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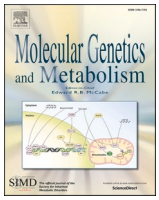

Review article

Inborn errors of the malate aspartate shuttle – Update on patients and cellular models

Jasmine Koch [a], Melissa H. Broeks [b], Matthias Gautschi [a,c], Judith Jans [b], Alexander Laemmle [a,c,*]

[a] Division of Pediatric Endocrinology, Diabetology and Metabolism, Department of Pediatrics, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland
[b] Department of Genetics, Section Metabolic Diagnostics, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, the Netherlands
[c] University Institute of Clinical Chemistry, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland

A B S T R A C T

The malate aspartate shuttle (MAS) plays a pivotal role in transporting cytosolic reducing equivalents – electrons – into the mitochondria for energy conversion at the electron transport chain (ETC) and in the process of oxidative phosphorylation. The MAS consists of two pairs of cytosolic and mitochondrial isoenzymes (malate dehydrogenases 1 and 2; and glutamate oxaloacetate transaminases 1 and 2) and two transporters (malate-2-oxoglutarate carrier and aspartate glutamate carrier (AGC), the latter of which has two tissue-dependent isoforms AGC1 and AGC2). While the inner mitochondrial membrane is impermeable to NADH, the MAS forms one of the main routes for mitochondrial electron uptake by promoting uptake of malate.

Inherited bi-allelic pathogenic variants in five of the seven components of the MAS have been described hitherto and cause a wide spectrum of symptoms including early-onset epileptic encephalopathy.

This review provides an overview of reported patients suffering from MAS deficiencies. In addition, we give an overview of diagnostic procedures and research performed on patient-derived cellular models and tissues. Current cellular models are briefly discussed and novel ways to achieve a better understanding of MAS deficiencies are highlighted.

1. Introduction

The malate aspartate shuttle (MAS), first described in the 1960s [1,2], is composed of four enzymes and two carriers (Fig. 1). First, cytosolic oxaloacetate is reduced to malate by cytosolic malate dehydrogenase (MDH1; OMIM *154200) with NADH as a co-factor regenerating cytosolic NAD^+. Consecutively malate is transported into the mitochondria by exchange of 2-oxoglutarate which is shuttled out of the mitochondria through the malate-2-oxoglutarate carrier (OGC; OMIM *604165). In the mitochondrial matrix, malate is re-oxidized to oxaloacetate by malate dehydrogenase 2 (MDH2; OMIM *154100) simultaneously generating NADH for the ETC [1,3]. In the mitochondrial matrix, oxaloacetate and glutamate further react in a transamination reaction by aspartate aminotransferase (GOT2; OMIM *138150) generating aspartate and 2-oxoglutarate. Next, aspartate is exported from the mitochondria into the cytosol via the aspartate glutamate carrier (AGC) in exchange with glutamate and a proton/H^+ [1,3–5]. AGC is regulated by cytosolic calcium and has two isoforms which are tissue-specific. *AGC1* (OMIM *603667) is mainly expressed in the central nervous system, skeletal muscle and heart whereas *AGC2* (OMIM *603859) is mainly expressed in the liver [6]. To complete the cycle of the MAS, the cytosolic aspartate aminotransferase (GOT1; OMIM *138180) converts aspartate into oxaloacetate, donating the amino group to 2-oxoglutarate to form glutamate [1,3] (Fig. 1).

A main role of the MAS is to transport cytosolic reducing equivalents - electrons - from the cytosol into the mitochondria to provide electrons for the electron transport chain (ETC) to generate ATP. The MAS links glycolysis, the tricarboxylic acid (TCA) cycle and the ETC (Fig. 2). As the inner mitochondrial membrane is impermeable for NADH, the MAS shuttles electrons into the mitochondria via malate [1–3,7–11]. Simultaneously the MAS regenerates cytosolic NAD^+ to uphold the cells' redox state and to enable continuation of glycolysis and/or the

Abbreviations: AGC, aspartate glutamate carrier; ETC, electron transport chain; GOT1, glutamate oxaloacetate transaminase 1; GOT2, glutamate oxaloacetate transaminase 2; G-3-P, glycerol-3-phosphate; hiPSCs, human induced pluripotent stem cells; hiPSC-Heps, human induced pluripotent stem cell-derived hepatocytes; IEMs, inborn errors of metabolism; LDH, lactate dehydrogenase; MRI, magnetic resonance imaging; MAS, malate aspartate shuttle; MD, mitochondrial disease; MDH1, malate dehydrogenase 1; MDH2, malate dehydrogenase 2; MEF, mouse embryonic fibroblasts; OGC, malate-2-oxoglutarate carrier; MeSH, medical subject headings; TCA cycle, tricarboxylic acid cycle; WES, whole exome sequencing; WT, wild-type.

* Corresponding author at: University Institute of Clinical Chemistry, Department of Pediatrics, Julie-von-Jenner-Haus, Freiburgstrasse 15, 3010 Bern, Switzerland.
E-mail address: alexander.laemmle@insel.ch (A. Laemmle).

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biosynthesis of nucleotides and specific amino acids [1,3]. Replenishment of NAD^+ for glycolysis is also maintained by the conversion of pyruvate to lactate by the enzyme lactate dehydrogenase (LDH). This is the major pathway in anaerobic conditions and is energetically far less efficient than full mitochondrial oxidation of glucose into CO_2 and H_2O and causes no net change in NAD^+/NADH ratio [3]. Maintaining a high cytosolic ratio of NAD^+/NADH is a key function of the MAS as it determines the direction of NAD^+/NADH-dependent reactions. Of note the NAD^+/NADH ratio in the cytosol (500–1000) is two orders of magnitude higher than in the mitochondria (5–10) [1,3,12,13]. Therefore importing NADH reducing equivalents into the mitochondria with a 10- to 100-fold difference in its NAD^+/NADH ratio is energetically unfavorable. It only takes place because AGC activity is coupled to the ETC via the electrical potential gradient across the inner mitochondrial membrane [3,10]. An alternative pathway to transfer cytosolic reducing equivalents into the mitochondria is via the glycerol-3-phosphate (G-3-P) shuttle [3,14,15]. A major difference between the MAS and the G-3-P shuttle is that the latter generates mitochondrial $FADH_2$ from cytosolic NADH thus resulting in less ATP production in comparison to the MAS [1,3,14].

The enzymes and carriers of the MAS are involved in other biochemical pathways. For instance, MDH2 has a dual role and in addition of being part of the MAS it is an enzyme of the TCA cycle. Further, enzymes and carriers of the MAS provide pivotal metabolites required for other pathways and enzyme reactions such as aspartate which is a substrate for the urea cycle enzyme argininosuccinate synthetase in the cytosol [16].

Several bi-allelic pathogenic variants cause MAS deficiencies, a group of ultra-rare disorders belonging to the inborn errors of metabolism (IEMs). As expected, a defect in any MAS component leads to impaired and inadequate energy supply of the affected organs. On a cellular level the lack of energy has various consequences predominantly in tissues with high energy demands such as the central nervous system, skeletal muscle and liver.

The clinical presentation of patients suffering from varying MAS deficiencies is diverse and dependent on the affected component. For example, a molecular defect in AGC2 causes citrin deficiency which is mainly associated with a hepatic clinical phenotype [17]. All other reported MAS deficiencies mainly manifest with severe neurological symptoms such as epileptic encephalopathy, developmental delay and generalized muscular hypotonia [3].

Hitherto, bi-allelic disease-causing variants in genes encoding MDH1, MDH2, GOT2 and AGC1 have been reported [3,17]. To the best of our knowledge thus far no patients with defects in the genes encoding GOT1 or OGC have been described. Our literature search performed on PubMed revealed a total number of 35 reported patients with MAS deficiencies – excluding patients affected by citrin deficiency (defect in AGC2) which is much more common and phenotypically clearly differs from the other MAS deficiencies.

The main focus of this review is to provide an up-to-date overview of all reported patient cases affected by a MAS deficiency. Since MAS deficiencies have only recently been discovered there is still a considerable gap in understanding the underlying pathophysiological mechanisms and disease consequences. Therefore, an additional goal of this review is to focus on insights from experimental models – specifically performed in affected patient-derived tissues and cellular disease models - to elucidate the pathophysiology of these inherited defects. Currently, treatment options for patients with MAS deficiencies are not very effective. Thus, a further aim of this work is to explore the literature regarding new and potentially promising treatment possibilities for MAS deficiencies.

2. Methods

We conducted a literature search on MEDLINE database via the PubMed interface for each individual component of the MAS (MDH1, MDH2, GOT1, GOT2, AGC1, AGC2 (citrin) and OGC). The main goal of our literature search was to identify all published reports on patients suffering from bi-allelic pathogenic variants causing MAS deficiencies. We excluded patients affected by AGC2 defects underlying citrin deficiency since the clinical presentation differs from the other MAS defects and several comprehensive reviews have been previously published [18,19]. Further, we focused our literature search on on-going research in the context of MAS defects. We specifically addressed the question, which diagnostic procedures and investigations had been performed in patient-derived tissues and cellular models as well as in selected

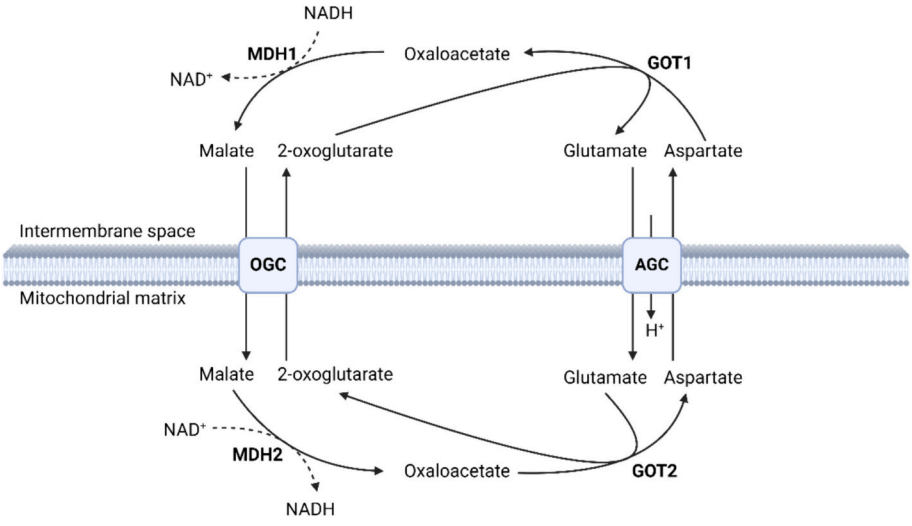

Fig. 1. Schematic of the malate aspartate shuttle.
The MAS is composed of four enzymes and two carriers. Cytosolic oxaloacetate is reduced to malate by malate dehydrogenase 1 (MDH1) regenerating NAD^+ from NADH. Consecutively malate is transported into the mitochondria by exchange of 2-oxoglutarate which is shuttled out of the mitochondria through the malate-2-oxoglutarate carrier (OGC). In the mitochondrial matrix, malate is re-oxidized to oxaloacetate by malate dehydrogenase 2 (MDH2) simultaneously generating NADH for ATP production. In the mitochondrial matrix, oxaloacetate and glutamate further react in a transamination reaction by glutamate oxaloacetate transaminase 2 (GOT2) generating aspartate and 2-oxoglutarate. To complete the MAS cycle, aspartate is exported from the mitochondria into the cytosol via the glutamate aspartate carrier (AGC) in exchange with glutamate and H^+. In the cytosol aspartate reacts with 2-oxoglutarate in a transamination reaction mediated by glutamate oxaloacetate 1 (GOT1) to form oxaloacetate and glutamate. Figure created with BioRender.com.

alternative models. Finally, our literature search focused on currently available treatments as well as on novel potential therapeutic strategies to treat patients with MAS deficiencies.

Conducting a basic literature search for each component of the MAS separately, yielded a high number of articles (Table 1). To narrow down our search and to focus on reported patient cases, additional search terms were applied containing controlled vocabulary (medical subject heading (MeSH)) regarding patient populations. Combining search terms for each individual MAS component in conjunction with the patient-centered search terms still resulted in a significant number of papers (Table 1). Next, we scanned the abstracts of these papers to identify all published case reports of patients suffering from bi-allelic pathogenic variants in one of the MAS components (Table 1). Further details regarding specific applied search strategies and search terms used in this review are found in supplementary materials (Tables S1 and S2).

3. Results

The main results from our literature review are summarized in Table 2. We identified patients with bi-allelic pathogenic variants in *MDH1, MDH2, GOT2* and *AGC1*. Table 2 provides an overview of the number of patients – listed separately for each component – as well as on the years the first patient reports were published. Further, Table 2 summarizes typical clinical presentations, diagnostic procedures including (specific) laboratory parameters and magnetic resonance imaging (MRI) brain scan findings, and current treatment approaches. The second part of the result section focuses on experiments conducted in patient-derived tissues and cellular models as well as selected alternative disease models. These results are summarized in Table 3 and they reveal insights into pathophysiological mechanisms and disease consequences underlying the individual MAS component deficiencies.

Table 1
Overview of literature search regarding MAS components and published reports of patients with MAS deficiencies.

MAS Components	Total number of articles found	Combined with search term for population of interest	Publications containing patient reports
MDH1	627	164	1
MDH2	717	174	4
GOT1	850	341	0
GOT2	572	215	2
OGC	419	250	0
AGC*	7432	3849	*
AGC1	366	228	10
Total	10,983	5221	17

Performing a literature search for each individual MAS component (first column) revealed the total amount of papers displayed in the second column. Conducting an additional patient-centered literature search applying specific patient-centered search terms (for details see Supplementary information, Tables S1 and S2) reduced the number of papers as shown in the third column. The abstracts of the papers identified in the third column were carefully screened for patients suffering from bi-allelic pathogenic variants affecting one of the MAS components resulting in the patient reports summarized in the fourth column. The search was performed as of 12th of February 2024. *AGC has two isoforms: AGC1 and AGC2. In this literature search we excluded patients suffering from AGC2 defects (citrin deficiency) and only focused on patients with AGC1 deficiency.

3.1. Reported patient cases with malate aspartate shuttle deficiencies

3.1.1. MDH1 deficiency

The first patient reported with MDH1 deficiency was a 25-month-old boy born to two healthy consanguineous parents [7]. He presented with global developmental delay, progressive microcephaly, facial dysmorphism, epilepsy, axial hypotonia and hypertonia of the extremities with hyperactive reflexes. MRI brain scan revealed agenesis of the corpus callosum and hypoplasia of the pons and the EEG showed a hypsarrhythmic pattern. The clinical picture was consistent with epileptic encephalopathy as found in the majority of patients presenting

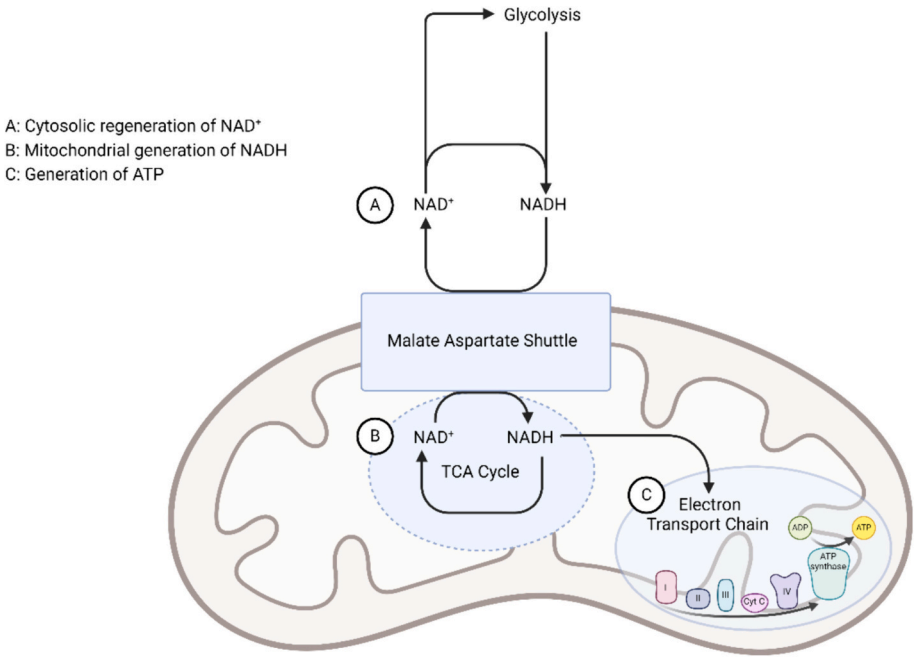

Fig. 2. Illustration showing how the malate aspartate shuttle links glycolysis, TCA cycle and electron transport chain.
The malate aspartate shuttle (MAS) links glycolysis, the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC). Because the inner mitochondrial membrane is impermeable for NADH, the MAS is required to shuttle electrons into the mitochondria. The MAS regenerates cytosolic NAD^+ enabling continuation of glycolysis (A) and generates mitochondrial NADH (B) which delivers its electrons to the ETC to ultimately generate ATP (C). Figure created with BioRender.com.

Table 2
Description and characterization of patients with MAS defects as extracted and summarized from published patient reports.

Disorder (OMIM)	Total number of reported patients	Year of first patient report	Number of patients per published report (references)	Clinical phenotype	Diagnostics	Treatments
MDH1 deficiency (618959)	2	2019	2 patients (Broeks et al.) [7]	Epileptic encephalopathy; global developmental delay; progressive microcephaly; facial dysmorphism	Plasma: lactate≈ MRI: findings from normal to thinning and hypogenesis of brain regions EEG: findings from normal to hypsarrhythmic pattern WES	Anticonvulsants
MDH2 deficiency (617339)	12	2017	3 patients (Ait-El-Mkadem et al.) [20]	Epileptic encephalopathy; global developmental delay; generalized muscular hypotonia	Plasma: lactate ↑, L/P ↑ Urine: fumarate and malate ↑ CSF: lactate ↑ MRI: brain atrophy and delayed myelination WES	Anticonvulsants Ketogenic diet
			1 patient (Laemmle et al.) [21]	Epileptic encephalopathy; global developmental delay; generalized muscular hypotonia; failure to thrive	Plasma: lactate ↑ CSF: lactate ↑ MRI: brain atrophy and delayed myelination WES	Anticonvulsants Triheptanoin
			1 patient (Ticci et al.) [22]	Epilepsy; global developmental delay; dilated cardiomyopathy	Plasma: lactate ≈ Urine: fumarate ↑ MRI: hyperintense brain regions WES	Anticonvulsants Heart transplant
			7 patients (Priestley et al.) [23]	Epilepsy; global developmental delay; muscular hypotonia	Plasma: lactate ↑, L/P ↑ Urine: malate, fumarate and succinate ↑ CSF: lactate ↑ MRI: brain atrophy EEG: variable abnormal findings NGS	Anticonvulsants Triheptanoin
GOT2 deficiency (618721)	5	2019	4 patients (Van Karnebeek et al.) [24]	Epilepsy; progressive microcephaly; global developmental delay; failure to thrive	Plasma: lactate ↑, ammonia ↑ Urine: organic acids ≈ MRI: mild cerebral atrophy EEG: tempoparietal or frontoparietal spikes WES	Anticonvulsants Pyridoxine L-serine supplementation
			1 patient (Çapan et al.) [25]	Epilepsy; microcephaly; global developmental delay; severe spastic tetraplegia	Plasma: lactate ↑, ammonia ↑, homocysteine ↑ Urine: organic acids ≈ MRI: diffuse cerebral-cerebellar atrophy EEG: diffuse severe background slowing, bilateral frontal and generalized epileptogenic potentials WES	Pyridoxine L-serine supplementation

(*continued on next page*)

Table 2 (*continued*)

Disorder (OMIM)	Total number of reported patients	Year of first patient report	Number of patients per published report (references)	Clinical phenotype	Diagnostics	Treatments
AGC1 deficiency (612949)	16	2009	1 patient (Wibom et al.) [30]	Epilepsy; global developmental delay; muscular hypotonia	Plasma: lactate ↑ MRI: global cerebral hypomyelination DNA sequence analysis kit	Anticonvulsants
			2 patients (Falk et al.) [29]	Epilepsy; global developmental delay; muscular hypotonia; multiple dysmorphic features	Plasma: lactate ≈ CSF: lactate ↑ MRI: cerebral hypomyelination EEG: non-specific findings suggestive of bilateral cerebral dysfunction WES	Anticonvulsants
			1 patient (Pronicka et al.) [26]	n.d.a.	WES	n.d.a.
			1 patient (Kavanaugh et al.) [31]	Epilepsy; global developmental delay; muscular hypotonia	MRI: brain atrophy EEG: abnormal findings due to epileptiform discharges WES	Anticonvulsants
			1 patient (Nashabat et al.) [27]	n.d.a.	EEG: findings suggestive for epileptic encephalopathy WES	Anticonvulsants
			1 patient (Pfeiffer et al.) [33]	Epilepsy; global developmental delay	MRI: brain atrophy WES	Ketogenic diet
			1 patient (Saleh et al.) [34]	Epilepsy; cerebral palsy; global developmental delay	Plasma: lactate ↑ MRI: brain atrophy WES	Anticonvulsants
			1 patient (Kose et al.) [32]	n.d.a.	WES	n.d.a.
			1 patient (Mir et al.) [28]	Epilepsy; global developmental delay; microcephaly; muscular hypertonia	MRI: brain atrophy, hypomyelination WES	n.d.a.
			6 patients (Bölsterli et al.) [17]	Epilepsy; global developmental delay; microcephaly; muscular hypotonia	Plasma: lactate ↑, L/P ↑ CSF: lactate ↑ MRI: brain atrophy, delayed myelination EEG: slowing or unspecific epileptic discharges WES	Anticonvulsants Ketogenic diet

Included in this table are all published reports of patients with MAS deficiencies (i.e. deficiencies in MDH1, MDH2, GOT2 and AGC1) except for patients with AGC2 deficiency which were excluded due to its differing clinical presentation and phenotype. Each MAS deficiency is listed separately. Columns contain the total number of published patient cases per MAS component defect. Further it shows the year of the first published patient report, and the number of patients published per article including the respective references. The main and characteristic clinical phenotypes as well as selected diagnostics and treatment approaches are further summarized in different columns. CSF, cerebrospinal fluid. L/P, lactate/pyruvate ratio. NGS, next-generation sequencing. WES, whole-exome sequencing. ≈, normal value. ↑, elevated value. n.d.a., no data available.

with a MAS deficiency. His 4-year-old female cousin was the second reported patient [7]. She presented with a similar phenotype. Her MRI brain scan showed a mild shortening of the corpus callosum and a normal pons. Her EEG was normal. Molecular genetic analysis performed by whole exome sequencing (WES) revealed homozygous variants (*MDH1*: NM_001199111: c.413C > T, p.(Ala138Val)) in both related patients. The male patient was treated with antiepileptic medications showing only partial response [7].

3.1.2. *MDH2 deficiency*

The first patients with MDH2 deficiency were described in 2017 by Ait-El-Mkadem et al. [20]. All three unrelated patients suffered from epileptic encephalopathy with refractory epilepsies and their MRI brain scans were abnormal revealing delayed myelination and brain atrophy. Molecular genetic analyses revealed bi-allelic pathogenic compound-heterozygous variants in *MDH2*. One allele expressing the same pathogenic variant c.398C > T, p.(Pro133Leu) was found in each of the three reported patients [20]. Whereas the other defective alleles were expressing the following different pathogenic variants: c.620C > T, p.

Table 3
Patient-derived cellular models and tissue of MAS deficient patients and alternative research models.

Disorder (OMIM)	Patient-derived cell models and tissues (references)	Alternative models (references)
MDH1 deficiency (618959)	Patient-derived lymphoblastoid cells and skin fibroblasts from both affected individuals for MDH1 protein expression analysis [7]	*MDH1* knockout HEK293 cell line generated by Crispr/Cas9 [7,37]
		• targeted and untargeted metabolomics approaches • [U-13C]-glucose tracing experiments
		MDH1 KO Jurkat cell line for cell proliferation assays [36]
		mTUNE cell line [38]
		• oxygen consumption and extracellular acidification rate measurements • proteomics • metabolomics • stable isotope tracing experiments
MDH2 deficiency (617339)	Patient-derived liver and muscle tissue for molecular screening [20]	*mdh1Δ*-null *Saccharomyces cerevisiae* for functional complementation assay [20]
	Patient-derived skin fibroblasts [20–23]	*MDH2* knockdown in HeLa cells [41]
	• MDH2 protein expression • MDH2 enzymatic activity assay • restoration experiments via lentiviral-based transduction systems	• MDH2 protein expression • MDH2 enzymatic activity assay • metabolomics
		In silico structural modeling [22,42]
	Patient-derived induced pluripotent stem cell-derived hepatocytes [40]	*MDH2* overexpression in mouse neuroblastoma N1E-115 cells for mRNA level measurements [43]
	• proteomics • metabolomics • functional studies using triheptanoin	*MDH2* knockdown and *MDH2* overexpression in HEK293 cell lines [43]
		• MDH2 protein expression • proteomics
		HepG2_MDH2$_{52Cys}$ and HepG2_MDH2$_{160Met}$ cells [42]
		• MDH2 protein expression • MDH2 enzymatic activity assay
		MIN6-K8 mouse insulinoma cells transduced with lentiviral constructs of MDH2$_{52Cys}$ and MDH2$_{160Met}$ [42]
		• MDH2 protein expression • MDH2 enzymatic activity assay
		mdh-2$_{56Cys}$ and *mdh-2*$_{164Met}$ *Caenorhabditis elegans* strains generated by Crispr/Cas9 for insulin secretion GFP experiments [42]
		Hemizygous *Mdh2* mutant Drosophila
		• metabolomics [39] • molecular screening [39]
GOT2 deficiency (618721)	Patient-derived skin fibroblasts [24]	*GOT2* knockout HEK293 cell lines generated by Crispr/Cas9 [24]
	• GOT2 protein expression • GOT2 enzymatic activity assay • restoration experiments via lentiviral-based transduction systems • Stable isotope tracing experiments analyzing de novo serine biosynthesis	• stable isotope tracing analyzing de novo serine biosynthesis • protein measurements • enzymatic activity assay
		Got2 knockout mice (heterozygous) generated by Crispr/Cas9 [24]
		got2a knockdown morpholino-based in zebrafish [24]
		• phenotype rescue measurements • EEG measurements
		In silico structural modeling [25]
AGC1 deficiency (612949)	Patient-derived muscle tissue [30]	Liposomes reconstituted with wt and mutant AGC1 (Q590R) [30] and (R353Q) [29]
	• mitochondrial ATP production in dependence of different substrates • respiratory chain activities • protein measurements	• substrate (glutamate and aspartate) uptake experiments
		Slc25a12$^{-/-}$ mice [44]
	Patient-derived skin fibroblasts [29,30]	• AGC1 protein expression in different tissues (brain, muscle, heart) • MAS activity and aspartate formation assays • oxygen uptake measurements • behavioral studies • determination of metabolites in brain and cultured neurons
	• AGC1 protein expression • co-localization experiments by fluorescence microscopy	
	Patient-derived lymphocytes [30]	
	• AGC1 protein expression	In silico structural modeling [27,31]

Table 3 contains information regarding patient-derived cellular models and tissue (e.g. gained by skin or muscle biopsies) separately listed by individual MAS component defects (deficiencies of MDH1, MDH2, GOT2 and AGC1). The column describing alternative models includes additional cell lines and organisms in which experiments were performed. GFP, green fluorescent protein.

(Pro207Leu) (subject 1); c.596delG, p.(Gly199Alafs*10) (subject 2) and c.109G > A, p.(Gly37Arg) (subject 3). They all received a ketogenic diet reducing seizure frequency in two of the three patients, whereas the third patient died shortly after installing therapy [20].

Laemmle et al. recently reported on a fourth patient with MDH2 deficiency, an 18-month-old girl that suffered from a stroke-like episode following a catabolic state in context of an intercurrent infection [21]. Thereafter she developed epileptic encephalopathy as seen in the other MDH2-deficient patients. MRI brain scan revealed atrophy, delayed myelination and slight enlargement of the ventricles. The patient had compound-heterozygous missense variants in *MDH2*. One allele was expressing the pathogenic variant c.398C > T, p.(Pro133Leu), which

had been identified previously in the above mentioned three MDH2-deficient patients [20,21]. The second allele was expressing the truncating variant c.445delinsACA, p.(Pro149Thrfs*22) that has not been described before. In this severely affected patient we initiated a treatment with triheptanoin at the age of 36 months [21]. Since this drug trial was well tolerated by the patient and her overall neurological phenotype improved, triheptanoin treatment has been continued and is currently still on-going in this now 7-year-old girl.

The fifth reported patient with MDH2 deficiency showed psychomotor delay and generalized onset seizures and additionally a severe dilated cardiomyopathy [22]. The boy was treated with antiepileptic medication and he required an orthotopic heart transplantation. He inherited compound-heterozygous variants, both not described before: c.641C > A, p.(Pro214His) and c.320-2 A > T. MRI brain scan revealed pathological hyperintense signals in the cerebellar vermis and the cranial region of the cerebellar hemispheres [22].

Priestley et al. recently reported on seven patient cases with bi-allelic variants in *MDH2* from six different families [23]. Five of these patients are the offspring of consanguineous parents. All seven patients suffered from epileptic seizures and motor delay. Muscular hypotonia was observed in four patients. Speech delay, abnormal EEG findings as well as abnormal MRI brain scans were present in six of seven patients. All patients were treated with antiepileptic medication and one patient received triheptanoin [23].

3.1.3. GOT2 deficiency

The first case series of GOT2 deficiency was published by van Karnebeek et al. in 2019 and reported on four patients derived from three unrelated families [24]. Parental consanguinity was evident in two of the three families. All four patients suffered from epileptic encephalopathy, progressive microcephaly, failure to thrive and feeding difficulties, intellectual and motor disabilities. MRI brain scan findings revealed abnormalities in white matter and cerebral atrophy. Main EEG findings were tempoparietal and frontotemporal spikes. Treatment with pyridoxine and L-serine supplementation considerably ameliorated seizure control in two of four patients [24]. The fifth published patient case suffered from seizures, microcephaly, global developmental delay and severe spastic tetraplegia [25]. The currently 29-year-old male patient is the offspring of healthy unrelated parents. The patient harbors compound-heterozygous variants in the *GOT2* gene. MRI brain scan showed diffuse cerebral-cerebellar atrophy. EEG revealed diffuse severe background slowing and bilateral frontal and generalized epileptogenic potentials. Treatment with pyridoxine and L-serine supplementation as described above [24] was stopped after three weeks due to persistent and uncontrollable episodes of vomiting [25].

3.1.4. AGC1 deficiency

A total of sixteen patient cases were reported for AGC1 deficiency [17,26–35]. The first published patient report in 2009 by Wibom et al. described a 3-year-old girl from distantly related parents presenting with seizures, global developmental delay and generalized muscular hypotonia [30]. MRI brain scan was abnormal showing global lack of myelination. The epilepsy was initially treated with antiepileptic medication [30] and from the age of six years on a ketogenic diet was initiated and dramatically improved the clinical as well as the neuroradiological picture [35].

Patients number two and three were two affected siblings from consanguineous parents. The following bi-allelic homozygous pathogenic missense variant was identified by WES: c.1058G > A, p.(Arg353Gln). MRI brain scans revealed signs of atrophy and delayed myelination. Non-specific EEG activity was suggestive for abnormal brain function. Both patients were treated with anticonvulsants [29].

A further patient was described by Kavanaugh et al. and the main focus was held on MRI findings at multiple ages [31] while Pfeiffer et al. found a beneficial effect of ketogenic diet in another patient [33].

Four patients with AGC1 deficiency were identified by WES performed retrospectively in a broader population of patients [26–28,32]. One of these four patients originate from a WES study performed in Polish pediatric patients in which Pronicka et al. selected patients with high suspicion of mitochondrial defects lacking diagnosis [26]. One patient with AGC1 deficiency was identified by a descriptive retrospective study performed by Nashabat et al. in which WES was conducted on Saudi Arabian patients with early infantile epileptic encephalopathy [27]. Thereby novel pathogenic variants in several different genes were identified, among them one patient with a homozygous variant in *AGC1* (NM_003705.4: c.1385C > T, p.(Thr462Met)) [27]. Another retrospective study performing molecular genetic sequencing on a Saudi Arabian pediatric population was reported by Mir et al. and revealed one patient with a novel homozygous pathogenic variant in *AGC1* (NM_003705.4: c.832 T > C, p.(Tyr278His)) [28]. Finally, one patient was identified by WES in a retrospectively performed study on patients suspected for mitochondrial diseases by Kose et al. [32].

Saleh et al. described a 7-year-old patient with early-onset epileptic encephalopathy [34] and Bölsterli et al. recently published six patients with an AGC1 deficiency with early onset encephalopathy, microcephaly, global developmental delay and muscular hypotonia [17]. MRI brain scans showed progressive cerebral volume loss and extensive abnormal signal characteristics suggesting hypomyelination. In all patients the EEG findings were abnormal revealing slowing or unspecific epileptic charges. All six patients received a ketogenic diet [17].

3.2. Patient-derived cellular and other models to study MAS deficiencies

3.2.1. MDH1 deficiency

To functionally confirm MDH1 deficiency in suspected patients, MDH1 protein expression was determined by western blotting and revealed significantly reduced levels in patient-derived fibroblasts and lymphoblastoid cells compared to controls [7].

To study the metabolic consequences of MDH1 deficiency, *MDH1* knockout (KO) HEK293 cell lines were generated with Crispr/Cas9. In line with other *MDH1* KO cell models, such as Jurkat cells [36], aspartate levels were elevated and fumarate levels decreased in *MDH1* KO HEK293 cells [7]. Insights from [U—13C]-glucose tracing experiments revealed disruption of glycolysis and consequently a lower metabolite flux into the TCA cycle [37]. The accumulation of aspartate may be explained by disrupted TCA cycle flux, where oxaloacetate is increasingly transaminated by GOT2 to form aspartate, rather than to proceed in the TCA cycle by combining with acetyl-CoA. These metabolic consequences were restored by the addition of pyruvate in HEK293 cell models. Moreover, [U—13C]-glucose tracing experiments revealed reduced de novo serine and glycine biosynthesis, elevated flux into G-3-P, and an increased conversion of pyruvate into lactate in *MDH1* KO HEK293 cells [37].

An essential role of MDH1 is the link between cytosolic reductive carboxylation of glutamine and glycolysis in cells with mitochondrial dysfunction. Isogenic mTUNE cell lines can be generated with varying degrees of mitochondrial function which is thought to provide a more physiological model compared to eradication of mitochondrial respiratory complexes [38]. To elucidate mitochondrial dysfunction mTUNE cell lines were investigated by performing metabolomics, proteomics and other protein measurements [38].

3.2.2. MDH2 deficiency

To functionally confirm MDH2 deficiency in suspected patients, MDH2 protein levels and enzymatic activity were assessed in patient-derived fibroblasts revealing significantly decreased expression and activity levels compared to the controls [20–23]. Restoration of MDH2 in patient-derived fibroblasts by lentiviral transduction normalized both, *MDH2* expression and enzymatic activity [20].

Targeted investigations of specific metabolites in MDH2-deficient patient-derived fibroblasts revealed elevated ratios of malate/citrate

and fumarate/citrate compared to control fibroblasts indicating disruption of TCA cycle [20]. Likewise, in *Mdh2* mutant drosophila fumarate and malate levels were significantly increased [39]. However, functional tests in MDH2-deficient fibroblasts demonstrated survival upon a galactose stress test implying proper function of the ETC [21].

Insights from [U-13C]-glucose tracing experiments in *MDH2* KO HEK293 cells revealed disruption of pyruvate metabolization in the TCA cycle, due to lack of oxaloacetate formation by the loss of MDH2, hence resulting in low levels of citrate [37].

In a recently published study, we investigated the effects of triheptanoin in induced pluripotent stem cell (hiPSC)-derived hepatocytes (hiPSC-Heps) from our previously reported MDH2-deficient patient [21,40]. Results from untargeted proteotyping revealed an overall upregulation of mitochondrial proteins in MDH2-deficient hiPSC-Heps, specifically of enzymes involved in the TCA cycle and fatty acid oxidation. In MDH2-deficient hiPSC-Heps malate, fumarate and aspartate were significantly increased compared to controls - in line with the results found in fibroblasts - and normalized upon treatment with triheptanoin [40].

MDH2 has an important role as a tumor suppressor gene. Pathogenic germline mutations in *MDH2* are associated with an increased (familial) risk of developing paragangliomas [41]. Investigations into the pathomechanism of *MDH2* as a tumor suppressor gene revealed similar findings as described above in patient-derived MDH2-deficient cells. *MDH2* knockdown in HeLa cells caused accumulation of malate and fumarate as a consequence of TCA cycle interruption [41].

In contrast to the aforementioned "loss of function" *MDH2* variants, recently two specific gain of function variants in *MDH2* gene were described and shown to cause familial forms of diabetes [42]. To elucidate the pathogenicity of increased MDH2 activity due to these two specific *MDH2* variants, HepG2 cells and mouse insulinoma MIN6-K8 cell lines expressing both variants were studied and revealed impaired insulin secretion in the latter cell line [42]. Similar effects were observed in vivo in the nematode *Caenorhabditis elegans* [42]. Further, in silico modeling software tools – previously applied to receive insight into structural variations of different bi-allelic variants causing MDH2 deficiency [22] - confirmed to influence MDH2 protein stability and function for these specific gain of function variants [42].

Recently, yet another potential role of MDH2 was established and suggested MDH2 as an RNA-binding protein in HEK293 and mouse neuroblastoma N1E-115 cells that regulates the expression of *SNC1A* on the posttranscriptional level [43].

3.2.3. GOT2 deficiency

To functionally confirm GOT2 deficiency, patient-derived dermal fibroblasts were used to determine GOT2 protein levels by western blot [24]. Measurements of GOT2 enzymatic activity in patient-derived fibroblasts revealed reduced activity. Lentiviral restoration of GOT2 in patient-derived fibroblasts resulted in rescue of GOT2 enzymatic activity [24]. Based on clinical findings in patients with GOT2 deficiency, de novo serine biosynthesis was assessed in patient-derived fibroblasts [24]. Further, *GOT2* KO HEK293 cells were supplemented with high concentrations of pyruvate in the cell culture medium thereby fully restoring serine biosynthesis [24]. Similar to *MDH1* KO HEK293 cells, [U-13C]-glucose tracing experiments in *GOT2* KO HEK293 cells revealed reduced de novo serine and glycine biosynthesis, elevated flux into G-3-P, and an increased conversion of pyruvate into lactate compared to control cells. In addition, due to diminished synthesis, aspartate levels were low. *Got2* KO in mice caused lethality beyond early pregnancy, therefore mice being heterozygous for the *Got2* gene were generated and mouse embryonic fibroblasts (MEFs; 14 days post coitum) were collected and genotyped. *got2a* knockdown zebrafish were used to test various concentrations and combinations of pyridoxine, serine, pyruvate and proline to evaluate their therapeutic potential for GOT2 deficiency [24].

In a recently published article, in silico software tools were applied to characterize novel disease-causing GOT2 pathogenic variants [25].

3.2.4. AGC1 deficiency

A thorough laboratory work-up using patient-derived tissues and cells of the first AGC1-deficient patient as well as additional models revealed the main pathophysiological mechanisms contributing to the neurological phenotype of this first described MAS defect affecting the central nervous system [30]. Enzymatic activities of the respiratory chain complexes determined in patient-derived muscle tissue were normal. However, determination of mitochondrial ATP production in muscle was drastically reduced when using glutamate as substrate for mitochondrial respiration due to impaired mitochondrial glutamate uptake [30]. Additional experiments in liposomes reconstituted with either WT or mutant AGC1 (Q590R) revealed severely affected uptake of glutamate and aspartate by mutant AGC1. Similar results were obtained by Falk et al. for the R353Q variant [29]. Using patient-derived fibroblasts and lymphocytes Wibom et al. further proved co-localization of AGC1 with mitochondria [30].

In contrast to *Got2*$^{-/-}$ mice which did not survive early pregnancy, some *Agc1*$^{-/-}$ mice lived until 15 days postnatal [44]. Experiments conducted in these mice revealed significantly reduced MAS activity in skeletal muscle and brain mitochondria [44]. To evaluate the potential structural changes caused by the different bi-allelic variants, in silico structural modeling studies were performed [30,31]. Analysis of different variants expressed by the *SLC25A12* gene by several in silico structural modeling tools reliably predicted pathogenicity [30,31].

4. Discussion

4.1. General remarks

MAS deficiencies belong to the group of IEMs. The first report of a patient suffering from a neurological MAS deficiency was published in 2009 and described an individual suffering from AGC1 deficiency [30]. In recent years several additional MAS deficiencies have been discovered and were reported in literature (as summarized in this work in Tables 1 and 2, and as recently reviewed [3,17]). While MAS deficiencies by definition belong to the group of ultra-rare diseases, it is likely that the true incidence of these inherited defects is considerably underestimated due to diagnostic challenges and limitations [45]. Remarkably, two potential MAS deficiencies – defects of OGC and GOT1 – have not been reported in patients hitherto to the best of our knowledge. This raises the question on whether these defects do or do not exist in humans, whether they are or are not compatible with life or whether they are simply challenging to diagnose. Likewise, for a long time it has been assumed that inherited bi-allelic disease-causing variants in TCA cycle enzymes are not compatible with life. However, in recent decades several inborn errors of the TCA cycle have been reported [46]. Thus, taken together and up for now the question on compatibility with life remains unanswered.

4.2. Diagnostic challenges

MAS deficiencies are challenging to diagnose not only because they are rare but also because specific and characteristic clinical symptoms and disease-relevant biomarkers are missing or are not recognized by clinicians. Neuroimaging findings greatly vary and range from Leigh syndrome and signs of metabolic stroke to structural abnormalities as recently nicely illustrated [23]. Typical symptoms inherent to MAS deficiencies are early onset epileptic encephalopathy, global developmental delay and generalized muscular hypotonia. These symptoms were reported in a majority of affected patients [3]. Main biochemical changes found in patients with MAS deficiencies are related to altered and disrupted intermediary metabolic pathways. Not only concentrations of metabolites directly associated with the MAS – such as aspartate or glutamate – are altered, but also metabolites that are indirectly linked

to the MAS or metabolites that are crucial for the energy conversion contained in reducing equivalents of NADH at the ETC to generate ATP. Thus, besides changes in several amino acid concentrations, several organic acid concentrations – specifically those involved in the TCA cycle – are dysregulated as well. In contrast, biochemical investigations of the ETC are mostly normal as assessed in MAS deficient patient-derived tissue such as muscle (Table 3).

The clinical and biochemical phenotype not only depends on specific bi-allelic pathogenic variants but also on the affected individual's genetic background. Additional modifying factors such as environmental stressors – e.g. frequency of minor infections during early childhood associated with a catabolic state - cause a large phenotypical spectrum of disease [47]. Thus, as described for other IEMs, in patients with MAS deficiencies caused by identical bi-allelic pathogenic variants, phenotypes still considerably differ (Table 2). Since the clinical presentation varies greatly, a thorough laboratory work-up including genome sequencing is essential and required to establish an early diagnosis and to initiate a rapid treatment. Only recently genes encoding MAS defects have been included in gene panels for epilepsies which should enhance and improve the diagnostic yield. Thus far most MAS-deficient patients were identified through exome sequencing. As far as we can conclude from the 35 reported patients, MAS defects are pan-ethnic diseases.

4.3. Therapeutic aspects

So far there is no curative therapy available for none of the MAS deficiencies. Patients with inherited defects in the MAS receive supportive therapies. In addition to anticonvulsant medication, pyridoxine and L-serine proved to be effective for seizure control in patients with GOT2 deficiency. In Got2-deficient zebrafish serine and pyridoxine treatment seemed to have a synergistic effect [24]. Further studies are required to prove utility as well as mode of action of these suggested therapeutic approaches. Caution is advised when using pyruvate supplementation which could increase lactate formation resulting in acidification [24].

In patients with MDH2 and AGC1 deficiencies ketogenic diet has been applied, specifically to control refractory epileptic seizures not responding to conventional anticonvulsant therapies [17,20,33]. Ketogenic diet is based on a high fat and low carbohydrate intake. Hence it is decreasing (cytosolic) NAD^+-dependent glycolysis and is thus able to partially circumvent MAS defects [17]. While there was a drastic clinical and radiological improvement in response to ketogenic diet in an AGC1-deficient patient [35], it's role and utility in MDH2 deficiency remains to be clarified as there was significant morbidity despite ketogenic diet [20]. Recently, the medium chain triglyceride triheptanoin has been used in an individual drug trial in a patient suffering from MDH2 deficiency revealing a partial response and improvement of the phenotype [21,23]. However - as a general note - more research and specific clinical trials are required to produce solid evidence for or against the efficacy of potential treatments.

4.4. Current cellular models

For diagnostic purposes as well as in context of basic research studies, patient-derived cellular models are increasingly used. Tissue and cells gained from skin and muscle biopsies are investigated to elucidate pathophysiological concepts underlying MAS deficiencies (summarized in Table 3). Confirming reduced protein expression and/or activity is a relevant component in establishing pathogenicity caused by specific mutant MAS variants. Experiments restoring a deficient MAS component and reverting or ameliorating the phenotype are further steps required in establishing a diagnosis.

4.5. Outlook – research perspective

Advancements in gene sequencing techniques have been tremendous and opened a whole new avenue in medical diagnostics. Simultaneously we start to realize that the more results from genetic analyses are available the more questions raise. The concept or paradigm that a specific gene defect causes a specific monogenic IEM is somehow at question. While undoubtedly monogenic diseases do exist, it seems obvious too that various disease-modifying aspects require our consideration. While specific bi-allelic pathogenic variants may cause harm in an individual of an affected family, the exact same mutations may be associated with a less severe phenotype in another "affected" family member. The concept of monogenic diseases might need some refinement. One strategy to overcome such challenges is to improve personalized medicine approaches. Patient-specific disease models are one step forward in the diagnostic and therapeutic path of affected individuals. Still a rather novel strategy to create human and patient-derived cellular disease models is the application of hiPSCs (Fig. 3). This technology of producing patient-derived and organ-specific cellular models has been established for several IEMs over the last one or two decades and has been proven useful to perform patient-specific drug screenings. For example, we recently published the first hiPSC-derived disease model of MDH2 deficiency and investigated the therapeutic effects of triheptanoin [40]. To the best of our knowledge, hiPSC technology has not yet been applied to model any other MAS deficiency.

5. Summary

This review provides an overview of the 35 reported patients suffering from MAS deficiencies. Inherited bi-allelic pathogenic variants in five of the seven components of the MAS have been described hitherto and cause a wide spectrum of symptoms including early-onset epileptic encephalopathy. While timely diagnosis remains challenging and specific clinical and/or disease-relevant biomarkers are missing or are not recognized, advances in applied gene sequencing methods should improve the diagnostic yield. Likewise, diagnostic advances in analyzing patient-derived samples with multiomics approaches as well as novel patient-derived cellular models should help to improve the diagnostic and therapeutic path of affected individuals.

6. Conclusion

MAS deficiencies remain challenging to diagnose and to treat. Currently a thorough and timely patient work-up including history, clinical and laboratory assessments remains key in the diagnostic path. Improving gene sequencing technologies as well as basic and translational research conducted on patient-derived samples from individuals with ultra-rare diseases such as MAS deficiencies should further advance our knowledge to diagnose and understand fundamental pathophysiological aspects as well as to treat these mostly devastating diseases.

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Jasmine Koch: Writing – original draft, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Melissa H. Broeks:** Writing – review & editing. **Matthias Gautschi:** Writing – review & editing. **Judith Jans:** Writing – review & editing. **Alexander Laemmle:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

None.

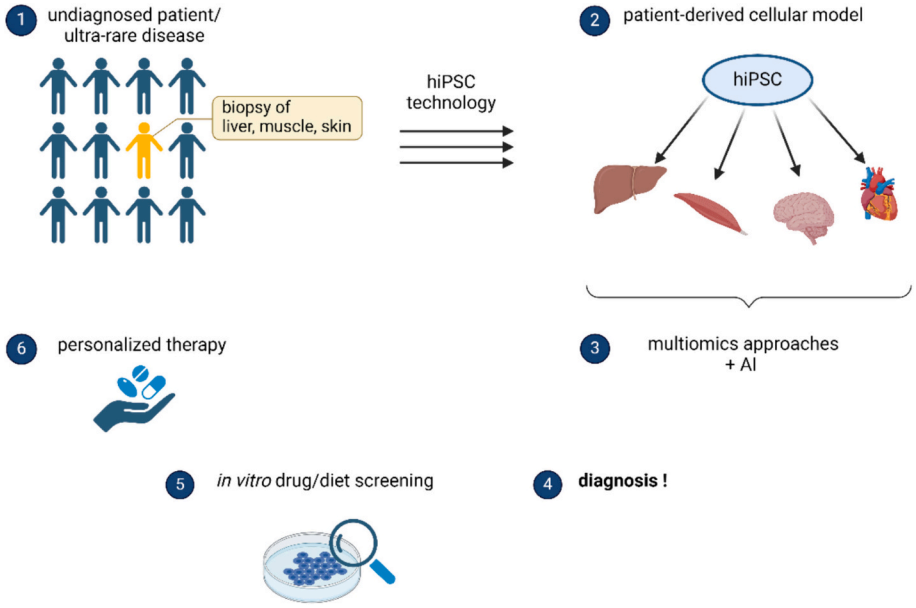

Fig. 3. Schematic process of patient-specific disease model using hiPSC technology.
Extraction of liver, muscle or skin biopsy of undiagnosed patient with suspected ultra-rare disease [1]. Application of hiPSC technology to generate patient-derived cellular models [2]. Multiomics approaches and artificial intelligence (AI) algorithms [3] are used to establish a diagnosis [4]. Patient-specific disease models are then used for in vitro drug/diet screenings [5] and to develop personalized therapies [6]. Figure created with BioRender.com.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2024.108520.

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