Inborn errors of metabolism associated with 3-methylglutaconic aciduria

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Abstract

A growing number of inborn errors of metabolism (IEM) associated with compromised mitochondrial energy metabolism manifest an unusual phenotypic feature: 3-methylglutaconic (3MGC) aciduria. Two major categories of 3MGC aciduria, primary and secondary, have been described. In primary 3MGC aciduria, IEMs in 3MGC CoA hydratase (AUH) or HMG CoA lyase block leucine catabolism, resulting in a buildup of pathway intermediates, including 3MGC CoA. Subsequent thioester hydrolysis yields 3MGC acid, which is excreted in urine. In secondary 3MGC aciduria, no deficiencies in leucine catabolism enzymes exist and 3MGC CoA is formed *de novo* from acetyl CoA. In the "acetyl CoA diversion pathway", when IEMs directly, or indirectly, interfere with TCA cycle activity, acetyl CoA accumulates in the matrix space. This leads to condensation of two acetyl CoA to form acetoacetyl CoA, followed by another condensation between acetyl CoA and acetoacetyl CoA to form 3-hydroxy, 3-methylglutaryl (HMG) CoA. Once formed, HMG CoA serves as a substrate for AUH, producing *trans*-3MGC CoA. Non enzymatic isomerization of *trans*-3MGC CoA to *cis*-3MGC CoA precedes intramolecular cyclization to *cis*-3MGC anhydride plus CoA. Subsequent hydrolysis of *cis*-3MGC anhydride gives rise to *cis*-3MGC acid, which is excreted in urine. In reviewing 20 discrete IEMs that manifest secondary 3MGC aciduria, evidence supporting the acetyl CoA diversion pathway was obtained. This biochemical pathway serves as an "overflow valve" in muscle / brain tissue to redirect acetyl CoA to 3MGC CoA when entry to the TCA cycle is impeded.

Keywords

3-methylglutaconic aciduria; inborn error of metabolism; mitochondria; energy metabolism

I. Introduction

Inborn errors of metabolism (IEM) are a heterogeneous collection of genetic disorders that, oftentimes, result in serious disease. Of interest to the present discussion is a

group of seemingly disparate IEMs that share a common and distinctive phenotypic feature: urinary excretion of 3-methylglutaconic (3MGC) acid. The metabolic origin of 3MGC aciduria in these disorders has been the subject of much speculation, and studies have revealed that two major categories of 3MGC aciduria exist, termed primary and secondary. Primary 3MGC aciduria is characterized by defects in the leucine catabolism pathway (Figure 1) due to mutations in either *AUH* [1; OMIM #250950] or *HMGCL* [2; OMIM *613898]. *AUH* encodes 3MGC CoA hydratase (AUH), which catalyzes the 5th step in the leucine catabolism pathway, converting *trans*-3MGC CoA to (*S*)-3-hydroxy-3-methylglutaryl (HMG) CoA. The *HMGCL* gene encodes the next enzyme in the pathway, HMG CoA lyase, which converts HMG CoA to acetoacetate and acetyl CoA. When either one of these enzymes is deficient, leucine metabolism is blocked and pathway intermediates upstream of the deficient enzyme accumulate. In these IEMs, *trans*-3MGC CoA is considered to be the precursor of 3MGC acid, which is excreted in urine as a waste product. Individuals with primary 3MGC aciduria excrete large amounts of 3MGC acid [3] and excretion levels increase in response to leucine ingestion [4].

By contrast, no defects in leucine catabolism pathway enzymes have been reported in subjects with secondary 3MGC aciduria and leucine loading does not lead to increased 3MGC acid excretion. Thus, in these IEMs, 3MGC aciduria does not arise from a block in the leucine catabolism pathway. In an effort to explain the biochemical origins of secondary 3MGC aciduria, various authors have proposed that it arises from aberrant cytosolic isoprenoid metabolism [5] or peroxisomal metabolism [6]. A lack of evidence supporting these models, however, has led to the proposal that secondary 3MGC aciduria is a byproduct of IEM-induced disruption of aerobic energy metabolism. Importantly, in nearly every known mutation associated with secondary 3MGC aciduria, the affected gene product localizes to mitochondria (Table 1). In several cases, IEMs that manifest secondary 3MGC aciduria are directly involved in electron transport chain (ETC) function, while others have an indirect connection. Herein, it is proposed that IEM-induced effects on the efficiency of aerobic respiration lead to inhibition of the TCA cycle. When this occurs in skeletal muscle, brain or cardiac tissue, the acetyl CoA diversion pathway is activated (Figure 2). In the first step, acetoacetyl CoA thiolase (T2 thiolase) condenses two equivalents of acetyl CoA to form acetoacetyl CoA. In this context, T2 thiolase functions in reverse of its normal direction in the fatty acid ß-oxidation pathway. Once formed, acetoacetyl CoA condenses with another equivalent of acetyl CoA to form HMG CoA in a reaction catalyzed by HMG CoA synthase 2. Subsequently, HMG CoA is dehydrated by AUH, generating *trans*-3MGC CoA plus H_2O [7, 8].

The next part of the acetyl CoA diversion pathway involves a series of three non-enzymatic chemical reactions. As *trans*-3MGC CoA is generated via AUH, it isomerizes to *cis*-3MGC CoA. Importantly, *cis*- and *trans*- 3MGC CoA are quite different in terms of their chemical reactivity [9]. *cis*-3MGC CoA, but not *trans*-3MGC CoA, is capable of intramolecular cyclization to form *cis*-3MGC anhydride and CoA. This chemical reaction is known to occur among terminally carboxylated short-chain acyl CoAs [10]. Although *trans*-3MGC CoA is the only diastereomer generated during intermediary metabolism, steric constraints prevent formation of *trans*-3MGC cyclic anhydride. Thus, isomerization of *trans*-3MGC CoA to *cis*-3MGC CoA is an essential step in formation of cyclic 3MGC anhydride. Once formed,

cis-3MGC anhydride is reactive and subject to hydrolytic cleavage, forming *cis*-3MGC acid. Alternatively, cyclic 3MGC anhydride can react with lysine side chain amino groups to 3MGCylate proteins [9]. When this occurs, 3MGCylated proteins can be deacylated through the action of the NAD$^+$ requiring deacylase, sirtuin 4 [11]. Whereas *cis*-3MGC acid is destined for excretion, Jones et al [12] reported that 3MGC acids can also isomerize under physiological conditions, albeit very slowly. Overall, *trans*-3MGC CoA isomerization provides an explanation for the ~2:1 *cis:trans* ratio of 3MGC acid diastereomers in urine of subjects with 3MGC aciduria [13]. Thus, the acetyl CoA diversion pathway is comprised of 2 stages (enzyme-mediated and non-enzyme mediated) that, together, result in conversion of acetyl CoA to a mixture of *cis*- and *trans*-3MGC acids.

To better define specific IEMs that give rise to secondary 3MGC aciduria, the nature of these IEMs is described. In every case, 3MGC aciduria is one of several disparate, yet characteristic, phenotypic features observed. These include dilated cardiomyopathy, neutropenia, optic atrophy, lactic acidemia, muscle weakness and more. In general, IEMs known to manifest secondary 3MGC aciduria can be grouped into several categories, including a) genes that encode proteins involved in mitochondrial lipid metabolism and membrane composition; b) genes that encode protein components of ETC complexes; c) mutations that alter mitochondrial DNA; d) genes that encode matrix metabolic enzymes. A fifth group includes those IEMs that manifest secondary 3MGC aciduria but don't fit neatly into these categories. By examining different IEMs that give rise to secondary 3MGC aciduria, insight into aberrant metabolic processes responsible for its production may be achieved.

II. IEMs that affect mitochondrial lipid metabolism / membrane structure

TAZ

Perhaps the best known secondary 3MGC aciduria, Barth Syndrome (OMIM #302060), is caused by mutations in the *TAZ* (OMIM *300394) gene (Table 1), which encodes the phospholipid transacylase, tafazzin [14]. Tafazzin is predominantly expressed in cardiac and skeletal muscle tissue and localizes to the inner mitochondrial membrane where it catalyzes remodeling of the unique anionic phospholipid, cardiolipin (CL). Through the action of taffazin, the heterogeneous acyl chain composition of nascent CL is remodeled to form tetralinoleoyl CL, a key component of cristae membranes [15]. CL's cone shaped structure stabilizes the highly curved cristae membranes that house complexes of the ETC. Phenotypically, Barth Syndrome is characterized by dilated cardiomyopathy, neutropenia, muscle weakness, 3MGC aciduria and other features [16]. Lack of tafazzin activity affects the content and composition of CL, resulting in morphological changes in cristae membrane structure and, thereby, reduced ETC function [17]. Decreased aerobic respiration and ATP production is considered a root cause of dilated cardiomyopathy associated with this disorder. Another consequence of *TAZ* mutation-induced disruption of ETC function is a reduced ability to oxidize cofactors (NADH and FADH$_2$) generated during oxidative metabolism of fuel molecules. When these reduced cofactors accumulate in the matrix space, metabolic flux through the TCA cycle declines [7]. Unable to enter the cycle, accumulating acetyl CoA is diverted to 3MGC acid and excreted in urine as a waste product.

SERAC1

The *SERAC1* (Serine active site containing 1; OMIM *614725) gene encodes the eponymous phosphatidylglycerol remodeling enzyme, SERAC1. In addition to its acyl chain remodeling function, SERAC1 is involved in biosynthesis of the unique lipid, bis(monoacylglycerol)phosphate, which affects cholesterol trafficking [18]. Moreover, SERAC1 functions in phospholipid exchange at the mitochondria - endoplasmic reticulum interface [19]. Interestingly, disruption of SERAC1 activity also affects CL metabolism, consistent with PG's role as a direct precursor of CL. Clinically, mutations in *SERAC1* are causative for "MEGDEL syndrome" (3-methylglutaconic aciduria with deafness, encephalopathy and Leigh-like features) [20]. Given its function in lipid trafficking and metabolism, it is reasonable to consider that loss of SERAC1 activity will result in defects in the structure, organization and integrity of cristae membranes. Consistent with this, studies indicate that altered CL subspecies composition, oxidative phosphorylation defects, increased serum lactate and 3MGC aciduria are associated with mutations in *SERAC1* [21].

AGK

The *AGK* gene encodes acylglycerol kinase (AGK; OMIM *610345), which phosphorylates diacylglycerol, forming phosphatidic acid, a key substrate in phospholipid biosynthesis. Deficiencies in AGK are causative for Sengers syndrome [22]. Curiously, loss of AGK activity results in decreased levels of adenine nucleotide translocator 1 (ANT1) in muscle mitochondria, although no mutations in the gene encoding ANT1 (*SLC25A4*; OMIM *103220) have been reported. Defects in AGK also affect fatty acid metabolism and glycogen accumulation in cardiac tissue. The finding that 70% of patients with mutations in *AGK* manifest 3MGC aciduria [23] is consistent with other phenotypic features associated with this disorder, including dilated cardiomyopathy and lactic acidemia.

C19orf70

The *C19orf70* (chromosome 19 open reading frame 70; OMIM *616658) gene encodes QIL1 (also known as MIC13), a subunit of the mitochondrial contact site and cristae organizing system (MICOS) complex involved in formation / stabilization of cristae junctions. MICOS is a multi-subunit complex that modulates the placement, distribution and number of cristae in mitochondria. It is noteworthy that cristae junctions play a crucial role in oxidative phosphorylation by separating contents of the inner cristae space from the adjacent inter-membrane space. Defects in QIL1 result in decreased cristae membrane potential due to loss of cristae junctions and disruption of cristae morphology [24]. Insofar as the inner cristae space is the site where H^+ accumulation generates the proton-motive force that drives oxidative phosphorylation, a breach in cristae junction integrity will result in unproductive dissipation of this proton gradient. Loss of membrane potential disrupts aerobic respiration and is consistent with the finding that subjects with mutations in QIL1 have increased levels of lactic acid and 3MGC aciduria [25]. Taken together, it may be considered that IEMs in *C19orf70* result in a breakdown of cristae junction structure [26] which, in turn, leads to disruption of cristae morphology and diminished efficiency of oxidative phosphorylation. Under these conditions, it is expected that pyruvate generated via

glycolysis will be converted to lactic acid while acetyl CoA, unable to undergo oxidative metabolism, will be diverted to 3MGC acid.

III. IEMs that affect ETC Complexes

ATPAF2

The IEM known as mitochondrial complex V deficiency nuclear type 1 is caused by homozygous mutations in the *ATPAF2* (OMIM *608918) gene, which encodes ATP12, a protein that functions in biosynthesis and assembly of Complex V of the ETC (i.e. F_1F_o ATPase). De Meirleir and coworkers reported that mutations in *ATPAF2* that lead to a deficiency in the ATP12 protein correlate with elevated 3MGC acid levels in urine [27]. The net effect of a deficiency in ATP12 is an inability to assemble the extrinsic F_1 domain of Complex V, thereby impairing ATP production efficiency via oxidative phosphorylation. Thus, it is anticipated that, during cellular respiration, mutation-induced disruption of Complex V structure / function leads to a diminished capacity of the ETC to oxidize NADH and $FADH_2$ generated during oxidative fuel metabolism. As a result, TCA cycle activity is inhibited and acetyl CoA is diverted to 3MGC acid.

TMEM70

Mitochondrial Complex V deficiency nuclear type 2 is caused by homozygous or compound heterozygous mutations in the *TMEM70* (OMIM *612418) gene. *TMEM70* encodes "transmembrane protein 70" (TMEM70), which localizes to the inner mitochondrial membrane and functions in assembly of the F_1 and F_o subunits of Complex V. Phenotypic features of this disorder include dysmorphia, hypertonia, cardiomyopathy, psychomotor retardation and 3MGC aciduria [28,29]. Recently, Sanchez-Caballero et al [30] reported that loss of TMEM70 leads to an accumulation of assembly intermediates, consistent with a block in the Complex V assembly pathway. Ultimately, defective TMEM70 function reduces Complex V activity, resulting in increased membrane potential, enhanced reactive oxygen species (ROS) production and diminished ATP production [31]. In addition, ultrastructural studies of tissues harboring mutations in *TMEM70* indicate aberrant cristae morphology [32].

ATP5F1E

Mitochondrial Complex V deficiency nuclear type 3 (OMIM #614053) is caused by mutations in the *ATP5F1E* (OMIM *606153) gene, which encodes ATP5F1E, another subunit of Complex V. Mutations in *ATP5F1E* that result in Complex V deficiency manifest phenotypic features that include neonatal-onset hypotonia, lactic acidemia, hyperammonemia, hypertrophic cardiomyopathy and 3MGC aciduria [33].

NFS1

The *NFS1* (OMIM *603485) gene encodes a cysteine desulfurase that catalyzes the first step of Fe/S cluster formation, converting cysteine to alanine and a sulfide intermediate [34]. The resulting inorganic sulfur binds to cysteine ligands that are supplied by Fe/S cluster scaffold protein. When complexed with iron, an Fe/S cluster is produced [35]. Once formed, Fe/S clusters are incorporated into proteins, most notably, components of ETC Complexes

I, II and III. Mutations in *NFS1* disrupt Fe/S cluster assembly, causing mitochondrial dysfunction in animal models [36,37]. Recently, Hershkovitz et al [35] reported a human subject harboring a homozygous *NFS1* pathogenic variant. In this individual, serum and cerebrospinal fluid amino acid analysis indicated elevated levels of alanine and glycine while urine organic acid analysis revealed increased lactic acid and 3MGC aciduria.

LYRM4

The *LYRM4* (OMIM *613311) gene encodes "iron-sulfur protein biogenesis, desulfurase-interacting protein 11" (Isd11), an 11 kDa mitochondrial protein that functions in Fe/S cluster assembly. Isd11 is a member of the "LYR" protein family, a recently identified superfamily characterized by small size (10–22 kDa), high positive charge, an invariant Phe residue and a Leu–Tyr–Arg (LYR) motif near the N terminus [38]. LYR proteins function as subunits or assembly factors for respiratory Complexes I, II, and III. Isd11 forms a tight complex with the cysteine desulfurase, NFS1 [39], and is required for Fe/S cluster biogenesis. Mutations in *LYRM4* adversely affect Isd11 activity, resulting in disease that manifests as cardiorespiratory arrest, lactic acidemia and 3MGC aciduria [40]. Lim et al [41] reported that mutations in *LYRM4* result in ETC complex deficiency, suggesting Isd11 functions in the early stages of Fe/S cluster assembly. Given this role, the finding that mutations in Isd11 manifest 3MGC aciduria is consistent with the role Fe/S clusters play in respiratory complex function. Moreover, ETC defects that result from mutations in *LYRM4* fit with the concept that, as reduced cofactors (i.e. NADH /FADH$_2$) accumulate, product inhibition of TCA cycle activity will result in diversion of acetyl CoA to 3MGC acid.

IV. IEMs affecting mitochondrial DNA

POLG1

The enzyme DNA polymerase subunit γ (POLG) is encoded by the *POLG1* (OMIM *174763) gene. The only polymerase that functions in mitochondria, POLG exists as a trimeric complex comprised of a catalytic subunit and a dimeric accessory subunit [42]. The catalytic subunit contains three enzymatic activities, a DNA polymerase activity, a 3'–5' exonuclease activity and a lyase activity required for base excision repair. Defects in POLG increase susceptibility to errors of replicated mitochondrial DNA which, in turn, adversely affect mitochondrial function. Wortmann et al [43] reported that a subset of patients harboring mutations in *POLG1* manifest 3MGC aciduria. Patients with this phenotypic feature often present with other maladies, including encephalopathy (epilepsy, psychosis, depression) and liver failure.

tRNA^Leu^ (UUR)—A mutation in the transfer RNA leucine (tRNA^Leu(UUR)^) coding region of mitochondrial DNA is associated with the disorder termed "mitochondrial encephalopathy, lactic acidosis and stroke-like episodes" (MELAS; OMIM #540000). The mutation, m.3243A>G (OMIM *590050), produces a MELAS disease phenotype. Of interest to the present discussion, Iwanicka-Pronicka et al [44] and Wortmann et al [45] reported that subjects with this mutation manifest 3MGC aciduria.

Pearson marrow-pancreas syndrome—Pearson marrow-pancreas syndrome (PS; OMIM #557000) is a rare mitochondrial disorder caused by single, large deletions (1– 10 kbp) of mitochondrial DNA. The most common deletion, which occurs in about 20 percent of affected individuals, is 4.9 kbp in length. Whereas PS is associated with impaired respiratory chain complexes, clinical features of this disorder vary widely, depending in the specific nature of the mitochondrial DNA deletion. Of interest to the present discussion, Sato et al [46] reported a PS patient that developed pancytopenia, lactic acidemia, steatorrhea, insulin-dependent diabetes mellitus, liver dysfunction, Fanconi syndrome and 3MGC aciduria. These authors identified a 5.4 kbp deletion of mitochondrial DNA in this subject. Analysis revealed the activity of Complexes I and IV were markedly reduced in liver and muscle and mildly reduced in skin fibroblasts and heart. Importantly, among patients diagnosed with PS, only a subset manifest 3MGC aciduria. However, given the fact that not all subjects diagnosed with PS carry the same DNA deletion, it is not surprising that they manifest variable phenotypic features.

V.   Metabolic enzymes

SUCLA2

The *SUCLA2* (OMIM *603921) gene encodes the ß-subunit of succinyl CoA ligase, also known as ADP-forming succinyl-CoA synthetase. In the TCA cycle, succinyl CoA synthetase catalyzes the conversion of succinyl-CoA plus GDP to succinate, CoA and GTP. Based on this key metabolic function, it may be anticipated that defective succinyl CoA synthetase activity will impede TCA cycle activity, resulting in a buildup of acetyl CoA. In this case, it is anticipated that the acetyl CoA diversion pathway would generate 3MGC acid as a byproduct. Consistent with this, Carrozzo R et al [47] reported that mutations in *SUCLA2* result in methyl malonic aciduria, lactic acidemia and 3MGC aciduria.

ECHS1

The *ECHS1* (OMIM *602292) gene encodes for short-chain enoyl CoA hydratase (ECH), which catalyzes hydration of the double bond in 2-*trans/cis*-enoyl-CoA substrates. In mitochondrial metabolism, ECH plays an essential role in fatty acid ß-oxidation. Phenotypic features of this IEM include delayed motor and cognitive development, as well as metabolic abnormalities including elevated lactic acid levels in plasma and cerebrospinal fluid and transient 3MGC aciduria [48].

CPS1

The *CPS1* (OMIM *608307) gene encodes carbamoyl phosphate synthase, a ligase located in mitochondria that functions in production of urea. Carbamoyl phosphate synthase functions to transfer NH_4^+ from glutamine or glutamate to bicarbonate, forming carbamoyl phosphate at the expense of 2 ATP. Carbamoyl phosphate then reacts with ornithine to form citrulline via ornithine transcarbamoylase. Subsequently, citrulline exits the mitochondria and is metabolized to yield urea and ornithine, which can react with carbamoyl phosphate to complete the cycle. Whereas Rokicki et al [49] detected 3MGC aciduria in 7 of 11 cases of *CPS1* IEMs, additional insight is required to define the underlying metabolic basis of 3MGC acid production in this disorder.

VI.   Other IEMs that affect mitochondrial function.

CLPB

The *CLPB* (OMIM *616254) gene encodes "caseinolytic peptidase B protein homolog" (ClpB), also known as suppressor of potassium transport defect 3. Studies indicate that ClpB is a mitochondrial AAA$^+$ protein containing an N-terminal ankyrin-repeat domain and a C-terminal nucleotide-binding domain. Recently, Cupo and Shorter [50] reported that ClpB functions as a protein disaggregase, capable of solubilizing aggregated proteins in the inter-membrane space. This activity is achieved by coupling protein disaggregase activity to ATP hydrolysis. Of interest to the present discussion is the observation that mutations in *CLPB* are associated with 3MGC aciduria [51–54]. Other phenotypic features associated with mutations in *CLPB* include progressive encephalopathy, cataracts and neutropenia. Importantly, a correlation exists between the site of mutation, the extent of disaggregase activity disruption and disease severity [50]. In experiments designed to determine the effect of ClpB on substrate protein solubility, these authors compared the relative solubility of mitochondrial proteins in wild-type and ClpB knockout human HAP1 cells. Consistent with localization of ClpB to the inter-membrane space, when ClpB was absent, an increased proportion of proteins in the inner mitochondrial membrane and inter-membrane space were recovered in the insoluble fraction. Examination of specific proteins affected by loss of ClpB revealed that proteins involved in Ca^{2+} import, chaperone-mediated protein transport, protein insertion into the inner membrane and respiratory chain complex assembly, were prone to insolubility. Based on these findings, it is conceivable that IEMs in *CLPB* lead to defects in aerobic respiration that adversely affect TCA cycle function, resulting in diversion of acetyl CoA to 3MGC acid.

TIMM50

The *TIMM50* (OMIM *607381) gene encodes a subunit of the translocase of the inner membrane (TIM23) complex. TIM23 functions to recognize proteins that possess a mitochondrial targeting signal and promote their transit into the mitochondrial matrix. Mutations in *TIMM50* result in apoptosis via cytochrome c release [55]. Deficiency in TIM23 has also been shown to cause severe mitochondrial dysfunction by targeting key aspects of mitochondrial physiology, cristae morphology, ETC Complex assembly and mitochondrial respiratory capacity [56]. Consistent with mutation-induced disruption of energy metabolism, IEMs in *TIMM50* have been reported to manifest 3MGC aciduria [57].

Duplication 5q—Zalopnik et al [58] published a case report of a 13-year-old boy harboring a duplication in chromosome 5 that manifested 3MGC aciduria. The duplicated region, 6.56 Mbp in length, contains 72 genes, including 4 genes [*HINT1* (OMIM *601314), *LYRM7* (*615831), *UQCRQ* (*612080), *PPP2CA* (*176915)] known to be associated with neurological and mitochondrial disease, respectively.

OPA3

Mutations in *OPA3* (OMIM *606580) are causative for Costeff optic-atrophy syndrome, (OMIM #258501) characterized by early bilateral optic atrophy with decreased visual acuity, spastic paraparesis, mild ataxia and 3MGC aciduria [59,60]. Although the function of

Opa3 remains unknown, experimental evidence on the pathogenicity of *OPA3* mutations are consistent with mitochondrial pathology. Anikster et al [61] reported that Opa3 deficiency leads to a reduction in lipid utilization in adipose tissue. Subsequently, Wells et al [62] discovered that Opa3 functions to control thermogenesis and abdominal fat mass accumulation. The mutant form of Opa3 studied by these authors (L122P) was also associated with increased levels of 3MGC acid in plasma. More recently, Navein et al [63] found that L122P Opa3 is associated with disrupted mitochondrial function and impaired skeletal integrity. Localization of Opa3 to mitochondria, together with the fact that mutations in this protein manifest altered mitochondrial metabolism, is consistent with production of 3MGC acid with this disorder.

DNAJC19

Mutations in *DNAJC19* (OMIM *608977), originally identified in the Canadian Dariusleut Hutterite population, are causative for dilated cardiomyopathy with ataxia (DCMA; OMIM #610198) syndrome [64], which is characterized by early onset dilated cardiomyopathy, non-progressive cerebellar ataxia, testicular dysgenesis, growth failure and 3MGC aciduria. The Dnajc19 protein [DnaJ heat shock protein family member c19] is homologous to yeast Pam18 / Tim14, a component of the mitochondrial machinery for import of nuclear-encoded proteins. Based on this homology, Davey et al [64] hypothesized that mutations in *DNAJC19* cause DCMA by interfering with protein import. In other studies, Richter-Dennerlein et al. [65] reported that Dnajc19 interacts with prohibitin (PHB) proteins. PHB 1 and 2 assemble into large ring-like structures that serve a scaffold function in cristae membranes. In studies of the PHB interactome, Dnajc19 was identified as a binding partner of PHB complexes. Moreover, gene silencing of either *DNAJC19* or *PHB2* (OMIM *610704) resulted in impaired cell growth and defective cristae morphogenesis. Curiously, disruption of the interaction between Dnajc19 and PHB affected cardiolipin remodeling / acyl chain maturation, resulting in accumulation of cardiolipin species with altered acyl chains. These changes in cardiolipin are reminiscent of those observed in Barth Syndrome. Based on this, it has been proposed [65] that PHB / Dnajc19 containing membrane domains modulate tafazzin-mediated cardiolipin remodeling, thereby providing an explanation for the similar phenotypes observed in these two inherited cardiomyopathies. How these distinct protein components of cristae membranes (Dnajc19, tafazzin and PHB) regulate membrane structure and integrity is not clear, but their pronounced effects on cristae membrane morphology are likely to have a negative impact on ETC function. By extension, compromised mitochondrial energy metabolism and impaired ATP production capacity will ensue, setting the stage for development of cardiomyopathy. Likewise, ETC defects will ultimately impede TCA cycle activity, resulting in acetyl CoA diversion to the dead-end product, 3MGC acid.

HTRA2

The *HTRA2* (OMIM *606441) gene encodes a member of the "high-temperature requirement" (HtrA) family of oligomeric serine proteases that possess a trypsin-like protease domain and one or more "PDZ" type interaction domains. HtrA2 participates in the cell death pathway, binding to the apoptosis inhibitory protein, baculoviral IAP repeat-containing 4 [66]. Deficiencies in HtrA2 are associated with severe neurodegenerative

effects, abnormal mitochondria, 3MGC aciduria and increased sensitivity to apoptosis [67–69].

HACE1

The *HACE1* (OMIM *610876) gene encodes Hace1 (HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1). Given its function as an E3 ubiquitin ligase, it is not surprising that Hace1 has been implicated in numerous processes, including Golgi biogenesis [70], tumor suppression [71], response to oxidative stress [72,73], autophagy [74] and targeting Ras-related C3 botulinum toxin substrate 1 for ubiquitination [75]. Ugarteburu et al reported on a patient harboring a mutation in *HACE1* who presented with brain atrophy, psychomotor retardation, and 3MGC aciduria [76]. In studies of fibroblasts from this subject, the authors observed high levels of lipid peroxidation, increased mitochondrial oxidative stress markers, diminished response to oxidative damage and a reduction in the mitophagic flux. Of interest, Ehrnhoefer et al [77] reported that genetic ablation of *hace1* in a mouse model of Huntington's disease accelerated motor deficits and exacerbated cognitive and psychiatric phenotypes. These authors also reported that mutant huntingtin protein expression combined with *hace1* gene disruption caused a deficit in astrocyte mitochondrial respiration. Thus, it appears that the far-ranging effects of this E3 ubiquitin ligase include deleterious effects on mitochondrial respiration. As such, detection of 3MGC aciduria in *HACE1* IEMs is consistent with the current paradigm.

VII. Discussion

The present review describes 20 discrete IEMs that manifest secondary 3MGC aciduria as a phenotypic feature (Table 1). A curious aspect of secondary 3MGC aciduria is that no mutations in leucine metabolic enzymes exist. The fact that, in human intermediary metabolism, 3MGC CoA is found only in this pathway strongly suggests secondary 3MGC aciduria arises from a unique biochemical route. Diagnostically, it is relatively easy to distinguish primary from secondary 3MGC aciduria. For example, in primary 3MGC aciduria, the amount of 3MGC acid excreted increases in response to leucine loading [4]. Likewise, in primary 3MGC aciduria, upstream pathway intermediates give rise to additional products, such as 3-hydroxyisovaleric acid [78] which are not observed in secondary 3MGC aciduria.

In secondary 3MGC aciduria, it is proposed that IEMs that affect mitochondrial energy metabolism, either directly or indirectly, can lead to an accumulation of acetyl CoA. This occurs because disturbances in ETC function, cristae membranes or other factors, lead to a decrease in the efficiency of TCA cycle activity. When this occurs in muscle or brain (tissues that rely on acetyl CoA oxidation for energy production), acetyl CoA has limited metabolic options. When IEMs affect the predominant metabolic pathway (acetyl CoA oxidation via the TCA cycle), acetyl CoA tends to accumulate in the matrix. At this point, the acetyl CoA diversion pathway converts acetyl CoA to HMG CoA via mass action, utilizing two known mitochondrial matrix enzymes, T2 thiolase and HMG CoA synthase 2. These enzymes catalyze sequential Claisen condensations between two equivalents of acetyl CoA and between acetoacetyl CoA and acetyl CoA, respectively, to yield HMG

CoA. Once HMG CoA is formed, it becomes a substrate for AUH. Unlike the role of this enzyme in the leucine degradation pathway, in this case AUH functions in the opposite direction, dehydrating HMG CoA to yield *trans*-3MGC CoA. Given that the equilibrium constant for AUH strongly favors the hydrated product [79], it is difficult to explain why the presumed precursor of 3MGC acid, 3MGC CoA, would accumulate instead of HMG CoA. Recent results have revealed that this occurs because *trans*-3MGC CoA isomerizes to *cis*-3MGC CoA [9]. When this occurs, the AUH substrate / product ratio is altered and, in order to re-establish equilibrium, more HMG CoA is dehydrated to *trans*-3MGC CoA. Once formed, *cis*-3MGC CoA is susceptible to intramolecular cyclization, forming *cis*-3MGC anhydride and CoA. The anhydride can then be hydrolyzed to *cis*-3MGC acid (Figure 2). Thus, directionality of the acetyl CoA diversion pathway relies on a molecular sink that leads to depletion of the HMG CoA pool. In addition, these non-enzymatic reactions provide an explanation why *cis*-3MGC acid is the predominant diastereomer recovered in urine of subjects with 3MGC aciduria [9, 13, 80]. It is worth mentioning that the enzyme that produces *trans*-3MGC CoA in the leucine degradation pathway, 3-methylcrotonyl CoA carboxylase, is unidirectional [81]. Thus, when *trans*-3MGC CoA is produced via the acetyl CoA diversion pathway, it cannot proceed further up the leucine degradation pathway.

In the present review, evidence supporting the conclusion that the acetyl CoA diversion pathway is responsible for secondary 3MGC aciduria is presented. To investigate this, we examined 20 discrete IEMs that manifest secondary 3MGC aciduria as a phenotypic feature. In studying these IEMs, a key question was whether the causative mutation, directly or indirectly, affects mitochondrial energy metabolism. The results obtained indicate that, in nearly every case, these discrete IEMs impact aspects of aerobic respiration, leading to dampening of ETC function and decreased acetyl CoA metabolism via the TCA cycle. We identified five categories of IEM associated with secondary 3MGC aciduria. In most cases, a specific gene product of known function in mitochondria is affected. In category 1, proteins involved in cristae membrane structure and function were identified. These include tafazzin, SERAC1, acylglycerol kinase and QIL1. Cristae membranes exist as narrow, elongated invaginations of the inner boundary membrane. Cristae membranes are cylindrical in shape [15] such that a continuously curved membrane envelops the inner cristae space, the site where hydrogen ion accumulation generates the proton motive force that drives ADP phosphorylation. The unique shape and function of cristae membranes requires specialized enzymes and proteins to form and maintain cristae integrity. IEMs in genes that encode phospholipid biosynthetic enzymes lead to defective membrane composition while mutations in protein components of the MICOS complex affect cristae junction formation and maintenance. Furthermore, although it appears that the inner cristae space and the inter-membrane space are confluent with each other, the MICOS complex, and other cristae membrane associated proteins, function as a diffusion barrier, preventing diffusion of inner cristae space contents into the inter-membrane space and vice versa. When this aspect of cristae membranes is disrupted or lost, the electrochemical gradient dissipates and ATP production efficiency decreases.

A second category of IEMs that give rise to secondary 3MGC aciduria directly affect protein components of the ETC. These proteins are embedded in cristae membranes such that their activity relies on the structural integrity of this membrane. When IEMs lead to deficiencies

in ETC Complex components, electron transport is less efficient and reduced cofactors generated via the TCA cycle activity are not oxidized by Complex I / II. This leads to a buildup of NADH in the matrix space and inhibition of TCA cycle activity. Thus, IEMs that interfere with ETC function prevent acetyl CoA metabolism via the TCA cycle, resulting in its diversion to 3MGC acid.

When IEMs affect mitochondrial DNA polymerase, mutant tRNAs may be produced or deletions in mitochondrial DNA may occur, leading to a broad range of potential complications. In addition, nuclear gene duplication can disrupt normal metabolic processes and impair aerobic respiration. IEMs in metabolic enzymes that localize to mitochondria are also associated with 3MGC aciduria. These include the TCA cycle enzyme succinyl CoA synthetase, the urea cycle enzyme, carbamoyl phosphate synthase and metabolic enzyme, short-chain enoyl CoA hydratase.

In addition to these examples, several other protein deficiencies exist that do not fit neatly into the categories described above. These include mutations in *CLPB, DNAJC19, OPA3, HACE1* and others. IEMs in these genes are known to adversely affect cristae membrane structure, protein stability or oxidative response. The important point is that each of these proteins plays a direct or ancillary role in mitochondrial energy metabolism and, when deficiencies arise, deleterious consequences can include aberrant metabolic processes, including the diversion of acetyl CoA to 3MGC acid.

Researchers have pondered the metabolic origins of 3MGC acid in secondary 3MGC aciduria for the past 30 years. Originally, it was proposed that 3MGC acid arises by shunting isoprene moieties from cytosol to mitochondria [5,78]. A basic flaw in this proposed pathway is that no explanation exists why 3MGC CoA arising in this manner would not be metabolized via the leucine degradation pathway. Also, as exemplified in the present investigation, IEMs that give rise to secondary 3MGC aciduria predominantly affect mitochondrial energy metabolism. Another recent study proposed that 3MGC acid is generated via peroxisomal metabolism [6]. This proposed route, however, fails to reconcile the fact that IEMs associated with 3MGC aciduria affect mitochondrial function.

Although the acetyl CoA diversion pathway has not previously been proposed in human metabolism, this pathway is known to exist in various microorganisms. Indeed, the biosynthetic route to iron chelating siderophores, iso-odd chain fatty acids and polyketide / nonribosomal peptide products employ this biochemical process. In these organisms, acetyl CoA is converted to 3MGC CoA, which then serves an intermediate in formation of larger structures [82]. As progress is made toward understanding the metabolic basis for 3MGC aciduria, the potential utility of this easily detected organic acid will be realized. Given that secondary 3MGC aciduria arises as a result of compromised mitochondrial energy metabolism, it is conceivable that evaluation of 3MGC acid levels in subjects harboring IEMs can provide a window into physiological processes that underlie disease manifestation. Toward that end, clinical trials on subjects with different IEMs that give rise to this phenotype may reveal a correlation between mitochondria impairment, physical exertion and 3MGC acid production.

Acknowledgements

Abbreviations:

AUH	3-methylglutaconyl CoA hydratase
3MGC	3-methylglutaconic
IEM	Inborn error of metabolism
HMG	3-hydroxy, 3-methylglutaryl
ETC	electron transport chain
PHB	prohibitin
SERAC1	Serine active site containing 1
AGK	acylglycerol kinase
ANT1	adenine nucleotide translocator 1
MICOS	mitochondrial contact site and cristae organizing system
TMEM70	transmembrane protein 70
Isd11	iron-sulfur protein biogenesis, desulfurase-interacting protein 11
POLG	DNA polymerase subunit γ
MELAS	mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
PS	Pearson marrow-pancreas syndrome
ECH	short-chain enoyl CoA hydratase
ClpB	caseinolytic peptidase B protein homolog
TIM23	subunit of the translocase of the inner membrane
DCMA	dilated cardiomyopathy with ataxia syndrome
Dnajc19	DnaJ heat shock protein family member c19
PHB	prohibitin
HtrA2	high temperature requirement A serine protease
Hect1	HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1
OMIM	Online Mendelian Inheritance in Man

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Highlights

- 3-methylglutaconic (3MGC) aciduria occurs in a number of inborn errors of metabolism

- Mutations associated with secondary 3MGC aciduria affect oxidative fuel metabolism

- Inhibition of TCA cycle activity leads to diversion of acetyl CoA to 3MGC acid

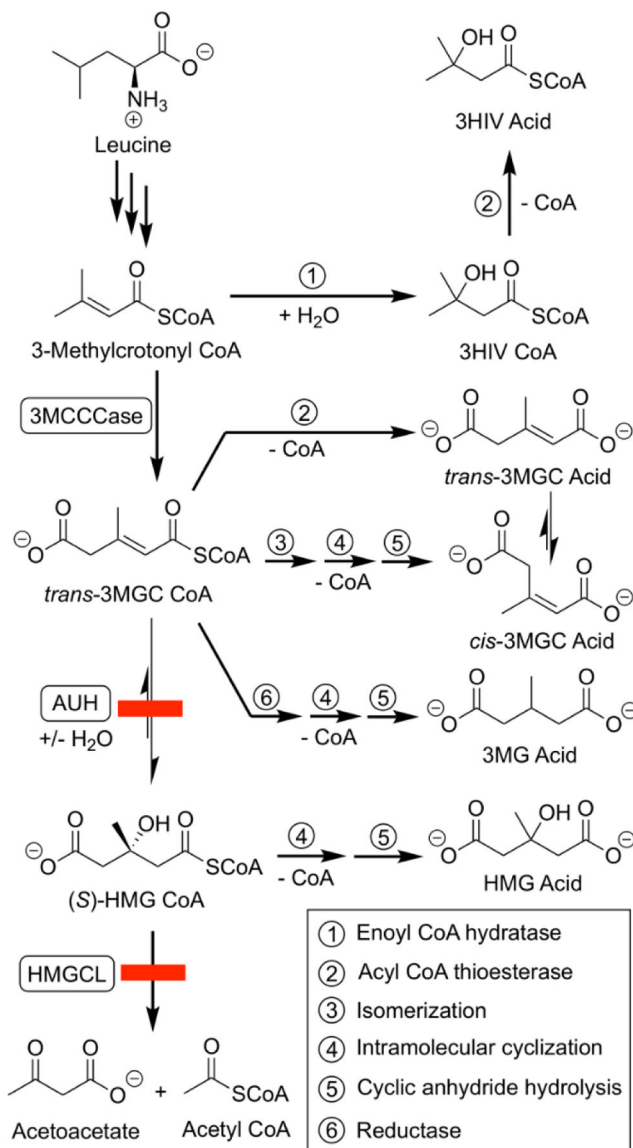- This pathway involves enzymatic and non-enzymatic chemical reactions

Figure 1. Leucine catabolism pathway defects lead to primary 3MGC aciduria.
IEMs affecting AUH or HMGCL (see red boxes) result in enzyme deficiencies that interfere with leucine catabolism. As leucine degradation proceeds, pathway intermediates accumulate, and are ultimately converted to organic acids. When AUH is deficient, 3-methylcrotonyl CoA is subject to hydroxylation (1) and thioester hydrolysis (2), forming 3-hydroxy-isovaleric (3-HIV) acid. Alternatively, 3-methylcrotonyl CoA can be carboxylated by 3-methylcrotonyl CoA carboxylase (3MCCCase), forming *trans*-3MGC CoA. This product can undergo isomerization (3), intramolecular cyclization (4) and hydrolysis (5), giving rise to *cis*-3MGC acid. *trans*-3MGC acid can be produced by isomerization (3) of *cis*-3MGC acid [12] or by acyl CoA thioesterase-mediated hydrolysis (2) of *trans*-3MGC CoA. In addition, *trans*-3MGC CoA can be reduced (6) to 3-methylglutaryl (3MG) CoA, and then converted to 3-methylglutaric acid via intramolecular cyclization (4) and hydrolysis (5). Alternatively, 3MG acid may be generated by thioesterase-mediated hydrolysis (2) of

3MG CoA [8]. When HMG CoA lyase (HMGCL) is deficient, an additional organic acid, 3MGC acid, is produced by intramolecular cyclization (4) and hydrolysis (5) of HMG CoA. Note: reaction 3,4 and 5 are non-enzyme-mediated chemical reactions.
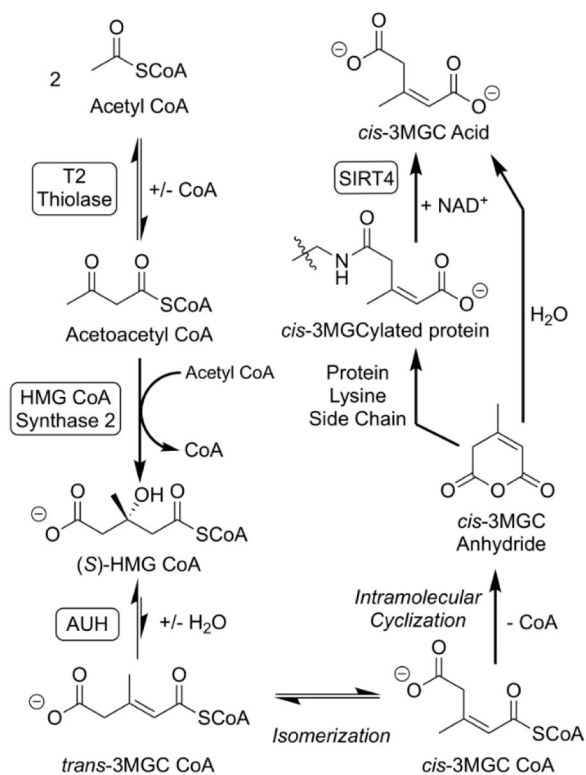
Figure 2. The acetyl CoA diversion pathway.

A sequence of three enzyme-mediated reactions that lead from acetyl CoA to to 3MGC CoA is depicted. Acetoacetyl CoA (T2) thiolase catalyzes the condensation of 2 acetyl CoA. Acetoacetyl CoA generated by this reaction reacts with another acetyl CoA in a second condensation reaction catalyzed by HMG CoA synthase 2. Subsequently AUH hydratase dehydrates HMG CoA to *trans*-3MGC CoA. Once formed, three non-enzymatic reactions follow, isomerization of *trans*-3MGC CoA to *cis*-3MGC CoA, intramolecular cyclization of *cis*-3MGC CoA to *cis*-3MGC anhydride, and hydrolysis to *cis*-3MGC acid. Alternatively, *cis*-3MGC anhydride can 3MGCylate protein lysine side chain amino groups. This latter product serves as a substrate for the NAD^+-dependent deacylase, sirtuin 4 (SIRT4), which produces *cis*-3MGC acid as a reaction product.

Table 1.

Gene mutations associated with secondary 3MGC aciduria

Gene	Gene product function	Location	Disorder / phenotype	Ref.
AGK	Lipid metabolism	Mitochondrial membrane	Sengers syndrome	22,23
ATP12	Biosynthesis and assembly of ATP synthase	Mitochondria	Complex V deficiency, nuclear type 1	27
ATP5E	Biosynthesis and assembly of ATP synthase	Mitochondria	Complex V deficiency, nuclear type 3	33
CLPB	ATP-dependent chaperone	Inter-membrane space	Increased protein aggregation	53
CPS1	Urea cycle enzyme	Matrix	Hyperammonemia	49
DNAJC19	Molecular chaperone	Cristae membrane	DCMA syndrome	64
ECHS1	Short chain enoyl coenzyme A hydratase	Matrix	Encephalopathy	48
HACE1	E3 ubiquitin ligase	Multiple locations	Enhanced protein degradation	76
HTRA2	Serine protease	Matrix	Caspase dependent apoptosis	67
LYRM4	Fe/S cluster assembly	Matrix	ETC Complex assembly defects	39,40
m.3243A>G	tRNA	Matrix	MELAS syndrome	44,45
NFS1	Cysteine desulfurase	Matrix	ETC complex assembly defects	35
OPA3	unknown	Mitochondria	Costeff optic atrophy	61,62
POLG1	mtDNA replication	Matrix	mtDNA deletions	42,43
QIL1/MIC13	Cristae junction assembly / maintenance	Mitochondrial inner membrane	defective cristae morphology	24,25
SERAC1	Phosphatidylglycerol remodeling	Mitochondrial outer membrane	MEGDEL syndrome	19,20
SUCLA2	Succinyl-CoA ligase β-subunit	Matrix	Metabolic defects	47
TAZ	Cardiolipin transacylase	Cristae membrane	Barth syndrome	14,15
TIMM50	Translocase subunit	Inner mitochondrial membrane	Impaired protein import	57
TMEM70	Biogenesis of mitochondrial ATP synthase	Mitochondria	Complex V deficiency, nuclear type 2	29