in complex I deficiencies [90,91]. Further studies have shown that mutations in *ACAD9* cause defects in both OXPHOS and β-FAO, suggesting ACAD9 plays an important role in both complex I assembly and as a fatty acyl-CoA dehydrogenase [91].

Additionally, the β-FAO proteins HADH and enoyl-CoA delta isomerase 1 (ECI1) are predicted to be involved in complex I biogenesis. Phylogenetic comparison of species with, or without, complex I has been used to generate a complex I phylogenetic profile (COPP) gene list of putative complex I assembly factors [95,96]. While HADH and ECI1 are both parts of the COPP list, they are yet to be experimentally confirmed as complex I assembly factors in the same way that other proteins such as NDUFAF5/C20orf7 [97], FOXRED1 [98], NDUFAF6/C8orf38 [99] and NDUFAF3/C3orf60 [100] have been.

ECHS1 deficiency

ECHS1D onset generally occurs during early infancy, often at birth. The median lifespan is 2 years; however, death can occur within the first 2 days of life in severe cases [1,6,8]. ECHS1D patients typically present with Leigh syndrome (subacute necrotizing encephalomyelopathy; LS) or Leigh-like syndrome (LLS), with symptoms including developmental delay, dystonia, cardiomyopathy, bilateral symmetric brain lesions, metabolic acidosis and apnoea [15,22,101]. Leigh syndrome is a neurodegenerative disease that is not typically observed in other β-FAO disorders, and is more commonly associated with primary OXPHOS deficiencies. Over 75 genes have been associated with LS/LLS, including those that encode OXPHOS complex subunits as well as genes involved in OXPHOS protein biogenesis and assembly [22].

Almost all reported cases of ECHS1D present with T2 bilateral hyperintensities, a hallmark of LS. In fact, it has been proposed that ECHS1D is a distinct form of LS, associated with progressive encephalopathy, mitochondrial dysfunction and bilateral brain lesions [9]. While LS and LLS are common in ECHS1D patients, other symptoms, including exercise-induced metabolic stress and growth deficiency, or mitochondrial encephalopathy with cardiac involvement, have also been reported [9,102]. In addition, two patients have presented with paroxysmal exercise-induced dyskinesia (PED), which is a milder form of ECHS1D with a more positive prognosis [11,14].

All currently identified ECHS1D patients have mutations in both *ECHS1* alleles, indicating autosomal recessive inheritance, with many different mutations identified [1–21] (Table 2). Patients who are homozygous for mutations in *ECHS1* have all been offspring of consanguineous relationships, resulting in two copies of the same rare mutation [1,6,7,9,12]. These mutations can affect the mitochondrial targeting sequence, intro/exon boundaries, splice sites, potential protein–protein interaction sites or encode premature stop codons that lead to non-sense-mediated decay of the mRNA [9,14,102]. Interestingly, two possible *ECHS1* founder mutations have been identified; p.Asn59Ser, which is present in multiple patients of Japanese descent [9,13,20,21], and p.Thr180Ala, identified in an Irish traveller family and in French–Canadian patients [7,19]. In addition, p.Gln159Arg has also been suggested as a founder mutation of Pakistani origin [7], although this mutation has also been identified in patients of German, Japanese and North American ancestry [9,19].

ECHS1 activity has found to be reduced whenever it has been measured in patients with confirmed pathogenic *ECHS1* mutations [4,6,9,10,15,16,20,21]. Interestingly, certain mutations appear to cause a more severe reduction in ECHS1 activity than others. Yamada *et al.* [21] explored this idea by expressing the p.Asn59Ser and p.Ala138Val variants of ECHS1 and measuring their activity compared to wild-type ECHS1. The p.Asn59Ser variant had almost no detectable ECHS1 activity, whereas the p.Ala138Val variant had approx. 30% activity compared to the control. Interestingly, when both mutations were expressed (as in patients III-2 and III-3), ECHS1 activity was 15% of wild-type levels [21]. Alternatively, other patients with the p.Ala158Asp mutation, in conjunction with a mutation affecting the splicing of ECHS1, had no detectable ECHS1 activity compared to controls [15]. These patients died within 8 months of birth, suggesting a correlation between disease severity and loss of ECHS1 activity [15]. However, as ECHS1 activity has not been reported for every patient with confirmed pathogenic *ECHS1* mutations, a definitive correlation between residual ECHS1 activity and clinical presentation remains elusive.

Similarly, attempts to correlate specific ECHS1D genotypes and phenotypes have proved difficult, and it is currently unclear what influences disease severity in ECHS1D patients. Notably, none of the identified pathogenic *ECHS1* mutations impact the key residues of Gly141 (substrate binding) or Glu164 (catalytic activity) (Table 2) [16]. Some genotypes have been linked to a more severe phenotype, such as p.Ala31Glufs*23 [1]. This mutation causes a frameshift and premature stop codon early in the *ECHS1* gene, with

Table 2. A clinical, biochemical and metabolic summary of all reported ECHS1D patients. T2 hyperintensity refers to regions of high intensity on T2 weighted magnetic resonance imaging scans of the brain. NR, not reported; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V; C3, malonylcarnitine; C4, butyrylcarnitine; C5, glutarylcarnitine; C6, hexanoylcarnitine; C10, decanoylcarnitine. Red highlighting refers to a potential founder mutation (p.Asn59Ser) in patients of Japanese ancestry, green highlighting refers to a founder mutation (p.Thr180Ala) found in patients of French/Canadian descent derived from an Irish traveller family, and blue highlighting refers to the commonly occurring p.Gln159Arg mutation.

Reference	Respective patient ID	Age of onset/diagnosis	Death	Sex	Parental consanguinity	Mutation (genetic level)	Mutation (protein effect)	Mutation (genetic level)	Mutation (protein effect)	T_2 hyper-intensity	Acylcarnitine profile	PDC activity	OXPHOS activity
Peters *et al.* [15]	1	Birth	4 months	F	No	c.473C>A	p.Ala158Asp	c.414+3G>C	splicing	Yes	NR	Reduced	NR
	2	3 months	8 months	M	No	c.473C>A	p.Ala158Asp	c.414+3G>C	splicing	NR	NR	Reduced	NR
Sakai *et al.* [16]	1	2 months	NR	M	No	c.2T>G	p.Met1Arg	c.5C>T	p.Ala2Val	Yes	Normal	NR	Reduced CI, CIII, and CIV in muscle Reduced CI, CIV and CV in immortalized myoblasts
Haack *et al.* [9]	F1,II:2	Birth	4 months	F	No	c.176A>G	p.Asn59Ser	c.476A>G	p.Gln159Arg	Yes	Normal	NR	Reduced CI in liver, normal in heart and muscle
	F2,II:1	Birth	11 months	M	No	c.197T>C	p.Ile66Thr	c.449A>G	p.Asp150Gly	Yes	Normal	Reduced	Normal in fibroblasts
	F3,II:6	Birth	3 years	F	Yes	c.476A>G	p.Gln159Arg	c.476A>G	p.Gln159Arg	Yes	Normal	NR	NR
	F4,II:1	Birth	7.5 years	M	No	c.161G>A	p.Arg54His	c.817A>G	p.Lys273Glu	NR	NR	NR	Normal in fibroblasts (but reduced overall ATP production)
	F5,II:3	Birth	Alive at 2.3 years	F	Yes	c.673T>C	p.Cys225Arg	c.673T>C	p.Cys225Arg	Yes	Normal	NR	Normal in fibroblasts
	F6,II:1	Birth	Alive at 3 years	F	No	c.98T>C	p.Phe33Ser	c.176A>G	p.Asn59Ser	Yes	Normal	NR	Reduced CIV in muscle
	F7,II:2	2 years	Alive at 5 years	F	No	c.268G>A	p.Gly90Arg	c.583G>A	p.Gly195Ser	Yes	NR	NR	Normal in fibroblasts
	F8,II:1	1 year	Alive at 8 years	F	No	c.161G>A	p.Arg54His	c.394G>A	p.Ala132Thr	NR	NR	NR	NR
	F9,II:2	Birth	Alive at 16 years	F	No	c.161G>A	p.Arg54His	c.431dup	p.Leu145Alafs*6	Yes	NR	Normal	Normal in fibroblasts
	F10, II:1	11 months	Alive at 31 years	F	No	c.229G>C	p.Glu77Gln	c.476A>G	p.Gln159Arg	Yes	NR	NR	Normal in fibroblasts
Ferdinandusse *et al.* [6]	1	Birth	24 h	F	Yes	c.817A>G	p.Lys273Glu	c.817A>G	p.Lys273Glu	NR	Normal	Reduced	Normal in fibroblasts
	2	Birth	2 days	F	Yes	c.817A>G	p.Lys273Glu	c.817A>G	p.Lys273Glu	Yes	Normal	Reduced	Normal in fibroblasts
	3	Early infancy	Alive at 7 years	F	No	c.433C>T	p.Leu145Phe	c.476A>G	p.Gln159Arg	NR	Normal	NR	NR
	4	1 year	3 years	M	No	c.673T>C	p.Cys225Arg	c.674G>C	p.Cys225Ser	Yes	Normal	NR	NR
Tetreault *et al.* [19]	1	2.5 months	10 months	F	No	c.538A>G	p.Thr180Ala	c.583G>A	p.Gly195Ser	Yes	Normal	Reduced	Normal in fibroblasts, mild reduction of CI and CIII in muscle
	2	2.9 years	Alive at 18 years	M	No	c.538A>G	p.Thr180Ala	c.713C>T	p.Ala238Val	Yes	Normal	Normal	Normal in fibroblasts and muscle
	3	10 months	Alive at 13 years	M	No	c.538A>G	p.Thr180Ala	c.713C>T	p.Ala238Val	Yes	Normal	NR	Normal in fibroblasts and muscle
	4	6 months	Alive at 12 years	F	No	c.538A>G	p.Thr180Ala	c.476A>G	p.Gln159Arg	Yes	Normal	Normal	Normal in fibroblasts and muscle
Yamada *et al.* [21]	III-2	10 months	Alive at 7 years	F	No	c.176A>G	p.Asn59Ser	c.413C>T	p.Ala138Val	Yes	Normal	NR	Normal in fibroblasts
	III-3	7 months	5 years	M	No	c.176A>G	p.Asn59Ser	c.413C>T	p.Ala138Val	Yes	Normal	NR	NR
Ganetzky *et al.* [8]	1	Prenatal	16 h	M	No	c.8C>A	p.Ala3Asp	c.389T>A	p.Val130Asp	NR	Mild C4 elevation	NR	NR
	2	Prenatal	24 h	F	No	c.8C>A	p.Ala3Asp	c.389T>A	p.Val130Asp	NR	Mild C4 elevation	NR	NR
Olgiati *et al.* [14]	II-1	3.5 years	Alive at 17 years	M	No	c.232G>T	p.Glu78Ter	c.518C>T	p.Ala173Val	Yes	NR	NR	NR
	II-2	4.5 years	Alive at 15 years	M	No	c.232G>T	p.Glu78Ter	c.518C>T	p.Ala173Val	Yes	NR	NR	NR

Table 2. (Continued).

Reference	Respective patient ID	Age of onset/diagnosis	Death	Sex	Parental consanguinity	Mutation (genetic level)	Mutation (protein effect)	Mutation (genetic level)	Mutation (protein effect)	T_2 hyper-intensity	Acylcarnitine profile	PDC activity	OXPHOS activity
Nair *et al.* [12]	1	Birth	24 h	F	Yes	c.842A>G	p.Glu281Gly	c.842A>G	p.Glu281Gly	NR	Elevated C4 and C6	NR	NR
Stark *et al.* [18]	1	11 months	NR	F	NR	c.160C>T	p.Arg54Cys	c.160C>T	p.Arg54Cys	NR	NR	NR	NR
Mahajan *et al.* [11]	1	8 years	Alive at 8 years	M	No	c.518C>T	p.Ala173Val	c.817A>G	p.Lys273Glu	Yes	NR	NR	NR
Al Mutairi *et al.* [1]	1	Birth	2 days	F	Yes	c.88+5G>A	p.Ala31Glufs*23	c.88+5G>A	p.Ala31Glufs*23	NR	Mild C3, C4, C5 and C10 elevation	Reduced	NR
	2	Birth	8 h	M	Yes	c.88+5G>A	p.Ala31Glufs*23	c.88+5G>A	p.Ala31Glufs*23	NR	Normal	Normal	Normal in fibroblasts
Balasubramaniam *et al.* [3]	1	17 months	Alive at 4.5 years	F	No	c.476A>G	p.Gln159Arg	c.538A>G	p.Thr180Ala	Yes	NR	NR	NR
Bedoyan *et al.* [4]	1	Birth	39 days	M	No	c.836T>C	p.Phe279Ser	c.8C>A	p.Ala3Asp	Yes	NR	Reduced	Reduced in fibroblasts
Huffnagel *et al.* [10]	1	6 weeks	Alive at 26 years	F	No	c.229G>C	p.Glu77Gln	c.563C>T	p.Ala188Val	Yes	Normal	NR	NR
Ogawa *et al.* [13]	Pt376	NR	NR	NR	No	c.98T>C	p.Phe33Ser	c.176A>G	p.Asn59Ser	NR	NR	NR	Reduced CIV in fibroblasts
	Pt536	NR	NR	NR	No	c.5C>T	p.Ala2Val	c.1A>G	p.Met1Val	NR	NR	NR	Normal in fibroblasts
	Pt1038	NR	NR	NR	No	c.5C>T	p.Ala2Val	c.176A>G	p.Asn59Ser	NR	NR	NR	Normal (reduced O_2 consumption rate)
	Pt1135	NR	NR	NR	No	c.5C>T	p.Ala2Val	c.176A>G	p.Asn59Ser	NR	NR	NR	Reduced CI in fibroblasts
Fitzsimons *et al.* [7]	1	5 months	3 years	M	Yes	c.476A>G	p.Gln159Arg	c.476A>G	p.Gln159Arg	Yes	Normal	Reduced	Normal in fibroblasts
	2	3 months	21 months	M	Yes	c.538A>G	p.Thr180Ala	c.538A>G	p.Thr180Ala	Yes	Normal	NR	NR
	3	5 months	28 months	F	Yes	c.538A>G	p.Thr180Ala	c.538A>G	p.Thr180Ala	Yes	Normal	Normal	Normal in fibroblasts, reduced CIII in muscle
	4	2 weeks	13 months	M	Yes	c.538A>G	p.Thr180Ala	c.538A>G	p.Thr180Ala	Yes	Normal	NR	NR
Carlston *et al.* [5]	1	NR	Alive at 8 years	M	No	c.79T>G	p.Phe27Val	c.789_790del	p.Phe263Leufs*7	NR	NR	NR	NR
Aretini *et al.* [2]	1	3 years	Alive at 19 years	M	No	c.713C>T	p.Ala238Val	~ 35 kb deletion in coding region	N/A	NR	NR	NR	NR
Uchino *et al.* [20]	1	NR	NR	F	No	c.23T>C	p.Leu8Pro	c.176A>G	p.Asn59Ser	Yes	NR	NR	NR

homozygous patients dying within 48 h of life [1] (Table 2).

Similarly, the p.Gln159Arg mutation appears to correlate with a severe disease phenotype [6,7,9,19]. Two patients homozygous for this mutation both died at 3 years of age [7,9], with a heterozygous patient (p.Asn59Ser; p.Gln159Arg) dying at 4 months of age [9]. The p.Gln159Arg mutation is highly conserved in vertebrates, with the resulting substitution from a polar, uncharged residue to a positively charged residue predicted to be 'possibly damaging' [103] and disease-causing [9,19]. However, other patients heterozygous for the p.Gln159Arg mutation have less severe clinical progression and remain alive at 4.5 years (p.Thre180Ala; p.Gln159Arg), 7 years (p.Leu145Phe; p.Gln159Arg), 12 years (p.Thre180Ala; p.Gln159Arg) and 31 years (p.Glu77Gln; p.Gln159Arg) of age at the time of reporting [6,7,9,19]. These findings suggest that the corresponding heterozygous mutations may be less pathogenic than the p.Gln159Arg mutation; however, it should be noted that all of these mutations are associated with LS (which in itself is a severe disorder) and that they are all predicted to be disease-causing [9,19].

Conversely, both reported PED patients share the p.Ala173Val mutation, suggesting that this mutation is associated with milder clinical symptoms [14]. In fact, alanine at residue 173 is not completely conserved, with valine (as well as other amino acids) present at this position in other species. However, the p.Ala173Val mutation *is* predicted to be 'possibly damaging' [103], and it should be noted that a sibling of one of the PED patients suffered from Leigh-like symptoms [14]. This discrepancy further highlights the clinical heterogeneity associated with specific mutations, as well as the difficulty in assigning genotype/phenotype correlations in ECHS1 deficiency.

Different genotype combinations may also influence biochemical dysfunction and clinical presentation. For example, Pt536 (p.Ala2Val; p.Met1Val) has a normal maximal respiration rate, whereas Pt1038's (p.Ala2Val; p.Asn59Ser) maximal respiration rate is reduced to only 51% of control levels [13]. This suggests that the p.Asn59Ser mutation in the aforementioned patient is the primary pathogenic contributor. Indeed, this mutation is predicted to be 'probably damaging' with a maximum score of 1.0 [103] and is also associated with early lethality in other patients (Patient F1, II:2) [9].

Conversely, the p.Met1Val and p.Ala2Val mutations may only be mildly pathogenic, which could be due to their location within the first 27 residues that form the mitochondrial targeting signal of ECHS1. Neither mutation is predicted to alter the amphipathic status of the targeting signal [104] and would therefore not disrupt its localization to the mitochondrial matrix (or the function of the mature ECHS1 protein once it has been processed). Conversely, some patients with mutations in this region (p.Ala3Asp) appear to have a more severe phenotype with early death [4,8]. This p.Ala3Asp mutation substitutes a hydrophobic side chain for a much larger uncharged polar side chain, which could impact mitochondrial import and subsequently ECHS1 expression within the mitochondria [104].

Biochemical and metabolic characterization of ECHS1D

While the clinical presentation of ECHS1D can vary, there are often common underlying biochemical and/or metabolic defects. Urinalysis frequently reveals elevated levels of *S*-(2-carboxypropyl) cysteine and *N*-acetyl-*S*-(2-carboxypropyl) cysteine, even in milder cases [21]. More severe cases also include the detection of methacrylate and 2-methyl-2,3-dihydroxybutyric metabolites [4,7,8,15]. Interestingly, only patients with severe clinical presentation have high acylcarnitine levels (C4 and C6 lengths) [12].

These findings support the use of urinary metabolite analysis for the diagnosis and prognosis of ECHS1D. Early diagnosis could be achieved through the detection of *S*-(2-carboxypropyl) cysteine and *N*-acetyl-*S*-(2-carboxypropyl) cysteine, while acylcarnitine profiling may serve as an indicator of ECHS1 disease severity.

ECHS1 is important in valine metabolism, where is converts methacrylyl-CoA and acryloyl-CoA to (*S*)-3-hydroxyisobutyryl-CoA and 3-hydroxypropionyl-CoA, respectively [105,106]. Loss of ECHS1 activity results in the accumulation of these highly reactive intermediates, which can become toxic *via* spontaneous reactions with sulphydryl groups, causing impairment of ATP production and metabolic acidosis [107] [reaction with free cysteine produces the *S*-(2-carboxypropyl) cysteine detected in ECHS1D patient urine]. Furthermore, methacrylyl-CoA and acryloyl-CoA can inhibit the function of the pyruvate dehydrogenase complex (PDC) by reacting with the E2 subunit's lipoyl domains [6]. This inhibition appears to be specific to the PDC, as no other lipoyl domain-containing enzymes within the mitochondria are reported to show inhibition due to methacrylyl-CoA and acryloyl-CoA accumulation [6].

Interestingly, there may be a correlation between ECHS1 phenotypic severity and PDC activity. Patients with low PDC activity also have high lactate levels, with several of these patients presenting with more

severe prognosis [7,9,15,19], including death within 48 h of birth [1,6]. In contrast, milder cases of ECHS1D do not exhibit reduced PDC activity or lactic acidosis [11,14,21].

Methacrylyl-CoA and acryloyl-CoA are the only metabolites identified to accumulate in ECHS1D, suggesting that ECHS1 is essential in valine metabolism, but not isoleucine or leucine metabolism [7].

Secondary OXPHOS defects in ECHS1D

ECHS1D patients can exhibit a wide range of OXPHOS dysfunction, with decreased activity of complex IV detected in three patients [9,13,16]. Two of these patients have the same ECHS1 mutations, p.Phe33Ser and p.Asn59Ser, suggesting a possible genotype/phenotype correlation between these mutations and complex IV dysfunction. Interestingly, the third patient, who has p.Met1Arg and p.Ala2Val ECHS1 mutations, exhibits a combined complex IV, complex I and complex III deficiency in muscle [16]. While the p.Ala2Val mutation is not predicted to impact the mitochondrial targeting signal (as described above), the p.Met1Arg mutation substitutes a hydrophobic side chain for a positively charged side chain, potentially disrupting an important hydrophobic region of the targeting peptide [104]. Consequently, the p.Met1Arg mutation would be highly pathogenic, causing secondary OXPHOS dysfunction due to the lack of mature ECHS1 protein within the mitochondria. Immortalized myoblasts derived from this third patient also exhibited a combined complexes I, IV and V deficiency, with the expression of exogenous wild-type ECHS1 restoring OXPHOS activities to normal levels [16]. This finding highlights the importance of ECHS1 expression within the mitochondrial matrix for maintaining normal OXPHOS function.

Complex I and complex III defects have also been observed independently in two other patients [7,13]. Reduced complex I activity was found in a patient with p.Ala2Val and p.Asn59Ser mutations, whereas reduced complex III activity was detected in muscle in a patient homozygous for the p.Thr180Ala mutation [9]. Notably, a patient with p.Thr180ala and p.Gly195-Ser mutations also exhibited complex III deficiency in muscle (as well as a complex I defect), suggesting a potential correlation between the p.Thr180Ala mutation and secondary muscle complex III defects.

While there is some correlation between *ECHS1* genotype and OXPHOS dysfunction (as described above), this is not the case for all of the *ECHS1* mutations that have been identified. For example, patients Pt1135 and Pt1038 have the same *ECHS1* genotype (p.Ala2Val; p.Asn59Ser) but exhibit either decreased complex I activity (Pt1135), or reduced O_2 consumption with normal complex I activity (Pt1038) [13]. This could be indicative of other unknown susceptibility factors that are modulating the effect of ECHS1 deficiency on OXPHOS function, such as genetic polymorphism of transcription factors or post-translational modification sites, or epigenetic regulation of ECHS1 expression.

Interestingly, one study has shown a reduction in the steady-state levels of OXPHOS complex IV in ECHS1D patient fibroblasts using blue native (BN)-PAGE [19]. Only one other study to date has investigated OXPHOS complex levels in ECHS1D, with no changes reported [16]. It is currently unclear why the levels of complex IV were reduced in the patient reported by Tetreault *et al*., but it can be hypothesized that ECHS1 may play some role in maintaining OXPHOS complex stability in a similar fashion to other FAO proteins such as MCAD and ACAD9 [24,91]. As such, the loss of ECHS1 expression may result in the degradation of OXPHOS complex IV due to the loss of stabilizing physical interactions. However, further research is required to elucidate if (and how) ECHS1 is involved in OXPHOS protein complex biogenesis.

Overall, these secondary OXPHOS defects may play an important role in ECHS1D pathogenesis; indeed, the LS or LLS presentation of ECHS1D is more commonly associated with OXPHOS defects than with β-FAO deficiencies. However, we need to develop a better understanding of how the OXPHOS and β-FAO pathways interact (in particular the interactions involving ECHS1), before we can determine how these secondary OXPHOS defects impact the pathology, clinical presentation and prognosis of ECHS1D.

Treatment of mitochondrial disease and ECHS1D

The phenotypic diversity of ECHS1D, in addition to the multiple enzymatic roles that ECHS1 performs, has resulted in difficulty in both diagnosing and treating ECHS1D [21]. No two ECHS1D patients, including siblings, have presented with the exact same set of clinical symptoms. Many patients with ECHS1D are initially diagnosed by their clinical presentation of LS or LLS, and do not undergo molecular diagnosis until later in life, or in some cases posthumously [1,2,8,12]. As such, they receive standard treatments that are used for LS, LLS and other mitochondrial disorders. These treatments are mostly nonspecific and include dietary

changes, exercise-related therapies or mitochondrial 'cocktails' that contain CoQ_{10}, vitamins C and E, riboflavine, creatine monohydrate and other antioxidants [108]. These treatments can help alleviate disease symptoms; however, only anecdotal evidence currently exists to support their use [11,15,109,110], with a Cochrane review finding no clear evidence to support the use of any current mitochondrial disease treatments [111].

Restricting the consumption of specific fatty acids has been trialled in several FAO disorders [112–114], aiming to reduce the build-up of potentially toxic intermediates [115]. In a similar fashion, ketogenic (keto) diets are designed to decrease the amount of long-chain fatty acids, reducing the burden on medium-chain and short-chain acyl-CoA dehydrogenases to limit the build-up of fatty acyl-CoA intermediates [116,117]. While keto diets may provide some benefit for patients with milder forms of ECHS1D, they were ineffective in improving symptoms in severe cases [4,6].

New treatment strategies, including the use of antioxidants, mitochondrial biogenesis stimulators and metabolic analogues, are now being developed for LS and could also prove effective for treating ECHS1D patients [118,119]. Idebenone, a CoQ_{10} derivative, can be taken up by cells and cross the blood–brain barrier more effectively than CoQ_{10} [120]. Initially used as a treatment for Leber Hereditary Optic Neuropathy, idebenone has also been investigated for treating LS, exhibiting improved mitochondrial uptake and increased mitochondrial ATP production, as well as increased abdominal and ribcage movement in a LS patient [120]. However, more studies are required to determine the efficacy of idebenone as a therapeutic for LS or ECHS1D [118].

EPI-473, a synthetic derivative of CoQ_{10} that can easily cross the blood–brain barrier, has also been trialled in patients with a range of mitochondrial diseases, including LS. While its exact mechanism of action is unclear, EPI-743 appears to target the repletion of reduced intracellular glutathione [121]. Initial results showed clinical improvements in all patients (except one whom died during the course of the trial due to natural disease progression) with no severe side effects observed [122]. A follow-up trial of EPI-743 in another ten LS patients resulted in improved clinical symptoms and reversal of disease progression in nine patients [123]. As EPI-743 is showing promise for treating multiple mitochondrial diseases, including LS, it may also be beneficial for ECHS1D patients.

Pyruvate therapy is another treatment that has been trialled in LS patients [124]. Pyruvate treatment reduces the cytoplasmic $NADH/NAD^+$ ratio, stimulating glycolysis to reduce the burden on OXPHOS to generate ATP [125]. LS patients treated with pyruvate show decreased lactate levels in blood and cerebrospinal fluid, as well as improved clinical symptoms (however, neurological symptoms did not improve in one patient) [124].

Pyruvate has also been shown to stimulate the PDC *via* its inhibition of pyruvate dehydrogenase kinase [125]. Apart from increasing glycolytic ATP production, this effect of pyruvate may provide additional benefit for the ECHS1D patients who display reduced PDC activity (Table 2). However, as the molecular basis for PDC deficiency in ECHS1D is unknown, further investigation is required before pyruvate therapy can be considered, as it may prove toxic if PDC activity is completely absent and cannot be restored in ECHS1D patients.

Rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), is another compound with therapeutic potential for treating mitochondrial disorders. Modelling in mitochondria-defective yeast showed that inhibition of mTOR *via* caloric restriction was sufficient to rescue lifespan [126]. Subsequent rapamycin treatment of NDUFS4 knockout mice, which model human LS, also exhibited increased lifespan with no development of LS-associated neurological lesions [127]. Interestingly, reduced ECHS1 expression in various cancer cell models results in the accumulation of branched-chain amino acids and fatty acids that activate mTOR signalling to induce apoptosis [128]. As such, rapamycin inhibition of mTOR may prove beneficial in ECHS1D patients, where a similar reduction in ECHS1 expression may also activate mTOR signalling. However, rapamycin treatment can cause serious side effects, such as immune suppression and hyperlipidaemia, which need to be addressed before it is suitable for any therapeutic use [129].

5-Aminolevulinic acid (5-ALA), in combination with sodium ferrous citrate, has also been proposed as a potential therapy for mitochondrial disease. 5-ALA is a precursor of haem, an important prosthetic group of OXPHOS complexes II, III and IV, as well as cytochrome *c*. 5-ALA has been trialled in LS patients under the age of 2 years, with ongoing clinical trials currently evaluating its efficacy [130].

An interesting therapeutic approach that targets overall mitochondrial function, rather than the underlying defect of disease, is the stimulation of mitochondrial biogenesis. Stimulating mitochondrial biogenesis aims to increase mitochondrial mass, allowing for the metabolic needs of the cell to be met. This technique has been tested in mice using compounds such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)

[132], in human fibroblasts using resveratrol [132] and in mitochondrial disease patients using bezafibrate [133]. AICAR stimulates mitochondrial biogenesis by activating the 'master regulator' of mitochondrial biogenesis, PGC-1α [131]. When trialled in a COX-defective mouse myopathy model, AICAR was shown to result in improving motor function and increased expression of OXPHOS and FAO genes [131]. Resveratrol is a naturally occurring compound found in red wine and has been shown to improve mitochondrial function *via* the stimulation of PGC-1α; however, the exact mechanism involved is unclear [134]. Resveratrol prevents lactate build-up and increases OXPHOS complex subunit levels and mitochondrial respiration in complex I or complex IV deficient fibroblasts [132]. These effects of resveratrol make it an interesting compound for potentially treating patients with LS and ECHS1D, where lactate levels can be elevated.

Bezafibrate has also been investigated for its potential to stimulate mitochondrial biogenesis *via* increased PPAR-γ expression. An initial trial of patients with CTP II and VLCAD deficiencies found that bezafibrate was unable to improve symptoms or FAO function during exercise, suggesting that previous *in vitro* findings would not translate to a clinical setting [135]. However, more recent *in vitro* and *in vivo* studies have shown greater therapeutic potential [133,136]. Bezafibrate treatment increased PGC-1α expression in human induced pluripotent stem cells, resulting in increased cell number and SDHA (OXPHOS complex II subunit) and COX-1 (OXPHOS complex IV subunit) levels [133]. MtDNA copy number, indicative of the amount of mitochondria present within the cell, was also increased in bezafibrate treated cells [133]. Interestingly, a follow-up study of patients with VLCAD or CPT II deficiencies treated with bezafibrate resulted in improved quality of life as well as increased physical functioning, confirming bezafibrate's therapeutic potential [136].

Stimulating mitochondrial biogenesis may prove effective for patients with residual ECHS1 activity, for example 15% of normal levels [21], which could be elevated to meet the requirements of the cell. However, it may not be appropriate for patients who are completely deficient in ECHS1, as activity cannot be increased in these cases. On the other hand, increasing mitochondrial mass would increase the MTP, which has some redundancy for ECHS1, providing enoyl-CoA hydratase activity for C6 fatty acids [137]. Increasing MTP levels would also allow for more C8-C16 fatty acyl-CoA esters to be metabolized, releasing NADH and $FADH_2$ for oxidation by the respiratory chain to create ATP. Additionally, if an OXPHOS defect is present, increasing mitochondrial mass may also help to alleviate the pressure on individual OXPHOS systems by increasing the amount of OXPHOS proteins able to produce ATP. As such, stimulating mitochondrial biogenesis may be able to increase ATP production in ECHS1D patients to alleviate disease symptoms, particularly if defects in both FAO and OXPHOS are present.

Concluding remarks and perspectives

Forty-six patients have been described with pathogenic mutations in *ECHS1* since the first identification of ECHS1D in 2014 [15]. Importantly, many ECHS1D patients present with Leigh syndrome (LS), a severe disorder traditionally associated with deficiencies of the OXPHOS system. While loss of ECHS1 function disrupts both β-FAO and valine metabolism, it has also been shown to cause secondary OXPHOS defects in some patients. These secondary defects may be linked to a more severe clinical ECHS1D phenotype; however, our understanding of how they contribute to ECHS1D pathogenesis is lacking.

While secondary OXPHOS defects can occur in part due to an accumulation of inhibitory fatty acid intermediates in β-FAO disorders, it is now evident that other mechanisms are also involved. Primary defects in β-FAO proteins may disrupt the activity, biogenesis and/or stability of the OXPHOS complexes, particularly *via* interaction with the OXPHOS supercomplex. As such, further research is required to improve our understanding of the mechanisms that cause secondary OXPHOS dysfunction in primary β-FAO deficiencies if we are to develop novel, targeted therapies to treat disorders such as ECHS1D.

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