The effect of deficiency and gain-of-function mutations in MDH2 and its relation with paragangliomas

By

Anouk Haasnoot

6156762

Writing Assignment

Biology of Disease

Faculty of Medicine

University of Utrecht

Supervisors:

Hannah German

Dr. Judith Jans

Dr. G.J.T. Zwartkruis

Prof. dr. Nanda Verhoeven-Duif

Content

List of abbreviations

2OG	2-oxoglutarate
3PG or G3P	Glycerate 3-phosphate or 3-phosphoglyceric acid
5hmc	5-hydroxy-methylcytosine
5mc	5-methylcytosine
ACG	Aspartate-glutamate carrier
Asp	Aspartate
ATP	Adenosine triphosphate
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
E4P	Erythrose 4-phospate
EPO	Erythropoietin
F6P	Fructose 6-phosphate
FAD	Flavin adenine dinucleotide
FADH	Flavin adenine dinucleotide hydrogen
FH	Fumarate hydratase
FIH	Factor inhibiting HIF
G6P	Glucose 6-phosphate
GA3P	Glyceraldehyde 3-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Glycerol 3-phosphate dehydrogenase
Glu	Glutamate
GLUT	Glucose transporter
GOT	Aspartate aminotransferase/glutamate oxaloacetate transaminase
HIF	Hypoxia-inducible factor
HIF-1α	Hypoxia-inducible factor 1 alpha
IDH	Isocitrate dehydrogenase
IDO	Indoleamine 2,3-dioxygenase
iPSC	Induced pluripotent stem cell
KD	Knock-down
KO	Knock-out
MAPK	Mitogen-activated protein kinase
MAS	Malate aspartate shuttle
MDH	Malate dehydrogenase
MDH2	Malate dehydrogenase 2
MODY	Maturity onset diabetes of the young
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
OCG	Oxoglutarate/malate carrier
PDH	Pyruvate dehydrogenase
PGL	Paraganglioma
PHGDH	Phosphoglycerate dehydrogenase
PPC	Pheochromocytoma
PPGL	Paragangliomas and pheochromocytomas
PSMA1	Proteasome subunit alpha type 1
R5P	Ribose 5-phosphate
RBP1	Retinol binding protein 1
RNA	Ribonucleic avid
SDH	Succinate dehydrogenase
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid
TDO	Tryptophan 2,3-dioxygenase
TET	Ten eleven translocation
Trp	Tryptophan
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
X5P	Xylulose 5-phosphate
α-KG	Alpha-ketoglutarate

Abstract

The malate-aspartate shuttle (MAS) consists of four enzymes and two mitochondrial carriers. The enzymes are malate dehydrogenase (MDH)1 and 2, and glutamate oxaloacetate transaminase (GOT)1 and 2, while the carriers are named aspartate-glutamate carrier (ACG) and oxoglutarate/malate carrier (OCG)1 and 2. MDH2 couples the MAS to the tricarboxylic acid (TCA) cycle. The MAS plays an important role in maintaining NAD^+ levels, both in the cytoplasm and the mitochondrial matrix. Due to its function in maintaining the NAD^+/NADH balance, it is also important for all NAD^+-dependent processes including glycolysis, serine production, pentose phosphate pathway, and the glycerol-3-phosphate shuttle. Patients with MDH2 deficiency presented at a young age with lethargy and neuropathologies, such as seizures. Moreover, MDH2 mutations are known to be involved in different cancer types, including paragangliomas and pheochromocytomas. With an MDH2 mutation, the paraganglioma or pheochromocytoma is more likely to be malignant. Several pathological pathways have been proposed, however, the pathogenic mechanism has not been elucidated yet.

Currently, two treatment options are being investigated for MAS deficiency, namely LOXCAT and triheptanoin. LOXCAT can normalise the cytosolic NAD^+/NADH ratio. Triheptanoin has shown a positive effect in a single patient. Therefore, more research is needed to either further test treatment options or to develop a new treatment option for MDH2 phenotypes. More in depth understanding of the pathways affected by MDH2 mutations as well as other MAS defects could lead to better understanding the pathogenic processes underlie MDH2 deficiency, and the way MDH2 affects the development of paragangliomas and pheochromocytomas.

This review will describe the function of MDH2, its role in disease, and research models for MDH2 abnormalities. In addition, an overview of the pathophysiological pathways involved in paragangliomas and pheochromocytomas will be presented.

Lay summary

A cell needs energy to function. The mitochondria are very important for the energy production of the cell. Within the mitochondria different proteins are responsible for different pathways which eventually result in energy production. Since these processes are very important, they are similar between different species. Nonetheless, in rare occasions, a mutation may occur in one of the mitochondrial proteins, such as malate dehydrogenase (MDH) 2. A mutation can have several consequences, either the protein becomes more active, less active, or functions entirely differently. First, in case of reduced activity, processes for energy production cannot happen as they normally do. This means that tissues that need a lot of energy, such as a brain or muscles, do not get enough. Therefore, patients may become weak and have trouble developing. Second, activating MDH2 mutations were found in two families with diabetes mellitus. It is not yet known how these specific mutations lead to diabetes mellitus. Third, energy production is usually disturbed in case of cancer. It is believed that this is because cancer grows fast and, therefore, needs a lot of energy. Normal energy production is slow, yet it harvests a lot of energy. However, cancer energy production is usually quick, but not as efficient compared to normal energy production. Thus, a problem with a protein that is important in the energy production can influence cancer development and its progression.

Each of the consequences of a different type of mutation has different symptoms that are expressed. Currently, for each type of symptoms there are treatment options. First, in case of reduced activity, patients are given a specific diet and, serine and vitamin B6 supplements. This is supportive, so there is no cure. Second, the mutations that leads to diabetes mellitus is treated as normal diabetes mellitus. Third, the mutation in MDH2 can lead to a more malignant cancer and treatment is generally ineffective. It is recommended to adjust the treatment to the specific type of mutation, and to test family members to see whether they also carry the same mutation.

To conclude, there are still many questions surrounding MDH2 mutations and the different phenotypes it causes. More research into the pathways causing this will lead to more understanding of the pathological pathways leading to these phenotypes. Hopefully, this better understanding will help in developing more treatments for the different MDH2 phenotypes.

Introduction

Mitochondrial metabolic disorders comprise of a wide range of clinical symptoms, from diabetes mellitus to neurological disorders. They mainly affect organs with high-energy requirements (1), such as the brain, liver, and muscles (2). The malate-aspartate shuttle is responsible for maintaining the nicotinamide adenine dinucleotide (NAD) and nicotinamide-adenine-dinucleotide hydrogen (NADH) ratio within cells. The NAD^+/NADH balance is important ensuring several metabolic pathways can occur, including serine synthesis, tryptophan metabolism, glycolysis, the tricarboxylic acid (TCA) cycle (2), and oxidative phosphorylation (3). Moreover, NAD^+-dependent processes are important in signalling pathways, transcriptional regulation and DNA repair (2). NAD^+ homeostasis is also associated with a prolonged lifespan, has a protective quality against inflammatory and infectious diseases, and is possibly important in resisting metabolic syndrome, neurodegenerative disease, and cancer (3). Moreover, a disturbed nuclear NAD^+/NADH ratio results in an increase of hypoxia-inducible factor 1 alpha (HIF-1α), which induces the Warburg effect, which is an important hallmark of cancer metabolism (3).

The malate-aspartate shuttle (MAS) consists of four enzymes and two transporters (2). The enzymes include malate dehydrogenase (MDH) and aspartate aminotransferase (GOT), both having a mitochondrial and cytosolic isoform. MDH1 and GOT1 encode the cytosolic isoforms, and MDH2 and GOT2 the mitochondrial isoforms, respectively (2,4,5). Both MDH2 and GOT2 are functionally coupled to the TCA cycle and adenosine triphosphate (ATP) production. MDH1 is highly expressed in the brain, heart, and skeletal muscle, whereas MDH2 is expressed more throughout the entire body (2), especially in skeletal muscle, liver, adipose tissue, endocrine pancreas and brain (5).

Within the central nervous system, MAS has an additional function, namely, the synthesis of aspartate and glutamate for neurotransmission (2). Additionally, tryptophan metabolism is important, since it is a building block for essential molecules, such as melatonin and serotonin, and it can serve as a precursor for NAD^+ (3). In a developing foetus, fatty acid metabolism is preferred over glucose metabolism, however, in infants this switches (6). In this period, the disturbance of MAS can lead to more detrimental phenotypes. Deficiencies in GOT2, MDH1, MDH2, and AGC1 all present with early infantile epileptic encephalopathy (2). However, there is still insufficient knowledge on these different enzymes. More research is needed to improve patient care and to better understand the metabolic pathways involved. This review will have a limited scope, only focussing on MDH2.

In 2017, the first patients with an MDH2 loss-of-function mutation were reported (1). Loss-of-function MDH2 mutations have been reported to cause early-onset severe encephalopathy. Nonetheless, no change in glucose homeostasis was observed (1). However, in gain-of-function MDH2 mutations, a phenotype similar to diabetes mellitus was observed, lacking the neuropathology seen in loss-of-function MDH2 mutation (5). This shows that different mutational mechanisms can lead to different phenotypes. Which mechanisms are responsible for the pathological symptoms is not well understood (4). Moreover, MDH2 has a suggested role in cancer and cancer metabolism (7). Therefore, further research is needed. To this end, this review will give an overview of possible pathophysiological pathways of MDH2 mutations, a short description of all identified patients so far, including symptoms and possible biomarkers, and different models that can be used to study MDH2 mutations.

Malate dehydrogenase 2

MDH2 is a protein encoded on chromosome 7, consisting of 10 exons (8) and is made up of 338 amino acids (figure 2) (9). The catalytic activity of MDH2 enables the reversible conversion of malate to oxaloacetate (10). During this reaction NAD^+ is reduced to NADH.

MDH is a homodimer. Each subunit contains two domains: the NAD-binding domain and the malate binding domain. The NAD-binding domain is localised in the amino-terminal half of MDH and consists of a parallel β-sheet structure, also known as the Rosman fold motif. The substrate binding domain is located at the carboxy-terminal domain. The active site of MDH2 is located in the cleft between the two domains (figure 1 + table 1) (5,10,11). It consists of mainly hydrophobic amino acids, which includes the binding sites for malate and the nicotinamide ring of the coenzyme. The active site also contains a histidine/aspartate pair required for proton transfer and an arginine which has an anchoring role towards malate (10).

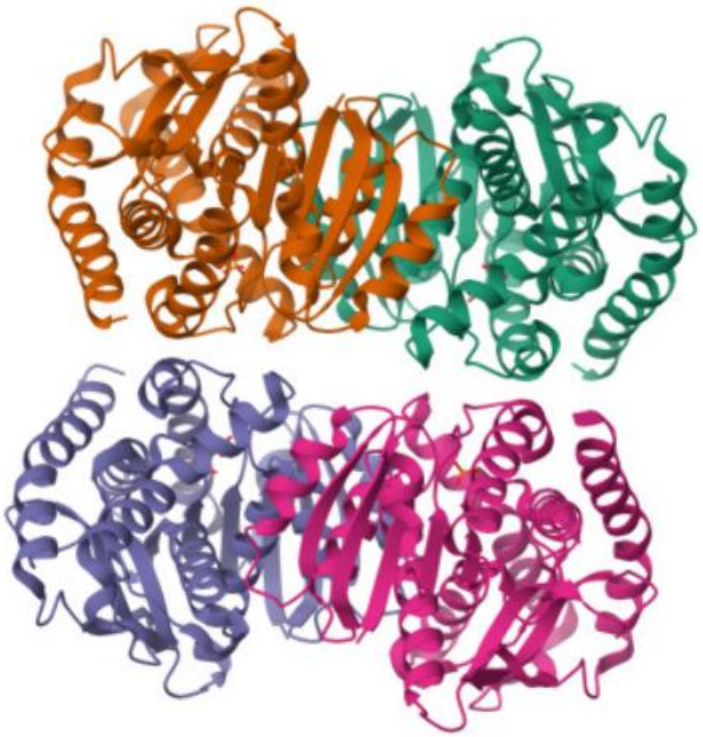

Figure 1. Crystal structure of L-Malate bound MDH2. Malate and NAD(H) are not visible. The N-terminus of the orange monomer is located at the right top, and the C-termius at the left bottom (11).

MDH2 is expressed in different tissues. MDH2 RNA is expressed in tissues with a central role in glucose metabolism, such as skeletal muscle, heart tissue, liver, adipose tissue, endocrine pancreas, and brain tissue (5,8).

MDH2 catalyses a reversible reaction. High concentration of the substrate, malate, increase the production of the product, oxaloacetate, and vice versa. MDH2 is allosterically regulated. Citrate inhibits malate oxidation, but only at low malate or NAD^+ concentrations. In case of high malate and NAD^+ concentrations, citrate stimulates MDH2 activity (10). MDH2 activity is also increased by acetylation (5).

Table 1. Different functional domains within the MDH2 molecule (5,9)

What?	What?	Amino acid number
NAD^+	Binding site	31-37, 52, 57, 117, 160, 251
Substrate	Binding site	104, 110, 142, 176
Proton acceptor	Active site	200
Malate	Binding site	213-235

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        10         20         30         40         50         60         70         80         90        100        110
MLSALARPAS AALRRSFSTS AQNNAKVAVL GASGGIGQPL SLLLKNSPLV SRLTLYDIAH TPGVAADLSH IETKAAVKGY LGPEQLPDCL KGCDVVVIPA GVPRKPGMTR

       120        130        140        150        160        170        180        190        200        210        220
DDLFNTNATI VATLTAACAQ HCPEAMICVI ANPVNSTIPI TAEVFKKHGV YNPNKIFGVT TLDIVRANTF VAELKGLDPA RVNVPVIGGH AGKTIIPLIS QCTPKVDFPQ

       230        240        250        260        270        280        290        300        310        320        330
DQLTALTGRI QEAGTEVVKA KAGAGSATLS MAYAGARFVF SLVDAMNGKE GVVECSFVKS QETECTYFST PLLLGKKGIE KNLGIGKVSS FEEKMISDAI PELKASIKKG

EDFVKTLK
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Figure 2. The amino acid sequence of human MDH2 (9).

Pathways

MDH2 is involved in multiple metabolic pathways. It is directly involved in the TCA cycle and the MAS, and indirectly in metabolic pathways which use NAD^+ or NADH. These pathways include serine synthesis, glycolysis, the glycerol-3-phosphate shuttle, and the pentose phosphate pathway. De novo tryptophan synthesis is a source of NAD^+. All the metabolic pathways named will be discussed below.

Malate-aspartate shuttle

The main function of the malate aspartate shuttle (MAS) is maintaining the cytosolic and mitochondrial NAD(H) levels. NADH and NAD^+ cannot pass the mitochondrial membrane. The MAS consists of four enzymes and two mitochondrial carriers. The four enzymes are MDH1, MDH2, aspartate aminotransferase (GOT) 1 and 2. The two carriers are aspartate-glutamate carrier (ACG) and the oxoglutarate/malate carrier (OGC). There are two isotypes of ACG, namely 1 and 2. All the carries within the MAS are located in the inner mitochondrial membrane (figure 3). The expression of the carriers is dependent on the type of tissue, AGC1 is highly expressed in the brain, heart, central nervous system, whereas AGC2 is expressed in the epithelial lining of the intestine and the liver. The MAS starts with the reduction of oxaloacetate to malate and the oxidation of NADH to NAD^+ by MDH1 in the cytosol. Next, the OGC exchanges malate for 2-oxoglutarate. In the mitochondrion, malate is converted to oxaloacetate and NAD^+ is converted to NADH by MDH2 (2). This is an ordered reaction. First, the NAD^+ binds to MDH2. Next, malate binds to MDH2. After the binding of malate to MDH2, MDH2 undergoes a conformational change where an external loop closes over the active site. (10). This process is similar for MDH1. After the reaction takes place, transamination of oxaloacetate and glutamate by GOT2 results in the formation of aspartate and 2-oxoglutarate. AGC exchanges aspartate for glutamate and a proton. Lastly, the aspartate and 2-oxoglutarate are converted into oxaloacetate and glutamate by GOT1 (2). After the description of the role of MDH2 in the TCA, I will review processes that are disturbed in case the NAD^+/NADH balance is not maintained.

TCA cycle

The tri-carboxylic acid (TCA) cycle is required for oxidation of pyruvate or fatty acids. Pyruvate, the product of aerobic glycolysis, is transported into the mitochondrial matrix. There, the NAD^+-dependent pyruvate dehydrogenase is responsible for the conversion of pyruvate into acetyl-CoA. The TCA cycle starts when an acetyl-CoA molecule is covalently bound to oxaloacetate, yielding citrate. Each round of the TCA cycle results in one GTP, one $FADH_2$, and three NADH molecules. Next, NADH is oxidized to NAD^+ by complex I of the electron transport chain. This results in protons being transported from the mitochondrial matrix into the cytosol. The proton gradient that is formed drive the formation of ATP via ATP synthase. In total, one glucose molecule yields 32 ATP molecules (12). Two molecules are also able to enter the TCA cycle, namely malate and 2-oxoglutarate (2).

Glycolysis

Aerobic glycolysis can occur in the presence of both oxygen and mitochondria. Without oxygen, mitochondria, or both, anaerobic glycolysis will take place (12). The glycolysis starts with a ten step NAD^+-dependent metabolic pathway converting glucose to pyruvate. The NAD^+-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) converts glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (2).

In case of the presence of oxygen and mitochondria, pyruvate enters the TCA cycle (13). In the absence of oxygen, mitochondria, or both, the pyruvate is converted to lactate by lactate dehydrogenase. During this reaction NADH is oxidised to NAD^+ (14) allowing glycolysis to continue.

De novo serine synthesis

Serine synthesis is a side chain of glycolysis. The first step of serine synthesis is catalysed by phosphoglycerate dehydrogenase (PHGDH), which is dependent on NAD^+ (2,15). Subsequent steps convert 3-phosphohydroxy pyruvate into serine, by the use of transamination by phosphoserine aminotransferase (PSAT)1 and phosphate ester transamination by phosphoserine phosphatase (PSPH) (15).

MAS defects result in reduced serine synthesis. This is caused by a reduced level of NAD^+. NAD^+ is part of the first step in de novo serine synthesis, and therefore, a shortage in NAD^+ causes a reduction in serine levels. Another effect that can be seen in case of MAS defects, is a drop in the pyruvate-lactate ratio. As a reaction, there is an increase in pyruvate synthesis. Serine can be used during pyruvate synthesis. This mechanism might also cause a drop in serine levels (16).

Pentose phosphate pathway

The pentose phosphate (PPP) is another branch of glycolysis. It starts right after the first committed step of glycose metabolism (6,17). The PPP is important for ribonucleotide synthesis and is a source of nicotinamide adenine dinucleotide phosphate (NADHP) (6). Multiple steps within the PPP are NAD^+-dependent (figure 3). (17). The PPP is important for cancer cells since it provides NADPH. The NADPH is required for fatty acid synthesis and resolving reactive oxygen species. This means that it helps glycolytic cancer cells with their anabolic demands and with combating oxidative stress (17).

Glycerol-3-phosphate shuttle

The glycerol 3-phosphate (G3P) shuttle is a side loop of glycolysis. It occurs in the cytosol. Glycerol 3-phosphate dehydrogenase (GDP)1 transfers the reducing equivalents of NADH to dihydroxyacetone phosphate (DHAP), and in the process also forms G3P and NAD^+. In turn, G3P is oxidized into DHAP, and flavin adenine dinucleotide (FAD) is reduced to flavin adenine dinucleotide hydrogen (FADH)2. FADH2 is then able to enter the oxidative phosphorylation (figure 3). Within the G3P shuttle, GDP2 is the rate limiting enzyme. In mammalian cells, GDP2 is expressed in brown adipose tissue, muscle, brain, and in lesser extent in the liver, heart and β-pancreatic cells (2).

The G3P shuttle is directly connected to cytosolic NAD^+ and NADH levels. Moreover, it functions as a connection between the glycolysis, electron transport chain, and the glycerol metabolism. Therefore, the G3P shuttle might of importance in MAS deficiencies (2).

De novo tryptophan metabolism

Tryptophan (trp) is an important substrate for the *de novo* synthesis of NAD^+. Trp can be converted to NAD^+ in an eight-step process. The rate limiting step is the first step, where Trp is converted to N-formylkynurenine by indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) (figure 3). The expression of the enzymes is dependent on the tissue, IDO is mostly expressed in the liver, whereas TDO is expressed in numerous cell types including microglia, astrocytes, neurons, and macrophages (3).

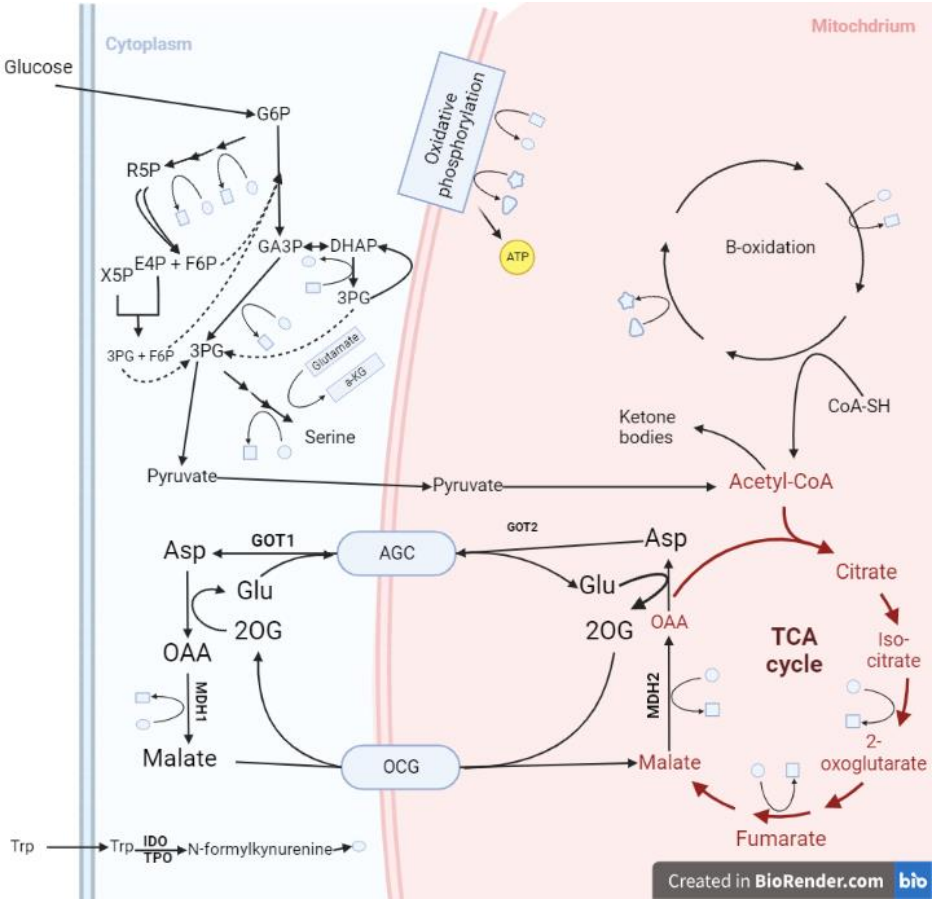

Figure 3. Overview of the different pathways either MDH2 is directly involved in, or indirectly by affecting the NAD^+/NADH balance. In the left top corner, glycolysis is shown, including the alternative pentose phosphate pathway. It is also shown which product in the glycolysis is important for the serine synthesis. In the right lower corner, the MAS is shown, coupled to the TCA cycle in the lower left corner. The upper right corner shows the β-oxidation. The oxidative phosphorylation with the electron transport chain is also shown. Each round is a NAD^+ molecule, each square is an NADH molecule. A triangle is FAD, where a star is $FADH_2$.DHAP = dihydroxyacetone phosphate, E4P = erythrose 4-phosphate, F6P = fructose 6-phosphate, GA3P = glyceraldehyde 3-phosphate, glu = glutamate, R5P = ribose 5-phosphate, X5P = xylulose 5-phosphate, 2OG = 2-oxoglytarate, trp = tryptophan, IDO = indoleamine 2,3-dioxygenase, TDO = tryptophan 2,3-dioxygenase, MDH = malate dehydrogenase, GOT = glutamate oxaloacetate transaminase, AGC = aspartate-glutamate carrier, OCG = oxoglutarate malate carrier, α-KG = alpha ketoglutarate. Created with Biorender. Based on (2–4,6,13,17).

MDH2 deficiency

Patients

Symptoms

Currently, 12 patients have been described with a MDH2 deficiency due to a loss-of-function mutation (table 2) (1,4,18,19). Most patients presented symptoms at two months of age. There was no skewed ratio between female and male. All patients presented with either hypotonia (decreased muscle tone) or ataxia (poor muscle control) and seizures or other neurological symptoms. All patients suffered from developmental delay. Moreover, patients presented with eye or vision abnormalities and/or obstipation. Only 2 patients had a family history with cancer (4).

Diagnosis

The diagnosis was made after either exome sequencing, multigene sequencing, or familial variant testing. Biomarker testing showed elevated lactate levels (11/12 patients), elevated lactate to pyruvate ratio (6/6 patients) and urinary excretion of malic acid (6/10 patients), fumaric acid (6/11 patients), and succinic acid (2/11 patients) (4). Brain magnetic resonance imaging (MRI) was aberrant (11/12), commonly showing abnormal brain ventricles. Priestly et al. (4) suggests that indicators of a MDH2 deficiency are patients presenting with lactatemia, with early-onset seizures, structural brain anomalies, and developmental delays. Other indicators are elevated levels of malic acid and fumaric acid in the analysis of urinary excretion of organic acids. To confirm the diagnosis a gene sequencing assay should be performed (4).

Ait-El-Mkadem et al. (1) used patient fibroblasts to establish the role of Pro133Leu and Pro207Leu, and of Gly37Arg and Pro133Leu in MDH2 deficiency. A western blot showed decreased levels of the MDH2 protein, and additionally, the enzymatic activity was decreased, thus, the mutation did not result in a null mutation. The parents with each one of the mutations also showed decreased enzymatic activity, which suggests each mutation has a deleterious effect. The combination results in a severe MDH2 deficiency (1).

Treatment

The current standard of care is supportive (4). For all MAS deficiencies, the current treatment includes a ketogenic diet (16), as well as serine and vitamin B6 supplementation (2). The ketogenic diet is low-carbohydrate and high-fat, resulting in a reduction of glycolysis and an increase in β-oxidation and the usage of ketone bodies. This seems to have a generally positive effect (16). Laemmle et al. reported a patient who improved after triheptanoin treatment in regard to her motor skills, she gained weight, and there was a lack of further metabolic decompensation (19).

Two treatment options for MDH2 deficiency are currently being investigated, namely LOXCAT and triheptanoin (19,20). LOXCAT is a fusion protein of the bacterial lactate oxidase and catalase, which is able to convert lactate and oxygen into pyruvate and water. It was shown that LOXCAT was able to normalise the cytosolic lactate/pyruvate and NAD^+/NADH ratio. However, the MAS is able to re-oxidise the NADH that is created in the cytosol. Therefore, the intra-mitochondrial NADH cannot be re-oxidised. Nonetheless, this could mean that LOXCAT holds potential as a treatment in MAS deficiencies (20). Triheptanoin has already shown positive results in one patient (19), further research on triheptanoin as a treatment for MDH2 deficiency is not yet published.

In short, loss-of-function usually results in neuropathological symptoms and loss of muscle strength at a young age. No specific biomarkers are available (4), and the standard care consists of a ketogenic diet (16).

Table 2. Overview of patients identified with MDH2 deficiency due to MDH2 mutation, with both allelic forms, whether the mutation is homo- or heterozygous, the age of onset, last follow-up date, their heritage, and sex. The two Afghani are brothers. * Indicates death of patient. Information from: (4).

Mutation	Homo or heterozygous	Age of onset symptoms	Last follow-up/death	Heritage	sex
p.Pro133Leu p.Pro207Leu	Compound heterozygous	5 months	5 years	French	Male
p.Pro133Leu p.Pro199Alafs*10	Compound heterozygous	2 months	1.5 years*	Dutch	Male
p.Gly37Arg p.pPo133Leu	Compound heterozygous	neonatal	12 years	French	Male
p.pro133Leu p.Pro149Hisfs*22	Compound heterozygous	18 months	3-4 years	European	Female
c.320-2A>T (splicing?) p.Pro214His	Compound heterozygous	18 months	7 years	Italian	Male
p.Pro133Leu p.Asp173Asn	Compound heterozygous	2 months	21 months	European, African American	Female
c.885+5G>A (intronic)	Homozygous	2 months	11 months	Indian	Female
c.885+5G>A (intronic)	Homozygous	Neonatal – day 2	19 months	Indian	Male
p.Gly306Ser	Homozygous	10 weeks	3 years	Indian	Female
p.Met251Val	Homozygous	1 month	3 years	Afghani	Male
p.Met251Val	Homozygous	2 months	23 months	Afghani	Male
p.Ala66Thr	Homozygous	Late infancy	21 years	Somali	Female

MDH2 gain-of-function

Patients

Symptoms

MDH2 gain-of-function mutations may lead to diabetes mellitus type 2 (T2DM). T2DM can be caused by genetic defects, such as maturity onset diabetes of the young (MODY). This usually is inherited in an autosomal dominant matter and mostly occurs in nonobese young individuals. Several causative genes have been identified so far (21). However, this does not explain all the different T2DM cases (5).

Diagnosis

Jungtrakoon et al. (5) studied 60 families, with a total of 1144 patients with early onset diabetes. They set out to discover more genes associated with MODY. The mutation p.Val150Met in MDH2, a missense mutation, was strongly associated with hyperglycaemia in an Italian family. In an American family, they found the p.Arg52Cys mutation in MDH2. Both families showed a median age of 40 for age of onset (table 3). There was no specific biomarker mentioned for MDH2-mediated T2DM (5).

Gain-of-function mutations are very rare in metabolic enzymes. To prove the gain-of-function mutation in MDH2 HepG2 human liver cells were transfected with cDNAs encoding WT MHD2, Arg52Cys MDH2 or Val150Met MDH2 to check expression levels and enzymatic activity. The expression levels were similar between the different types of MDH2. The enzymatic activity was determined by quantifying the oxidation of malate to oxaloacetate. The transfected cells all had increased levels of oxaloacetate, compared to untransfected cells. The mutated variants showed an increase over the WT transfection (5).

Table 3. Overview of the carriers of the two families with their respective diagnosis and number of patients per diagnosis, age at diagnosis or examination, sex and their mutation. In the American family there is one age unknown in the diabetes group and one in the prediabetic group. If disregarding the outlier in the American diabetes group, the average age is 42. All mutations are heterozygous (5).

Number of patients	Diagnosis	Age at diagnosis/examination	Sex	Mutation
American Family				
6	Diabetes	34 or 42 (2-46)	1F:5M	Arg52Cys
3	Prediabetic	44 (30-58)	3F	
1	No diagnosis	16	1M	
Italian Family				
3	Diabetes	40 (34-40)	1F:2M	Val150Met
1	Prediabetic	34	1F	
6	No diagnosis	32.5 (23-64)	4F:2M	

Gain-of-function MDH2 mutations have a less clear phenotype compared to the loss-of-function phenotype. The onset of symptoms in gain-of-function MDH2 T2DM seems to be age dependent.

Treatment

The patients are treated as a normal T2DM patient (5).

Paraganglioma and pheochromocytoma

Pheochromocytoma and paragangliomas (PPGLs) are neuroendocrine tumours (22). Paragangliomas originate from non-chromaffin cells of the parasympathetic ganglia in the hands and/or head, or from chromaffin cells of the sympathetic nervous system in the chest, abdomen, or pelvis. Pheochromocytomas are paragangliomas located in the adrenal medulla (7,23). Paragangliomas show much genetic and clinical diversity (22), and are considered to be the tumour most frequently inherited (7). More details can be found in table 4.

Multiple genes have been identified that increase PPGL susceptibility. An overview of different papers and their respective susceptibility genes can be found in table x. However, there are still patients with the clinical indicators of an inherited tumour (i.e. family history, multiple tumour, and/or young age) that are not explained by mutations in the susceptibility genes as named by Cascón et al. (table 4) (24).

Susceptibility genes	Paper
SDHx, FH, MAX, NF1, RET, TMEM127, VHL	(24)
SDHx, *FH*, EPAS1, HRAS, MAX, NF1, RET, TMEM127, VHL	(22)
* **GOT2,** ATRX, BAP1, BRAF, CSDE1, EGLN1/PHD2, EGLN2/PHD1, EZH2, FGFR1, H3F3A, IDH1, IDH3B, JMJD1C, KDM2B, KIF1B, KMT2D/MLL2, MEN1, MERTK, MET, MITF, SETD2,TP53	(22)
SDHx, *FH*, ***MDH2,*** **GOT2**, **SLC25A11 (OCG)**, DLST, EPAS1, MAX, NF1, RET, TMEM127, VHL,	(7)
SDHx, *FH*, ***MDH2,*** ATRX, HIF2A, HRAS, MAX, NF1, RET, TMEM127, VHL	(23)
SDHx, FH, HIF2A, IDH, KIF1Bb, MAX, NF1, PHD2, RET, TMEM127, VHL	(25)

Table 4. Overview of the susceptibility genes according to the different papers. SDHx = SDHA/B/C/D/AF2. FH = * = susceptibility genes for which the contribution to the disease remains unclear. In bolt are genes within the MAS, in italic are genes within the TCA cycle (7,22–25).

As can be seen from the different susceptibility genes (table 4), there are multiple genes encoding proteins that are important in the TCA cycle (24), namely SHDx, FH, and MDH2. Moreover, genes from the MAS can also affect the development of PPGLs. These genes include MDH2, GOT2, and OCG. This part of the review will focus on the relationship between PPGLs and MDH2, and the similarities and differences between MDH2-mediated PPGLs and SHDx/FH-mediated PPGLs.

Cluster PPGL

The mutations of PPGLs are mainly classified into two clusters. Cluster 1 includes mutations in PDH2, VHL, SDHx, IDH, HIF2A and FH. They are involved in the pseudo-hypoxic pathway. Cluster 2 includes mutations in RET, NF1, KIF1B, MAX, and TMEM127, and is associated with abnormal activation of kinase signalling pathways. (25). Both benign and malignant cluster 1 tumours show an increase in vascularisation and the expression of vascular endothelial growth factor (VEGF) and its receptors (figure 4) (23). SHDx, FH, and MDH2 are all cluster 1 genes, therefore, this review will only describe the tumourigenesis of cluster 1.

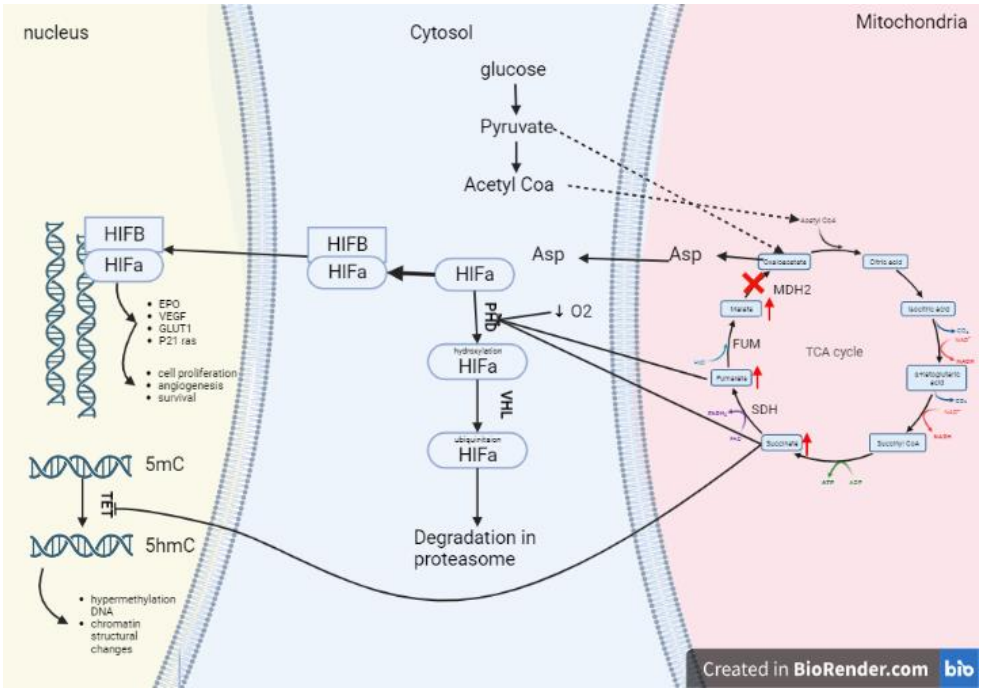

Figure 4. Overview of the hypothesized pathways that influence cancer development. This is mainly due to accumulation of the intermediates of the TCA cycle. Asp = aspartate, HIF = Hypoxia inducible factor, PHD = prolyl hydroxylase domain, VHL = von Hippel-Lindau, EPO = erythropoietin, VEGF = vascular endothelial growth factor, GLUT = glucose transporter, 5mC = 5-methylcytosine, 5hmc = 5-hydroxy-methylcytosine, TET = ten eleven translocation, FUM = fumarase, SDH = succinate dehydrogenase. Created with Biorender. Based on (7,23,25)

Cluster 1 PPGL

Within cluster 1, there are multiple different pathways that affect tumourigenesis. Since both SDHx and FH are part of the TCA cycle like MDH2, this part of the review will focus on cluster 1 tumourigenesis, specifically of FH- and SHDx-mediated PPGLs and the relationship between MDH2 and PPGL.

In FH- and SDHx-mediated tumours there is an accumulation of fumarate and succinate, the respective substrates of FH and SDH (figure 4). HIF consists of the constitutionally HIFβ and the hypoxia induced HIF1/2α. Normally, HIF1/2α is broken down quickly due to hydroxylation by prolyl hydroxylase domain (PHD). In case of hypoxia, pyruvate dehydrogenase can no longer hydroxylate HIF1/2α and HIF1/2α is stabilised and translocated to the nucleus (7). The accumulation of fumarate and succinate has three main effects in the tumourigenesis.

First, accumulated fumarate and succinate inhibits PHD activity, as they are competitive inhibitors of PHD. This results in pseudohypoxia (23). Second, accumulation of fumarate and succinate inhibits factor inhibiting HIF (FIH). FIH can hydroxylate an asparagine on the transactivation domain of HIF. This prevents the recruitment of coactivators and renders the HIF inactive. The inhibition of FIH results in less inhibition for HIF. This includes the regulation of angiogenesis, tumour growth, energy metabolism, and cell survival (23). Third, there is also methylation on CpG sites in the DNA (24), leading to reprogramming of transcription on a genomic scale, mostly by repressing cancer stem cell identity gene expression (23,24). This is done by ten eleven translocation (TET) enzymes, which hydroxylate 5-methylcytosine (5mC) into 5-hydroxy-methylcytosine (5hmC). Inhibition of TET enzymes by succinate leads to DNA methylation and low DNA hydroxymethylation. This results in hypermethylation of CpG islands, which is associated with repression of transcription. This repression of transcription also affects gene expression related to angiogenesis, tumour growth, energy metabolism, and cell survival. Next to the epigenetic alterations, miRNAs are also dysregulated (figure 4) (23).

SDH-mediated PPGL

SDHx genes (SDHA, SDHB, SDHC, SDHD, and SDHAF2) were the first mitochondrial proteins implicated in the development of cancer. This supported the hypothesis of the Warburg effect (23). The Warburg effect states that cancer cells take up 4- to 10-fold more glucose. In normal cells, lactate is only produced during hypoxia. However, in cancer cells lactate is also produced during normal oxygen levels, resulting in a lower ATP yield, then if the glucose would be used in the TCA cycle. However, the ATP yield is faster (figure 5) (13).

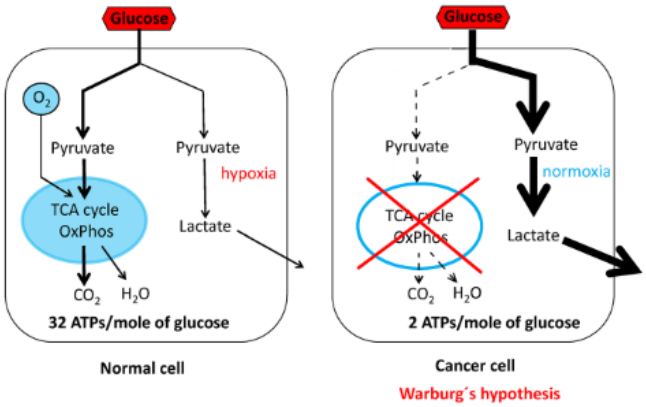

Figure 5. Warburg effect. In case of normal oxygen levels, aerobic metabolism is preferred over anaerobic (left). During lack of oxygen the cell would switch to anaerobic metabolism. This is sometimes also seen in cells with normal oxygen levels. This is called the Warburg effect (left) (13).

Succinate dehydrogenase (SDH) is an enzyme located in the inner mitochondrial membrane. It is part of the TCA cycle (figure 4), where it oxidizes succinate into fumarate and transfers electrons to the respiratory chain. SDH consists of two catalytic proteins (SDHA and SDHB), two anchoring proteins (SDHC and SDHD), and one protein essential for the assembly of the complex (SDHAF2) (23). SDHx genes are tumour suppressor genes, and combined with loss-of-heterozygosity, can lead to complete loss of SDH activity (7).

The different SDH subunits lead to different phenotypes of PPGL (table x). A mutation in SDHA leads to Leigh syndrome, SDHB and SDHD have the highest mutation rate, and SDHB occurs most often in a malignant tumour (7,22–25).

FH-mediated PPGL

Fumarate hydratase (FH) in an enzyme within the TCA cycle that catalysis the hydration of fumarate to malate (figure 4) (7). FH mutations predispose to PPGL by a hypermethylator phenotype. FH-mediated PPGLs are most similar to malignant SDHB-mediated tumours (25)

MDH2-mediated PPGL

It is known MDH2 dysfunction increases the risk for PPGLs (22), and MDH2-mediated tumours have a similar transcriptional profile as SDH-mediated PPGLs. Moreover, MDH2- mediated PPGLs also have methylation on CpG sites in the DNA, similar to other cluster 1 PPGLs. This phenotype is also linked to TCA cycle disruption (24).

Malate inhibits HIF1a, however, there was no malate accumulation found in the MDH2-mediated PPGLs. However, there was a high fumarate-succinate ratio in the MDH2-mediated PPGLs, suggesting fumarate accumulation. This fumarate accumulation could be responsible for the CpG site DNA methylation phenotype (24).

Patients

Cascón et al. (24) described a patient with PPGL and a mutation in MDH2. This 55-year-old patient had five single nucleotide substitutions and 11 insertions or deletions in his MDH2 gene. One of the mutations found in MDH2 was c.429+1G>T, which affects the splicing of the protein. This meant that 20 additional amino acids were added into the MDH2 protein including a premature stop codon. The $MDH2_{c.429+1G>T}$ had a six to fourteen-fold lower levels of MDH2 expression (table 4). This patient had 4 different tumours, of which two had loss of heterozygosity for the WT-allele of MDH2. This led to the expression of $MDH2_{c.429+1G>T}$,

resulting in significant lower expression levels of MDH2. There was no malate accumulation seen within the tumour tissues. Afterwards, Cascón et al. (24) looked at five relatives of the patient and found two carried $MDH2_{c.429+1G>T}$. Clinical testing showed that one of the $MDH2_{c.429+1G>T}$ carriers already had PPGL (24).

Tumourigenesis

Calsina et al. (22) further investigated the role of MDH2 in PPGL patients. Five mutations with possible pathogenic effects were identified in MDH2. One of the mutations was found in Arg104, of which neighbouring residues are related to other types of cancers. All amino acids in table 5 are conserved between species, and mutations in locations close to all those described in table 5 have already been linked to other cancers. No altered MDH2 localisation was seen due to the mutated protein (22).

Table 5. Overview of the effects of different mutations in MDH2 in case of cancer phenotypes. RBP = retinol binding protein. (22).

Mutation	Effects
p.Arg104Gly	Functional impact Decreased expression of RBP1 Decreased enzymatic activity of MDH2
p.Val160Met	Conformational change affecting dynamics of substrate-binding site Decreased expression of RBP1 Tendency of decreased enzymatic activity of MDH2, in case of low levels of malate, or malate and NAD^+
p.Ala256Thr	Conformational change affecting neighbouring residues Tendency of decreased enzymatic activity of MDH2, in case of low levels of malate, or malate and NAD^+
p.Lys314del	Functional impact Decreased expression of RBP1
c.429+1G>T	Different protein due to affected splicing

Treatment PPGL

Current treatment of PPGL is generally ineffective, especially for metastatic forms (23). Recommended treatment for PPGL is to adjust the treatment to the specific phenotype of PPGL (7). In case of a mutation causing the PPGL, the family should also be informed and if possible, checked for the mutation. This way, the PPGL can be detected earlier (7,24). PPGL with an MDH2 mutations are not treated differently compared to other PPGL (7).

Table 5. Overview of the main symptoms seen in PPGLs, and in SDH-, FH- and MDH2-mediated PPGLs. When a certain part is blank, there is no clear information about that specific type available, or due to the several subtypes, there are no general phenotypes (7,22–25)

	Mean age of onset/diagnosis	Penetrance/ Incidence	Phenotype	Mutations	Malignancy	Life expectancy
PPGL in general	30-50 years	1 per 100.000-300.000		40% germline mutation in 1 of 13 predisposing genes	10-15% PPGLs 10% of pheochromocytoma 40% of paraganglioma	Benign: up to 90% → 10-year survival of 96% Malignant → 5-year survival below 50%
MDH2		Incomplete		1% of PPGLs in patients without other pathogenic variants	40% malignancy	
FH	45 years		Accumulation of succinate and fumarate Inactivating mutations predispose to renal cell cancer	0.83-1% of PPGLs 70-90% in renal cancer	60% malignancy	
SDHx	36 years	Incomplete	Accumulation of succinate and fumarate Implicated in renal (0.05-0.2% - most frequent SDHB) and gastro-intestinal cancer	20% in paraganglioma 0.05-0.2% in renal cancer SDHD (87.1%) SDHAF2 (6.7%) SDHB (5.9%) SDHC (0.3%)		Survival metastasis non-SDHB = 244 months
SDHA			Leigh syndrome Predispose to abdominal PPGL and cervical PPGL	Mutations are rare	Rare	
SDHB		50% penetrance at age 50	60% abdominal PGGL 40% cervical PPGL 20% has multiple tumours	>60% (together with SDHD)	36-40% malignancy	Survival after diagnosis first

						metastasis= 42 months
SDHC	38 years		This syndrome is characterised by head and neck PGLs. Also, they are more likely to be benign and do not have multiple tumours No PCC Predisposes to cervical PPGL, quarter of patients have family history	Mutations are rare	Rare	
SDHD		86% penetrance at age 50	Associated with development of multiple head and neck PPGL Family history of PPGL in the paternal branch 97% of cervical PPGL	>60% (together with SDHB) 80.5% mutated SDHx	Rare No metastasis has been described.	
SDHAF2	33 years		Predisposes to head and neck PPGL No PCC	Loss of SDHAF2 → loss-of-function SDH Parasympathetic PPGL	Rare	

Lessons from and in model organisms

MDH2 is highly conserved between different animal models, making it possible to study in different models, such as nematodes (5), mice (16), frogs, drosophila, and C. elegans (26). In most models, a loss-of-function MDH2 mutation is compatible with life. A complete null mutation in MDH2 is expected to be lethal, as is shown in mice (27).

Wang et al. (28) researched the function of MDH2 in Drosophila during development. They showed that whole body ATP levels drop, indicating an energy deficiency. Moreover, an accumulation of succinate, fumarate, and malate could be seen in *mdh2* mutants (28). This is expected as it is in line with the phenotype seen in MDH2 deficiency.

Saccharomyces cerevisiae, a yeast strain, contains three types of MDH, MDH1 for the mitochondrial MDH, MDH2 for the cytoplasmic MDH, and MDH3 for the peroxisomal MDH. This means the ortholog of the human MDH2 in yeast is MDH1. Yeast cells lacking MDH1 can only grow on fermentable carbon sources, which is in line with a function of MDH1 in the TCA cycle. The MDH1 yeast mutant is also useful for analysis of human MDH2 variants. Ait-El-Mkadem (1) used Saccharomyces cerevisiae, to assess the severity of three MDH2 mutations found in human patients. As the three amino acids found mutated in human patients are conserved in yeast, the corresponding mutations were introduced into yeast MDH1 and introduced into a yeast MDH1 deficient strain. In contrast to wild type MDH1 that was able to rescue, G30R mdh1, P128L mdh1, and P202L mdh1 all showed a severe growth defect (1). This indicates yeast as a suitable model system to study the effect of MDH2 lack-of-function mutations.

There is already some research into the function and pathway of MDH2. Chen et al. (29) showed that MDH2 is able to bind RNA, which affect the expression of a sodium channel, and Moliné et al. (30) showed that MDH2 is also part of the regulatory system of the respiratory chain. However, this part of the review will focus on research models used to research MDH2 deficiency, MDH2 gain-of-function, or the relation between MDH2 and PPGL. This is shown in table 5.

MDH2 deficiency

Ait-El-Mkadem et al. (1) used patient fibroblasts to establish the deleterious effect of Pro133Leu, Pro207Leu, and Gly37Arg in MDH2 deficiency. It was shown that transfection with WT MDH2 could rescue both the MDH2 levels and the enzymatic activity. Laemmle et al. (19) also used patient fibroblasts to determine MDH2 enzymatic activity. Here, the combination of Pro133Leu and Pro149Hisfs*22 showed to express very low levels of enzymatic activity. Ticci et al. (18) and Priestley et al. (4) used a similar approach with patient fibroblasts establishing the their variants to cause MDH2 deficiency. Moreover, patient liver cells did show a slight decrease of activity within the respiratory chain (1), which indicates different effects of MDH2 mutations in different tissues.

As of date, no research on neuronal cells of patients with MDH2 deficiency has been conducted. Using fibroblast to dedifferentiate the cells to neuronal cells via induced pluripotent stem cells (iPSCs) could be a possibility. However, iPSCs and neuronal cells are very hard to culture (31).

MDH2 gain-of-function

As of to date, Jungtrakoon et al. (5) is the only paper reporting on the MDH2 gain-of-function. They used human pancreatic islets to test MDH2 expression and insulin secretion. Moreover, HepG2 cells were used to determine expression levels and enzymatic activity and MIN6-K8 cells were used to determine glucose-stimulated release. Caenorhabditis Elegans was used to investigate DAF-28 expression and secretion (5).

MDH2 expression and insulin secretion

Research in the pancreatic islets showed that in diabetic individuals, MDH2 expression and glucose-stimulated insulin secretion is lower compared to non-diabetic individuals. Next, they investigated the correlation between MDH2 expression and glucose-stimulated insulin secretion in islets from non-diabetic individuals, to correct for a possible effect of hyperglycaemia. This indicated that MDH2 could play an essential role in insulin secretion (5).

MDH2 expression levels and enzymatic activity

The immortal cell line HepG2 was used to investigate the impact of the mutations p.His52Cys and p.Val160Met. HepG2 cells were transfected with cDNA for either WT MDH2, His52Cys MDH2, or Val160Met. The variant did not affect the expression level of MDH2. However, when checked for MDH2 enzymatic activity, by measuring the oxidation of malate to oxaloacetate, this was increased after transfection by WT MDH2. The enzymatic activity was even more increased after transfection with His52Cys MDH2 or Val160Met MDH2 (5).

Glucose-stimulated insulin secretion

The immortal cell line MIN6-K9 was used to determine the impact of His52Cys and Val160Met on insulin secretion. The MIN6-K9 cells were transfected with either variant and the expression was determined. The variant did not affect the expression level of MDH2. There was a significant increase of glucose-stimulated insulin secretion in WT MDH2 transfected cells, however, there was no significant difference between non-transfected cells and cells transfected with His52Cys. Compared to WT MDH2 transfected cells, both His52Cys and Val160Met show decreased glucose-stimulated insulin secretion (5).

It is known that glucose stimulates insulin secretion via enhance ATP production, leading to depolarization of insulin secreting cells. This seems at odds with the claim that the mutations found are actual gain of function mutations, as a decrease in glucose-stimulated insulin secretion is seen. However, with a gain-of-function mutation one would expect an increase in ATP production, and therefore, an increase in glucose-stimulated insulin secretion (figure 6). *In vitro* assays could further elucidate if the enzymatic activity of the His52Cys and Val159Met mutations indeed are more active.

Caenorhabditis Elegans expresses an MDH2 ortholog, *mdh-2*. This has a 60% sequence overlay and 76% conservation of human MDH2. C. Elegans does not express insulin, but an insulin-like peptide, namely DAF-28. CRISPR-Cas9 has been used to create C. Elegans carrying the orthologs of His52Cys and Val160Met, namely H56C and V164M. C. Elegans expressing H56C and V164M *mdh-2* variants showed an increased glucose-stimulated DAF-28 expression compared to controls. The downside of using C. elegans is that *mdh2* mutant animals exhibit a phenotype where the eggs are retained within the body, and this results in internal hatching. This is also called bagging or bag-of-worms phenotype (5).

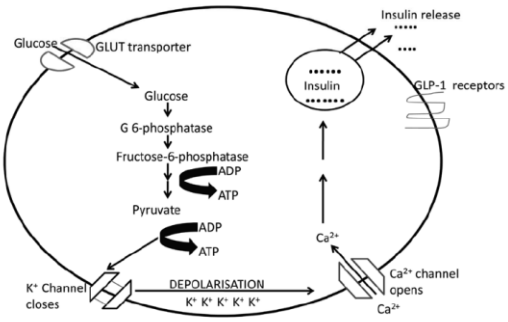

Figure 6. Pathway of glucose-dependent insulin secretion (39).

MDH2 and PPGL

Cascón et al. (24) described the first PPGL patient related to MDH2 variants. First, tumour tissue was used for whole-exome sequencing. This showed a slicing variant, c.430G>T. A Western blot showed that the splicing variant of MDH2 did result in a shorter version of MDH2, which was also present in tumour tissue. They also used HeLa cells in which a knock-down of MDH2 was induced. The HeLa cells also have increased malate and fumarate levels. Afterwards, transfection with the human MDH2 gene was able to reverse the knock-down (24).

Calsina et al. (22) looked for patients without a mutation in the PPGL driver genes (see table 4). Their patient material was either DNA samples from blood, from the tumour, or from both. These samples were screened for MDH2 mutations. The results from these screenings were determined by computational prediction models for functional impact. Moreover, knock-down MDH2 HeLa cells were transfected with cDNA of either WT MDH2, Arg104Gly MDH2, or an empty vector. Compared to the transfection with WT MDH2, Arg104Gly had a significantly lower expression (22)

Discussion

The current review offers an overview of protein characteristics, disease characteristics, and lessons from research models for MDH2 deficiencies and gain-of-function mutations, and some of the implications of MDH2 mutations in PPGLs. More clarity on MDH2 is needed to be able to treat patients with affected MDH2 proteins and to gain more knowledge on different metabolic pathways.

MDH is a homodimer with each subunit consisting of a NAD-binding domain and a substrate binding domain (5,10,11). Its function in the TCA cycle as well as in the malate-aspartate shuttle have well been established and rely on the mitochondrial location. Interestingly, MDH2 might also function outside mitochondria. He et al. indicate that MDH2 could be part of a scaffold (32). It was shown that a lncRNA is directly bound to both MDH2 and proteasome subunit alpha type 1 (PSMA1). Altered expression of PSMA1 leads to changes in proliferation, migration and invasion of glioma cells (32). Additionally, Yong-Hong et al. (29) showed a regulatory function of MDH2. MDH2 is able to bind to two out of four conserved regions in the 3'UTR of an ion channel. KD of MDH2 led to an increased expression of a reporter gene with the 3'UTR of the ion channel, whereas a decreased resulted in a lower expression (29). This indicates that MDH2 may have several functions, next to its metabolic functions.

To date, 12 patients with MDH2 deficiency have been identified (table 2). Since the patients are not monitored for a prolonged period of time, it is not known what the long-term effects of these mutations are. Moreover, due to the very limited number of published patients, it is difficult to determine the effects of a specific mutation. It is difficult to determine whether the phenotype is purely caused by the mutation, or if personal factors like genetic modifiers and diet are involved.

In MDH2 deficient patients, only loss of both functional alleles results in a phenotype. Therefore, it can be assumed that one functional allele is sufficient for proper function of the MAS. The specific effects of the mutations are outside the scope of this review. It can be assumed that the mutations affect the functional domains or they can lead to a conformational change affecting the function of the protein (5,22). More in dept research is needed into the effects of each single mutation and its implications on the conformation and functionality of the protein. For this, in vitro assays monitoring the conversion of malate to oxaloacetate would be the most direct read out.

The most ideal model would be one with the specific patient mutations. However, the downside of using any model that are not human cells is that it can be difficult to translate the results from model to human. This does not mean that research into metabolic pathways and the effect of MDH2 mutations should not be researched. To investigate the different pathways, yeast, patient derived iPSCs (33), and patient derived cells (34) are good models. Next, the C. elegans is a good localisation model since it is transparent. Last, animals should only be used after they have been tested for suitability and when it is clear how the metabolic pathways are influenced by MDH2 mutations. One factor to take into account is that even though the pathways are conserved between species, differences still might occur, and therefore, the model only mimics the human pathways (35). Xenopus tropicalis and Rattus norvegicus both have clearly orthologs of human MDH2 (26), and could therefore be used as research models into MDH2.

Immortal cells lines are not an ideal model to study metabolism, due to their disrupted metabolism. This will make it more difficult to study the normal physiological pathways. This is the case for most immortal cell lines, due to their usual cancerous origin. A good alternative would be the use of organoids. Organoid cultures of wild type cells can be genetically modified

using CRISPR/Cas9 methods to encode patient-specific isoforms. An advantage of this would is that organoids from different tissues can be used, making it possible to address the malfunctioning of MDH2 in various tissue types. Another possibility is to start with induced pluripotent cells from patients. Here also, various tissue types can be mimicked. In addition, the genetic make up is identical to the patient that the iPSC was derived from.

The pathways which influence the phenotype in patients with MDH2 deficiency remain unclear. However, during development the metabolism changes. The patients are diagnosed between the neonatal stage and late infancy, but most are diagnosed before the age of six months (9/12). Changes within the metabolism during development could explain the age of onset (table 2) (4). At the foetal stage, ketone bodies are an essential energy source for neurodevelopment (6). In this stage of development, ketone bodies are preferred over glucose in metabolism. Moreover, it was shown that in neonates lactate is preferred over glucose in brain metabolism (6). After the neonatal period, glucose consumption and metabolism rapidly increase. This indicates the patient will get more dependent on their glucose metabolism. However, this is disturbed due to the MDH2 mutation. In case of MDH2 deficiency, patients experience elevated lactate levels, and excrete malic acid, fumaric acid, succinic acid (4). This indicates that there is either a problem with the switch, or there is a problem with the glucose metabolism. Another reason for the difference in disease onset could be that the mutations differ in their severity, with partial loss of function mutations leading to later onset.

Two families with T2DM have been described with mutations in MDH2 affecting their phenotype. Two mutations have been identified that affect metabolism and are likely partly responsible for the development of T2DM, namely arg52cys and val150met. The specific effects of the mutations are outside the scope of this review (5). Since the phenotype of patients with diabetes is less severe compared to the phenotype of MDH2-deficient patients, it is likely that more patients with gain-of-function mutations exist, but this claim needs further experimental evidence. Similar to gain-of-function patients, there is a high likelihood that other mutations cause a less severe phenotype, and therefore, these patients have not been identified as of now (7).

The exact pathway of how gain-of-function MDH2 mutations can lead to T2DM still has to be elucidated. It is hypothesised that accumulation of mitotoxic metabolites promote dysfunction within the mitochondria of pancreatic β-cells, leading to stress signalling and apoptosis (36). This could be caused by the dysfunctional MDH2, leading to an accumulation of metabolic intermediates.

Moreover, T2DM is usually associated with chronic low-grade inflammation. There are increased levels of circulating free fatty acids, due to a bad diet or overeating. This upregulates G6PD in adipose tissue, which in turn results in an increased expression of resistin and tumour necrosis factor alpha (TNFα), both proinflammatory molecules (37). Products from the pentose phosphate pathway are NADHP and R5P, both of which important in regulating DNA damage responses, metabolism, and proliferation of cancer cells. NADPH is also important in the secretion of insulin from pancreatic β-cells. Since the pentose phosphate pathway is an important source of NADPH, either too little or too much NADPH negatively impacts the secretion of insulin (37). TCA intermediates remain within the mitochondria, however, under stress conditions the mitochondria can become disrupted, and intermediates can leak out. These intermediates affect many cellular processes, including immune responses. However, not much is known about the immunological role of malate (20).

Disruption of metabolism, especially the TCA cycle, is a hallmark of cancer (1,24). This can result in an increase in the anaerobic metabolism under normal oxygen conditions. This is also called the Warburg effect and seen in many types of cancer (13). During cancer, metabolism

is disrupted, mostly to increase glucose consumption. This also results in an increase of the pentose phosphate pathway (37). NADPH is important in fatty acid metabolism, and therefore, is important in glycolytic cancers cells to combat oxidative stress and avoid the TCA cycle (17). Due to the accumulation of mutations in cancer, it can be difficult to determine the effect of a single mutation. However, the fact that MDH2 mutations predispose to PPGL indicate that loss of their primary function in the TCA cycle and/or MAS supports tumorigenesis. (23–25). This function may be to indirectly stimulate HIF1 alpha breakdown by inhibiting PHD proteins and von Hippel-Lindau (VHL) (38). VHL is not affected directly by MDH2, but it is in the pathway to break down HIF, resulting in higher levels of HIF, similar to PDH (7,23,25). Ultimately, there is a change in transcription of genes important for cell proliferation, angiogenesis and survival of cells (23). Since the malignancy rate of MDH2-mediated PPGLs is 40%, it is important to screen all patients with MDH2 mutations for (malignant) PPGLs, regardless of phenotype of the mutation, next to their standard treatment (7).

References

1. Ait-El-Mkadem S, Dayem-Quere M, Gusic M, Chaussenot A, Bannwarth S, François B, et al. Mutations in MDH2, Encoding a Krebs Cycle Enzyme, Cause Early-Onset Severe Encephalopathy. Am J Hum Genet [Internet]. 2017;100(1):151–9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5223029/

2. Broeks MH, van Karnebeek CDM, Wanders RJA, Jans JJM, Verhoeven-Duif NM. Inborn disorders of the malate aspartate shuttle. J Inherit Metab Dis [Internet]. 2021 [cited 2023 Mar 3];44(4):792–808. Available from: https://pubmed.ncbi.nlm.nih.gov/33990986/

3. Yang Y, Sauve AA. NAD+ metabolism: Bioenergetics, signaling and manipulation for therapy. Biochim Biophys Acta. 2016;1864(12):1787–800.

4. Priestley JRC, Pace LM, Sen K, Aggarwal A, Alves CAPF, Campbell IM, et al. Malate dehydrogenase 2 deficiency is an emerging cause of pediatric epileptic encephalopathy with a recognizable biochemical signature. Mol Genet Metab reports [Internet]. 2022 Dec 1 [cited 2023 Mar 3];33. Available from: https://pubmed.ncbi.nlm.nih.gov/36420423/

5. Jungtrakoon Thamtarana P, Marucci A, Pannone L, Bonnefond A, Pezzilli S, Biagini T, et al. Gain of Function of Malate Dehydrogenase 2 and Familial Hyperglycemia. J Clin Endocrinol Metab [Internet]. 2022 Mar 1 [cited 2023 Mar 3];107(3):668–84. Available from: https://pubmed.ncbi.nlm.nih.gov/34718610/

6. Oyarzábal A, Musokhranova U, Barros LF, García-Cazorla A. Energy metabolism in childhood neurodevelopmental disorders. EBioMedicine. 2021;69.

7. Buffet A, Burnichon N, Favier J, Gimenez-Roqueplo AP. An overview of 20 years of genetic studies in pheochromocytoma and paraganglioma. Best Pract Res Clin Endocrinol Metab [Internet]. 2020;34(2):101416. Available from: https://doi.org/10.1016/j.beem.2020.101416

8. MDH2 malate dehydrogenase 2 [Homo sapiens (human)] - Gene - NCBI [Internet]. [cited 2023 May 18]. Available from: https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=4191

9. UniProt [Internet]. Available from: https://www.uniprot.org/

10. Minárik P, Tomásková N, Kollárová M, Antalík M. Malate Dehydrogenases - Structure and Function. Gen Physiol Biophys. 2002;257–65.

11. RCSB PDB: Homepage [Internet]. [cited 2023 May 18]. Available from: https://www.rcsb.org/

12. Naifeh J, Dimri M, Varacallo M. Biochemistry, Aerobic Glycolysis. StatPearls [Internet]. 2023 Apr 9 [cited 2023 Jul 3]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK470170/

13. Vaupel P, Multhoff G. Revisiting the Warburg effect: historical dogma versus current understanding. J Physiol. 2021;599(6):1745–57.

14. Melkonian EA, Schury MP. Biochemistry, Anaerobic Glycolysis. StatPearls [Internet]. 2022 Aug 8 [cited 2023 Jul 3]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK546695/

15. Amelio I, Cutruzzolá F, Antonov A, Agostini M, Melino G. Serine and glycine metabolism in cancer. Trends Biochem Sci. 2014;39(4):191–8.

16. Bölsterli BK, Boltshauser E, Palmieri L, Spenger J, Brunner-Krainz M, Distelmaier F, et

al. Ketogenic Diet Treatment of Defects in the Mitochondrial Malate Aspartate Shuttle and Pyruvate Carrier. Nutrients. 2022;14(17):1–30.

17. Patra KC, Hay N. The pentose phosphate pathway and cancer. Trends Biochem Sci [Internet]. 2014;39(8):347–54. Available from: http://dx.doi.org/10.1016/j.tibs.2014.06.005

18. Ticci C, Nesti C, Rubegni A, Doccini S, Baldacci J, Dal Canto F, et al. Bi-allelic variants in MDH2: Expanding the clinical phenotype. Clin Genet [Internet]. 2022 Feb 1 [cited 2023 Apr 11];101(2):260–4. Available from: https://pubmed.ncbi.nlm.nih.gov/34766628/

19. Laemmle A, Steck AL, Schaller A, Kurth S, Perret Hoigné E, Felser AD, et al. Triheptanoin – Novel therapeutic approach for the ultra-rare disease mitochondrial malate dehydrogenase deficiency. Mol Genet Metab Reports. 2021;29.

20. Wanders RJA, Karnebeek CDM, Jans JJM, Verhoeven NM, Houten SM. News and views. J Inherit Metab Dis. 2020;43(4):647–50.

21. Doria A, Yang Y, Malecki M, Scotti S, Dreyfus J, O'Keeffe C, et al. Phenotypic characteristics of early-onset autosomal-dominant type 2 diabetes unlinked to known maturity-onset diabetes of the young (MODY) genes. Diabetes Care [Internet]. 1999;22(2):253–61. Available from: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed4&NEWS=N&AN=1999043459

22. Calsina B, Currás-Freixes M, Buffet A, Pons T, Contreras L, Letón R, et al. Role of MDH2 pathogenic variant in pheochromocytoma and paraganglioma patients. Genet Med. 2018;20(12):1652–62.

23. Lussey-Lepoutre C, Buffet A, Gimenez-Roqueplo AP, Favier J. Mitochondrial deficiencies in the predisposition to paraganglioma. Metabolites. 2017;7(2):1–13.

24. Cascón A, Comino-Méndez I, Currás-Freixes M, De Cubas AA, Contreras L, Richter S, et al. Whole-exome sequencing identifies MDH2 as a new familial paraganglioma gene. J Natl Cancer Inst. 2015;107(5):1–5.

25. Pillai S, Gopalan V, Smith RA, Lam AKY. Updates on the genetics and the clinical impacts on phaeochromocytoma and paraganglioma in the new era. Crit Rev Oncol Hematol [Internet]. 2016;100:190–208. Available from: http://dx.doi.org/10.1016/j.critrevonc.2016.01.022

26. mdh2 | Danio rerio gene | Alliance of Genome Resources [Internet]. [cited 2023 May 15]. Available from: https://www.alliancegenome.org/gene/ZFIN:ZDB-GENE-040426-2143#orthology

27. IMPC Search | International Mouse Phenotyping Consortium [Internet]. [cited 2023 Jul 13]. Available from: https://www.mousephenotype.org/data/search?term=mdh2&type=gene

28. Wang L, Lam G, Thummel CS. Med24 and Mdh2 are required for Drosophila larval salivary gland cell death. Dev Dyn. 2010;239(3):954–64.

29. Chen YH, Liu SJ, Gao MM, Zeng T, Lin GW, Tan NN, et al. MDH2 is an RNA binding protein involved in downregulation of sodium channel Scn1a expression under seizure condition. Biochim Biophys Acta - Mol Basis Dis. 2017 Jun 1;1863(6):1492–9.

30. Molinié T, Cougouilles E, David C, Cahoreau E, Portais JC, Mourier A. MDH2 produced OAA is a metabolic switch rewiring the fuelling of respiratory chain and TCA cycle. Biochim Biophys Acta - Bioenerg. 2022 Mar 1;1863(3):148532.

31. Logan S, Arzua T, Canfield SG, Seminary ER, Sison SL, Ebert AD, et al. Studying Human Neurological Disorders Using Induced Pluripotent Stem Cells: From 2D Monolayer to 3D Organoid and Blood Brain Barrier Models. Compr Physiol. 2019;9(2):565–611.

32. He D, Xin T, Pang B, Sun J, Liu ZH, Qin Z, et al. A novel lncRNA MDHDH suppresses glioblastoma multiforme by acting as a scaffold for MDH2 and PSMA1 to regulate NAD+ metabolism and autophagy. J Exp Clin Cancer Res [Internet]. 2022;41(1):1–26. Available from: https://doi.org/10.1186/s13046-022-02543-7

33. The Human Protein Atlas [Internet]. [cited 2023 May 15]. Available from: https://www.proteinatlas.org/

34. Bell WC, Sexton KC, Grizzle WE. How to efficiently obtain human tissues to support specific biomedical research projects. Cancer Epidemiol Biomarkers Prev. 2009;18(6):1676–9.

35. Mager WH, Winderickx J. Yeast as a model for medical and medicinal research. Trends Pharmacol Sci. 2005;26(5):265–73.

36. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol [Internet]. 2014 [cited 2023 May 31];10(12):723. Available from: /pmc/articles/PMC4424797/

37. Ge T, Yang J, Zhou S, Wang Y, Li Y, Tong X. The Role of the Pentose Phosphate Pathway in Diabetes and Cancer. Front Endocrinol (Lausanne). 2020;11(June):1–11.

38. Kaelin WG. von Hippel-Lindau Disease. 2007;

39. Gunton J, Girgis CM. Pancreatic beta-cell failure in the pathogenesis of type 2 diabetes. Sydney Univ Press [Internet]. 2012 [cited 2023 Jul 13];(December):87–104. Available from: https://www.researchgate.net/publication/269984917_Pancreatic_beta-cell_failure_in_the_pathogenesis_of_type_2_diabetes