Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

STUDIES OF NUCLEAR ENCODED MITOCHONDRIAL PROTEINS IN METABOLISM

Qian Zhao

Karolinska Institutet

Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-410-8

STUDIES OF NUCLEAR ENCODED MITOCHONDRIAL PROTEINS IN METABOLISM
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Qian Zhao

The thesis will be defended live online (a zoom link will be provided) at Karolinska Institutet, Thursday, December 16th, 2021 at 13h.

Principal Supervisor:
Anna Karlsson
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Co-supervisor(s):
Sophie Curbo
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Xiaoshan Zhou
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Opponent:
Docent Liya Wang
Swedish University of Agricultural Sciences
Department of Anatomy, Physiology and Biochemistry

Examination Board:
Professor Maria Eriksson
Karolinska Institutet
Department of Biosciences and Nutrition

Professor Martin Ott
Stockholm University
Department of Biochemistry and Biophysics

Professor Andrei Chabes
Umeå University
Department of Medical Biochemistry and Biophysics

ABSTRACT

Mitochondrial function is vital for human health. Inherited genetic disease can cause mitochondrial DNA (mtDNA) deficiency, disorders with generally poor prognosis. The majority of the genes involved in keeping a normal mitochondrial function are nuclear encoded. Deficiency in the nuclear encoded enzymes that provide building blocks for mtDNA synthesis, thymidine kinase 2 (TK2) and deoxyguanosine kinase (DGUOK), cause myopathy, encephalomyopathy and hepatocerebral disorders in humans. The nuclear encoded SLC25A10 is located in the mitochondrial inner membrane and is involved in regulation of cell metabolism.

In the first study we hypothesized that SLC25A10 had a regulatory role in cancer metabolism. Since the antidiabetic drug metformin was known to reduce the risk for cancer and to alter cell energy production, we used the siSLC25A10 model to investigate effects of metformin. In the siSLC25A10 cell line, metformin significantly downregulated the SLC25A10 carrier, especially at low glucose conditions, at both mRNA and protein levels. Since SLC25A10 is a mitochondrial transporter, this lower expression affects the exchange of nutrients with the potential to alter metabolic pathways of cancer cells.

In addition to cell culture studies, animal models are important tools to study mitochondrial functions. We constructed a DGUOK complete knockout mouse model to investigate the phenotype with the aim to find a model for mechanistic studies and treatment strategies. Interestingly, the *Dguok*$^{-/-}$ mice survived for more than 20 weeks despite very low mtDNA levels in liver tissue. Lipid metabolism as well as the *de novo* serine synthesis and the folate cycle were altered in the long surviving *Dguok*$^{-/-}$ mice. Two pyruvate kinase genes, PKLR and PKM, were active to supply pyruvate for the mitochondrial citric acid cycle (TCA cycle), which may be an explanation for the long-term survival although severely affected mitochondrial function.

We also constructed a skeletal and cardiac muscle specific TK2 knockout mouse (mTk2 KO) and a liver specific TK2 knockout mouse (livTK2 KO). The mTk2 KO mice showed dilated hearts and markedly reduced adipose tissue, but livTK2 KO mice were not different compared to the control group. A severe decrease of mtDNA was found only in skeletal muscle and heart tissue in the mTk2 KO mice. The mTk2 KO mice survived for maximum 16 weeks, but livTK2 KO mice survived for more than one and a half years. The data suggested that TK2 was vital for mtDNA maintenance in cardiac and skeletal muscle, while Tk2 deficiency in liver could be compensated for. Despite low mtDNA levels in the liver of the livTK2 KO mice we did not observe any difference compared to the control mice. The receptor for angiotensin-converting enzyme 2 (ACE2), was also affected by mtDNA deficiency in mTk2 KO mice. Since ACE2 is a receptor for the SARS-CoV-2 virus, its regulation in relation to mitochondrial function may have important clinical implications.

LIST OF SCIENTIFIC PAPERS

I. **Zhao, Q**; Zhou, X; Curbo, S; Karlsson, A. Metformin downregulates the mitochondrial carrier SLC25A10 in a glucose dependent manner. Biochemical Pharmacology; 444-450, Volume 156, (2018)

II. Zhou, X; Curbo, S; **Zhao, Q**; Krishnan, S; Kuiper, R; Karlsson, A. Severe mtDNA depletion and dependency on catabolic lipid metabolism in DGUOK knockout mice. Human Molecular Genetics; 2874-2884, Volume 28 Issue 17, (2019)

III. Zhou, X, Mikaeloff, F; Curbo S, **Zhao, Q**, Kuiper R, Végvári A, Neogi U and Karlsson A. Coordinated pyruvate kinase activity is crucial for metabolic adaptation and cell survival during mitochondrial dysfunction. *In press 2021,* Human Molecular Genetics

IV. **Zhao, Q**; Zhou, X; Kuiper, R; Curbo, S; Karlsson, A. Mitochondrial dysfunction is associated with lipid metabolism disorder and upregulation of angiotensin-converting enzyme 2. *Submitted*

V. **Zhao, Q**; Zhou, X; Kuiper, R; Curbo, S; and Karlsson, A. Specific depletion of thymidine kinase 2 in liver tissue does not affect mice survival. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

araC	arabinofuranosylcytosine
ATP	adenosine triphosphate
CD36	cluster of differentiation 36
CoA	coenzyme A
CPEO	chronic progressive external ophthalmoplegia
dAdo	deoxyadenosine
dCK	deoxycytidine kinase
dCyd	deoxycytidine
dFdC	difluorodeoxycytidine
dGK/DGUOK	deoxyguanosine kinase
dGuo	deoxyguanosine
dIno	deoxyinosine
Dm-dNK	*Drosophila melanogaster* deoxyribonucleoside kinase
DNA	deoxyribonucleic acid
dNKs	deoxyribonucleoside kinases
dNTP	deoxyribonucleotide triphosphate
dThd	deoxythymidine
dU	deoxyuridine
FABP	fatty acids binding protein plasma membrane
FADH2	reduced form of flavin adenine dinucleotide
FATP	fatty acids transport protein
FFAs	free fatty acids
HDL	high-density lipoproteins
HSV TK	herpes simplex virus thymidine kinase
IDL	intermediate-density lipoproteins

LDL	low-density lipoproteins
LPL	lipoprotein lipase
MDS	mitochondrial DNA depletion syndromes
MELAS	mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
MNGIE	neurogastrointestinal encephalopathy
mtDNA	mitochondrial DNA
NADH	reduced form of nicotinamide adenine dinucleotide
nDNA	nuclear DNA
NDPK	nucleoside diphosphate kinase
NMPKs	nucleoside monophosphate kinases
OXPHOS	oxidative phosphorylation
PEO	progressive external ophthalmoplegia
PRPP	5-phosphoribosyl-1-pyrophosphate
RAS	renin-angiotensin system
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SLC25A10	solute carrier family 25 member 10
TK1	thymidine kinase 1
TK2	thymidine kinase 2
tRNA	transfer ribonucleic acid
VLDL	very-low-density lipoproteins

1 INTRODUCTION

Mitochondria are the powerhouse (ATP production) of the cells and are also involved in other activities like creating heat, generation of radical oxygen species (ROS) and contribution to lipid metabolism. A dysfunction of mitochondria usually results in diseases with poor prognosis. Among mitochondrial disorders are genetically inherited diseases with altered gene function of either nuclear genes or genes encoded by mitochondrial DNA. Phenotypically heterogenous syndromes with severe reduction in mitochondrial DNA (mtDNA) copy number are called mitochondrial DNA depletion syndromes (MDS). Studies in the present thesis mainly focus on enzymes that phosphorylate precursors for mtDNA. Deficiency of these enzymes results in MDS with a broad range of clinical presentations.

Depletion of the nuclear encoded mitochondrial nucleoside kinases thymidine kinase 2 (TK2) and deoxyguanosine kinase (DGUOK) was investigated in mouse models. TK2 phosphorylates deoxythymidine (dThd), deoxycytidine (dCyd), and deoxyuridine (dU) to their corresponding monophosphate. DGUOK phosphorylates deoxyguanosine (dGuo), deoxyadenosine (dAdo), and deoxyinosine (dIno) to their monophosphate form. A TK2 complete knockout mouse model was generated in our group, and showed short survival of 2-4 weeks [1], and with severe beta oxidation impairment in liver tissue [2]. The lifetime expectancy of the TK2 complete knockout mice was not long enough to allow studies of additional symptoms and metabolic changes. TK2 deficiency in humans causes muscle related symptoms. To enable further studies of this, our group established a TK2 cardiac and skeletal muscle specific knockout mouse model. The study demonstrated a specific reduction of mtDNA in heart and skeletal muscle and the knockout mice had longer life expectancy, about 16 weeks and, interestingly, showed a marked change in metabolism. In another mouse strain constructed, TK2 was specifically deleted in liver tissue. These mice did not show an apparent phenotype although the mtDNA levels in liver were markedly reduced.

The other mitochondrial nucleoside kinase is DGUOK. Deficiency of DGUOK in humans results in a hepatocerebral form of mtDNA deficiency. Until now, there is no effective treatment for these genetic diseases. Our group established a DGUOK complete knockout mouse model and found a systemic deficiency of mtDNA. The DGUOK deficient mice were also found to have alterations in metabolism.

Mitochondrial dysfunction often results from genetic inherited diseases with different clinical presentations. There are myopathic, encephalomyopathic, hepatocerebral and neurogastrointestinal forms. Mitochondrial alterations are also found in cancer cells where metabolic changes are a landmark for cancer [3]. Metformin, mainly a traditional diabetes type 2 medicine, has been found to have effects in cancer treatment. Previous studies indicated metformin to be involved in the metabolism of cancer cells, and to be strongly related to the glucose level in blood. Cancer cell experiments *in vitro* have been performed to address questions on the effects of metformin on cancer cell metabolism.

2 LITERATURE REVIEW

2.1 MITOCHONDRIA

Mitochondria have evolved from aerobic bacteria to become organelles in eukaryotic cells in an endosymbiotic event [4, 5]. Mitochondria are double-membrane cell organelles essential for cellular ATP and carbohydrate intermediate production through the process of TCA cycle and oxidative phosphorylation [3]. Mitochondria number per cell has a very large range in eukaryotes from a single mitochondrion to thousands of mitochondria, depending on the cell type [6]. The mitochondrial DNA copy number is related to the energy requirement of the specific cell type. The requirement of energy of a specific cell will determine the turnover of mitochondria. The most common form of cellular energy is adenosine triphosphate (ATP), and the main site of ATP production is through the electron transport chain and ATP synthase in the inner mitochondrial membrane [3]. The outer membrane has pores that are big enough for ions and small molecules to pass. In contrast, the inner mitochondrial membrane has a permeability limit which keep the TCA cycle substrates and other functional molecules inside the mitochondria. Other important functions of mitochondria, in addition to ATP production, are the metabolism of sugars and fatty acids, calcium storage [7] and signaling and regulation of the cellular redox state [3]. Mitochondria divide independently of the cell cycle and thus the mitochondrial DNA synthesis is not coupled to cell division.

2.1.1 Mitochondrial DNA

Most mitochondrial proteins are encoded by nuclear genes in the cell nucleus and transported into the mitochondria [8-10]. Mitochondrial DNA (mtDNA) has a size of 16569 bp in humans and encodes 37 genes. These genes encode 22 tRNAs, 2 rRNAs and 13 of the proteins involved in oxidative phosphorylation [11]. Usually, each mitochondrion contains 2 to 10 mtDNA copies, but there is a great variation depending on the type of tissue [12]. Since the replication and repair of mitochondrial DNA proceed throughout the cell cycle, mitochondria need a continuous supply of DNA precursors. Mitochondrial DNA depletion syndromes (MDS) are genetic disorders characterized by decreased mtDNA levels and may affect different tissues including muscle, liver, and brain [13]. We mainly discuss the nuclear encoded genes that cause mitochondrial DNA depletion, which are summarized in table 2.

2.1.2 Deoxyribonucleotides for mitochondrial DNA synthesis

Deoxyribonucleotides (dNTPs) cannot freely cross the cell membrane due to their negative charge and there are only known transporters of deoxyribonucleosides [14]. For the nucleotide biosynthesis, there are two pathways. The *de novo* pathway only exists outside mitochondria, while the salvage pathway functions both outside and inside mitochondria by using different phosphate transfer enzymes (figure 1). Both purines and pyrimidines can be synthesized *de novo* from 5-phoshoribosyl-1-pyrophosphate (PRPP)[15]. IMP is the first product in the purine biosynthesis pathway and IMP is subsequently converted into AMP or GMP. The monophosphates can be further phosphorylated to the di- and triphosphate forms by

monophosphate kinases (NMPKs) and nucleoside diphosphate kinase (NDPK)[15]. The committed step in *de novo* dNTP synthesis is the reduction of ribonucleotides by ribonucleotide reductase (RNR) [16]. RNR contains two subunits, R1 and R2 [17]. R1 is constantly expressed in the dividing cell [18], while R2 is regulated by the synthesis phase (S phase) of the cell cycle [19, 20]. RNR can reduce all four ribonucleotides to their corresponding deoxyribonucleotides [21]. RNR activity results in a balanced supply of dNTPs sufficient for both nuclear and mitochondrial DNA in dividing cells [22].

The situation is different in non-dividing cells. A similar R2 subunit, which is called p53R2, is encoded by RRM2B and induced by p53 [23]. This p53R2, together with the R1 subunit, contributes to mtDNA synthesis and DNA repair in both proliferating cells and non-proliferating cells [24, 25]. The *de novo* and salvage pathways together support the demand of DNA building blocks during all cell cycles. Deoxyribonucleosides can enter the cells from external sources or be recycled in catabolic processes within the cells and phosphorylated by deoxyribonucleoside kinases (dNKs) to produce deoxyribonucleotides. There are four dNKs in mammalian cells, cytosolic thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK), and the mitochondrial thymidine kinase 2 (TK2) and deoxyguanosine kinase (DGUOK) [26] (Table 2). TK2 and DGUOK are encoded by nuclear DNA with an N-terminal mitochondrial targeting sequence, and thus these proteins are transported to the mitochondrial compartment. TK1 is strictly active in dividing cells and as such it is used as a proliferation marker. The sequence related enzyme dCK, have overlapping substrate specificity to mitochondrial TK2 and DGUOK, but without a mitochondrial targeting signal. The activity of dCK is of clinical importance since dCK phosphorylates several clinically important nucleoside analogs used in cancer treatment, such as arabinofuranosylcytosine (araC, cytarabine) and difluorodeoxycytidine (dFdC, gemcitabine)[27, 28].

Deoxyribonucleoside kinase	Natural substrates	Expression phase
TK1	dThd, dUrd	S phase
TK2	dThd, dUrd, dCyd	constitutively
DGUOK	dGuo, dAdo, dIno	constitutively
dCK	dCyd, dAdo, dGuo	constitutively

Table 1. Substrates and expression of deoxyribonucleoside kinases

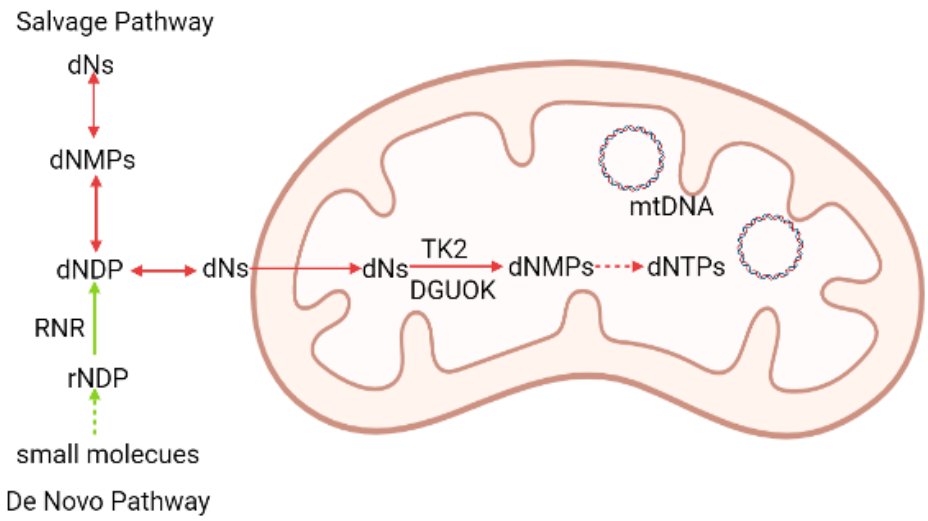

Figure 1. Nucleotide biosynthesis

TK2 is a nuclear gene located on chromosome 16q22 and the enzyme has a peptide sequence with an N-terminal mitochondrial targeting sequence [29]. TK2 is constitutively expressed throughout the cell cycle and its expression level is correlated to the mitochondrial content of the specific cell. The substrates phosphorylated by TK2 to their corresponding monophosphates are dThd and dCyd. TK2 is expressed at much lower levels than the cytosolic TK1 and thus is not regarded as a significant enzyme in proliferating cells. In contrast, TK2 plays an important role in post-mitotic tissues [15], since TK1 is not expressed outside the S phase. TK2 is genetically related to DGUOK, dCK, and herpes simplex virus thymidine kinase (HSV TK), but not to TK1 [30]. The primary sequences show that the deoxyribonucleoside kinases can be divided into two distinct families. TK2 belongs to a homologous family together with DGUOK, dCK, HSV TK and the multisubstrate nucleoside kinase from *Drosophila melanogaster* (*Dm*-dNK) [31, 32]. *Dm*-dNK is the only identified deoxyribonucleoside kinase that can phosphorylate all four deoxyribonucleosides (A,T,C,G) and with higher catalytic efficiency compared to the related mammalian enzymes [33].

2.1.3 TK2 mutation related disorders

Mutations of the TK2 gene results in mtDNA depletion and is predominately associated with a myopatic form of MDS [34, 35]. The first diagnose of TK2 mutations causing MDS was presented in 2001 [36]. Two mutations, His90Asn and Ile181Asn were identified in four patients with mtDNA depletion, skeletal myopathy, and early age death [36]. Since then, several additional TK2 mutations have been identified, among these many that result in multi organ involvement, and a common cause of early death is respiratory failure [37-40]. There are also reports on later onset and milder forms of TK2 associated MDS which usually is related to preserved residual TK2 activity [41]. The severity of the TK2 deficiency correlates to the clinical picture with a close to complete loss of TK2 activity that results in severe

encephalomyopathy and a partial reduction of TK2 activity that is associated with myopathy and sometimes a later onset of disease [42].

For studies of the mechanisms of TK2 deficiency two mouse models have been reported [1, 43]. A complete TK2 knockout mouse model and a TK2 knock in of mutation H126N (analogous to human H121N) showed similar pathology with progressive mtDNA depletion in skeletal muscle, liver, heart, and spleen [43]. The TK2 knockout mice also showed severe ataxia, neurodegeneration and died within 2-4 weeks [1]. In an attempt to rescue the TK2 depletion mice, the *Dm*-dNK which is related to dNK, was transgene expressed in the TK2 KO mice [44]. The *Dm*-dNK expression resulted in a very large dTTP pool and a complete rescue of the TK2 deficient phenotype, but interesting the mice did not have adipose tissue stored as wild type mice [44, 45]. The study demonstrated that nucleosides phosphorylated outside the mitochondria could support mtDNA synthesis in all tissues affected by TK2 deficiency and also that very unbalanced dNTP pools were not mutagenic in this specific mouse model.

2.1.4 DGUOK related disorders

The DGUOK gene is located on chromosome 2 and like TK2 the peptide contains an N-terminal positively charged mitochondrial targeting signal [46]. Similar to TK2, DGUOK is constitutively expressed throughout the cell cycle and its expression level is correlated to the mitochondrial content of the specific cell. The DNA precursor substrates phosphorylated by DGUOK to their corresponding monophosphates are dGuo and dAdo. DGUOK and TK2 are closely sequence related and together they account for the synthesis of all four dNTPs in quiescent cells. In proliferating cells, the TK1 activity is high and makes TK2 activity of little importance in the co-presence of TK1. There is no corresponding S-phase specific nucleoside kinase for the substrates of DGUOK where instead both dCK and RNR probably accounts for the increased demand of the DGUOK specific substrates.

The most common phenotype of DGUOK deficiency in humans presents as a neonatal multisystem disorder. Common symptoms of affected individuals are lactic acidosis, hepatic disease, hypoglycemia, and neurologic dysfunction [47]. Late onset of this disease has also been reported with a clinical presentation of liver failure but without neurological symptoms [48]. Individuals with symptoms restricted to the liver have been treated with liver transplantation and some of them responded well to the procedure [47, 49-51]. There have been no previous DGUOK deficient mice model presented for mechanistic and treatment studies of DGUOK deficiency.

2.1.5 Other mutations related to mitochondrial disorders

The first report of multiple mtDNA deletions was in muscle tissue, with autosomal dominant progressive external ophthalmoplegia (adPEO) in 1989 [52], and the initial description of profound depletion of mtDNA presenting as infantile myopathy, hepatopathy and nephropathy [53]. A TK2 deficiency related disorder was first diagnosed in children in 2001[36]. In 2013, an adult form of disease linked to TK2 deficiency was reported and was

found because of advanced genetic testing [42]. Until now, many genes have been linked to mitochondrial DNA depletion syndromes (Table 2).

Table 2. Summary of mitochondrial DNA depletion syndromes

Gene	Protein	Clinical phenotype	Reference
TK2	Thymidine kinase 2	Myopathy	[36]
DGUOK	Deoxyguanosine kinase	Hepatopathy, neurological symptoms	[54]
MPV17	MPV17	Hepatopathy, neurohepatopathy, neuropathy, leukoencephalopathy, Charcot-Marie-Tooth	[55]
POLG1	Polymerase gamma	Encephalohepatopathy, epilepsy, ataxia, neuropathy	[56]
C10orf2	Twinkle	Ataxia, neuropathy, ophthalmoplegia, hearing impairment, epilepsy, liver involvement	[57]
SUCLA2	Beta subunit of succinyl-CoA ligase	Dystonic motor disability, deafness, hypotonia	[58]
SUCLG1	Alpha subunit of succinyl-CoA ligase	Metabolic crisis, dystonic motor disability, deafness, hypotonia	[59]
RRM2B	p53-R2	Hypotonia, developmental delay, tubulopathy	[60]
TYMP	Thymidine phosphorylase	Ophthalmoparesis, ptosis, gastrointestinal dysmotility, cachexia, neuropathy, ataxia, leukoencephalopathy, mitochondrial neurogastrointestinal encephalopathy (MNGIE)	[61]
MGME1	Mitochondrial genome maintenance exonuclease 1	Progressive external ophthalmoplegia (PEO), emaciation and respiratory failure	[62]
RNASEH1	Ribonuclease H1	Chronic progressive external ophthalmoplegia (CPEO), muscle weakness, dysphagia, spinocerebellar signs	[63]
TFAM	Mitochondrial transcription factor A	Hyperbilirubinemia and hypoglycemia with progression to liver failure	[64]

SSBP1	Mitochondrial single strand binding protein	Optic atrophy, liver failure, neurological syndrome, retinopathy	[65]
LIG3	Ligase III	Severe gut dysmotility and neurological abnormalities, including leukoencephalopathy, epilepsy, migraine, stroke-like episodes, and neurogenic bladder.	[66]
MFN2	Mitofusin-2	Optic atrophy, myopathy, axonal neuropathy, Charcot-Marie-Tooth	[67]
MSTO1	Mitochondrial fusion	Muscular dystrophy with cerebellar involvement, myopathy, ataxia	[68]
MICOS13	MICOS complex subunit MIC13	Hepato-encephalopathy	[69]
SLC25A21	Mitochondrial 2-oxodicarboxylate carrier	Spinal muscular atrophy-like	[70]
SLC25A10	Mitochondrial dicarboxylate carrier	Epileptic encephalopathy	[71]
AGK	Acylglycerol kinase	Congenital cataract, hypertrophic cardiomyopathy, skeletal myopathy and lactic acidosis, Sengers syndrome	[72]
FBXL4	Protein homeostasis	Encephalomyopathy	[73]
ABAT	Aminotransferase	Encephalomyopathy	[74]
MRM2	Mito rRNA maturation	Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome	[75]

2.1.6 Mitochondria in cell metabolism

Mitochondria are key organelles in cellular metabolism and known as the powerhouse of the cell. They break down nutrients and produce energy rich molecules such as ATP. In the biochemical process known as cellular respiration glucose and other nutrients are converted into energy necessary for movements and growth. Glucose is first converted to pyruvate in the cytosol either under aerobic or anerobic conditions. Pyruvate is transported into the mitochondrial matrix and subsequently metabolized into acetyl coenzyme A (acetyl CoA).

Acetyl CoA enters the tricarboxylic acid cycle and generates dicarboxylic intermediates and NADH. High ATP demands is met by increasing mitochondrial mass and more OXPHOS and endurance exercise can thus increase mitochondrial mass and activity [76]. In this metabolic pathway NADH is converted to NAD+, FADH2 is converted to FAD+, due to those chemical reaction, electrons are handed over to O_2 with the help of other mobile electron carriers. The release of energy is used to produce ATP in a highly efficient way. In humans, certain cells are more dependent on mitochondria energy supply, like hepatocytes, while other cells are more likely to use glycolysis, like immune cells and embryonic cells when they are in the resting state [77]. In hepatocytes, mitochondria not only provide energy, but also support substrates to generate glycogen and lipids for use in the entire body. Low blood lipid levels induce ketogenesis in liver cells and starvation triggers lipolysis in adipose tissues to provide peripheral tissues with fuels [78, 79].

2.2 MITOCHONDRIA AND AGING

Mitochondrial loss of function in aging is of increasing interest. Dysfunction of mitochondria is affecting the metabolism of the aging body. There are some hypotheses of mechanisms that link the mitochondrial DNA variation/mutations, copy numbers and longevity.

2.2.1 mtDNA variation/mutations and aging

The mtDNA is predominantly maternally inherited, although there are recent reports of biparental inheritance [80]. The mtDNA may contribute to the heritability of estimated lifespan [81, 82]. Studies suggest that mtDNA polymorphisms is associated with people's longevity in Finnish [83, 84] and Japanese populations [84]. The mtDNA point mutations and deletions accumulate in an age-dependent manner [85]. In addition, deletions of mtDNA are flanked with repeats, and often found in elderly living individuals of many species [86-88]. The number of longer repeats in mtDNA has been shown to restrict the life span in 61 mammalian species [89].

2.2.2 mtDNA copy numbers and aging

The maintenance of a certain mtDNA copy number is vital for mitochondrial function. Changes in mtDNA copy number have been linked to life expectancy in humans [90]. A study shows that mtDNA copy number in peripheral blood mononuclear cells averagely reduce 0.4 copies each year [91]. The mtDNA copy number decrease age-related in skeletal muscle and this is more pronounced in males [92]. In contrast, mtDNA increase in liver during aging [92]. High mtDNA copy number in peripheral blood cells is associated with better health and survival in elderly people and low mtDNA copy number has poorer prognosis related to cognitive ability, physical strength, health, and higher mortality [93]. Studies have shown that the mtDNA copy number and age are strongly correlated with cognitive performance in women, and furthermore, the mtDNA quantity has been proposed as an early marker of dementia [94].

2.3 ANGIOTENSIN-CONVERTING ENZYME 2 (ACE2)

ACE2 is a membrane-anchored carboxypeptidase, and its discovery was first reported in 2000 [95, 96]. ACE2 converts angiotensin II to angiotensin-(1-7) [97]. The carboxypeptidase activity both function as an enzyme and as a trafficking regulator for transfer of neutral amino acids in the intestine [98]. Lack of ACE2 leads to tryptophan depletion in blood [99]. Recently, ACE2 was also found to be the functional receptor of the SARS coronavirus[100].

2.3.1 ACE2 in cardiovascular system

ACE2 is the main enzyme converting angiotensin II to angiotensin-(1-7). The biological relevance has been shown by administration of encapsulated angiotensin-(1-7) in a slow release form in animal models, which showed its benefits on cardiovascular function and metabolism, including antithrombogenic effects [101]. The administration showed several effects including protection of postinfarction cardiac dysfunction [102, 103], a reduction of the lesion area, antihypertensive effects [104], and ameliorating type II diabetes mellitus. The heterozygous ACE2 knockout mice described several cardiac contractility defects and increased angiotensin II levels [105].

2.3.2 ACE2 expression in different organs in animal models

There are several studies of animal models inhibiting ACE2 or overexpressing ACE2 protein [106]. Ablation/deletion of ACE2 in the gut of mice resulted in dysregulation of intestinal antimicrobial peptides, alterations of amino acid homeostasis, and changes of the gut microbiome [99]. Loss of ACE2 in mice resulted in a decrease of insulin secretion when exposed to glucose and a cumulative disability of glucose tolerance [107]. Increased human cardiac ACE2 expression in mice resulted in ventricular tachycardia and sudden death of the mice [108]. Overexpression of human ACE2 in the kidney had the opposite effect compared to the heart, with a protective function of ACE2 in podocytes in diabetic nephropathy [109].

ACE2 also plays a particularly important role in the central nervous system. Brain specific overexpression of human ACE2 in mice attenuated neurogenic hypertension and reduced angiotensin II-mediated oxidative stress in neuron cells [110], protected heart function in chronic heart failure through attenuating sympathetic outflow [111], and protected the brain from ischemia-induced damage [112, 113].

2.4 LIPID METABOLIDM

In recent years, lipid metabolism has gained more attention as important for liver disease, cancer, and even stem cells metabolism.

2.4.1 Lipid composition

Exogenous lipids through food consumption include triglycerides, cholesterol, free fatty acids (FFAs), fat-soluble vitamins and phospholipids. Endogenous lipids are synthesized within cells, and include for example the phospholipids in cell membranes and triacylglycerols (TAGs) in adipose tissues [114]. Exogenous lipids usually form chylomicrons in the intestine

and are transported into the body for further use of the lipids or storage (Figure 2) [115]. Lipids within the body are usually transported by lipoproteins, which includes very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Lipoproteins are synthesized in the liver, and then circulated through the blood. Lipoprotein carries endogenous triglycerides and cholesterol to support energy for other organs and tissues [115].

Chylomicrons transport triglycerides and cholesterol to the peripheral tissues [116]. VLDL export triglycerides from the liver. IDL are the product of lipoprotein lipase processing VLDL. LDL is the product of VLDL and IDL metabolism. Both IDL and LDL are cleared away by the liver. LDL can also be taken up by macrophages. HDL are synthesized in both enterocytes and in the liver [116].

2.4.2 Liver function in lipid metabolism

2.4.2.1 Acquisition of lipids

The uptake of lipids and fatty acids of hepatocytes are mainly from two sources: 1) the dietary fats are packed into chylomicrons derived from enterocytes in gut, 20% of the chylomicrons will be transport to the liver, and the liver will hydrolyze the esterified fatty acids into triglycerides from the chylomicrons [117]. 2) adipose tissue releases the non-esterified free fatty acids, which will be subjected to lipolysis by the liver during fasting. The hepatocytes can also generate fatty acids from glucose (de novo lipogenesis) when there is more than enough glucose in the liver [118].

2.4.2.2 Lipid storage

Triglyceride molecules represent the major form of storage, which are mainly synthesized in the liver and adipose tissue although partly in the small intestines as well [119]. Triglycerides synthesized in the liver are stored in cytoplasmic lipid droplets in the form of neutral lipids or secreted into the bloodstream as VLDL particles [119]. The lipids from the intestine will form chylomicrons which will also be transported into the liver and skeletal muscle for storage (figure 2) [120]. Circulating VLDL particles can deliver triglycerides to adipose tissue and muscle for storage via lipoprotein lipase (LPL) on the capillary endothelial cells [120].

2.4.2.3 Lipid consumption

Very long-chain fatty acids are oxidized within the peroxisomes, while short-chain, medium-chain, and long-chain fatty acids are oxidized within mitochondria [121]. Mitochondrial oxidation includes several steps and may not be complete. Incomplete beta oxidation of fatty acids leads to the formation of ketone bodies [122]. Fatty acid form fatty acyl-CoA in the endoplasmic reticulum or the outer mitochondrial membrane. Fatty acyl-CoA step by step finally generates acetyl-CoA through the beta-oxidation pathways. The acetyl-CoA will be oxidized to carbon dioxide (CO_2) in the tricarboxylic acid cycle (TCA). Take palmitic acid (16 carbons) as an example, the whole process finally yields 129 ATP.

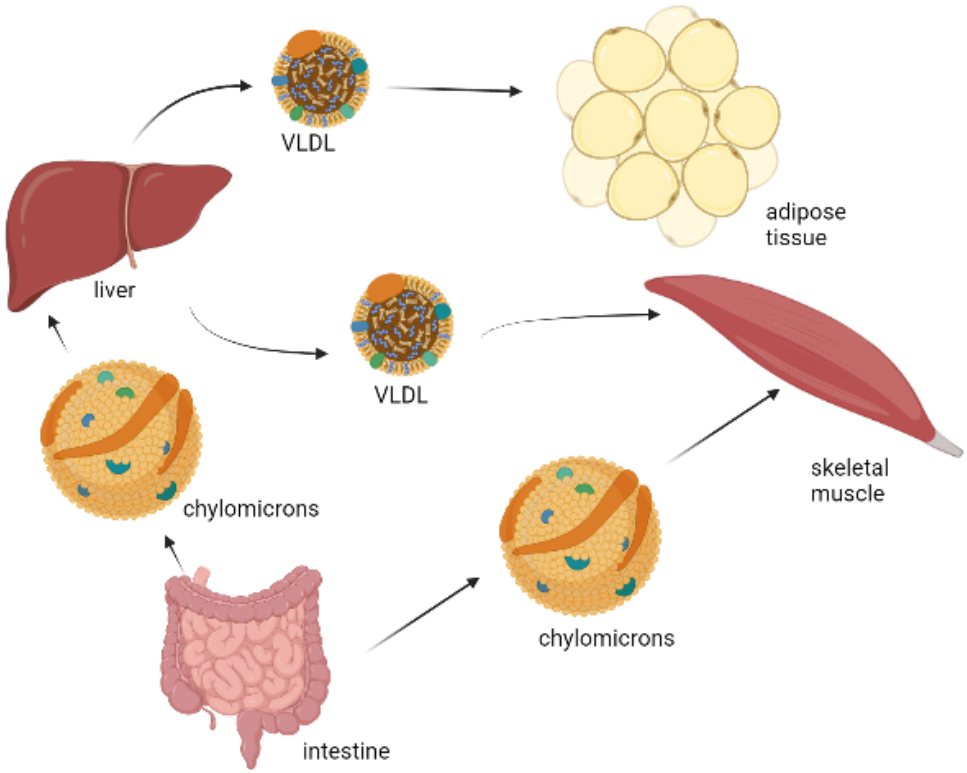

Figure 2. Lipid acquisition and storage

2.4.3 Myocardial lipid metabolism

To sustain continuous contractions, the human heart needs an exceptional amount of energy per gram of tissue, about 6-30 kg of ATP per day [123, 124]. The healthy heart can adapt fast during exercise or fever, while the failing heart cannot because of depletion of fuel [125]. In resting condition, around 70% of the ATP production in the heart is generated from mitochondrial oxidative phosphorylation of fatty acids [126].

Cardiac cells take up fatty acids in the form of free fatty acids or triacylglycerol within chylomicrons or VLDL [127]. To facilitate fatty acids uptake, there are three fatty acid transporters in cardiac cells: cluster of differentiation 36 (CD36), fatty acids transport protein (FATP), and the fatty acids binding protein plasma membrane ($FABP_{pm}$)[128].

2.4.4 Lipoprotein lipase

Apoprotein C-II in the capillaries of adipose tissue and muscle tissue, can activate endothelial LPL which is on the endothelial surface, converting triglycerides to fatty acids and glycerol. Then the adipocytes and muscle cells take the fatty acids and glycerol for energy or storage. LPL cardiomyocytes specific transgene mice developed cardiomyopathy due to uptake of excess amounts of lipids in heart[129]. These results seem to suggest the heart does need a large amount of lipids for energy, but too much lipids will cause damage to the cardiac cells.

2.4.5 Skeletal muscle function in lipid metabolism

Triglycerides (TGs) is stored in adipose tissue and within muscle fibres. Metabolism demand during exercise, recovery from exercise, and the lipid availability will decide the fatty acids uptake in the skeletal muscle [130, 131]. When there are more fatty acids available, the mitochondria will take the fatty acids as the energy source in priority [132, 133]. In a normal condition, how much fatty acids enter the cell decides the rate of mitochondrial oxidation[134]. There are also three proteins that support transport of fatty acids from plasma to the skeletal muscle fiber cells, CD36, FATP[135], and FABP[136]. A study showed that exercise do not have any effect on the CD36 protein expression when doing regular training no matter women or men[137]. However, another study showed that the CD36 content increased 36% after moderate daily aerobic exercise[138]. Later, it was found that exercise does increase the CD36 content in mitochondria but not in sarcolemma[139]. The high fat food intake will upregulate the CD36 mRNA and protein expression, and thereby stimulate the translocation of skeletal muscle fatty acids [140]. When the fatty acids enter the skeletal muscle fibers, they will either be stored as triglycerides or go to mitochondrial oxidation depending on the muscle condition[141]. Studies show that 8 weeks endurance training will enhance the fatty acid usage in type 1 fibers (slow twitch fiber)[142]. The whole process is shown in figure 3.

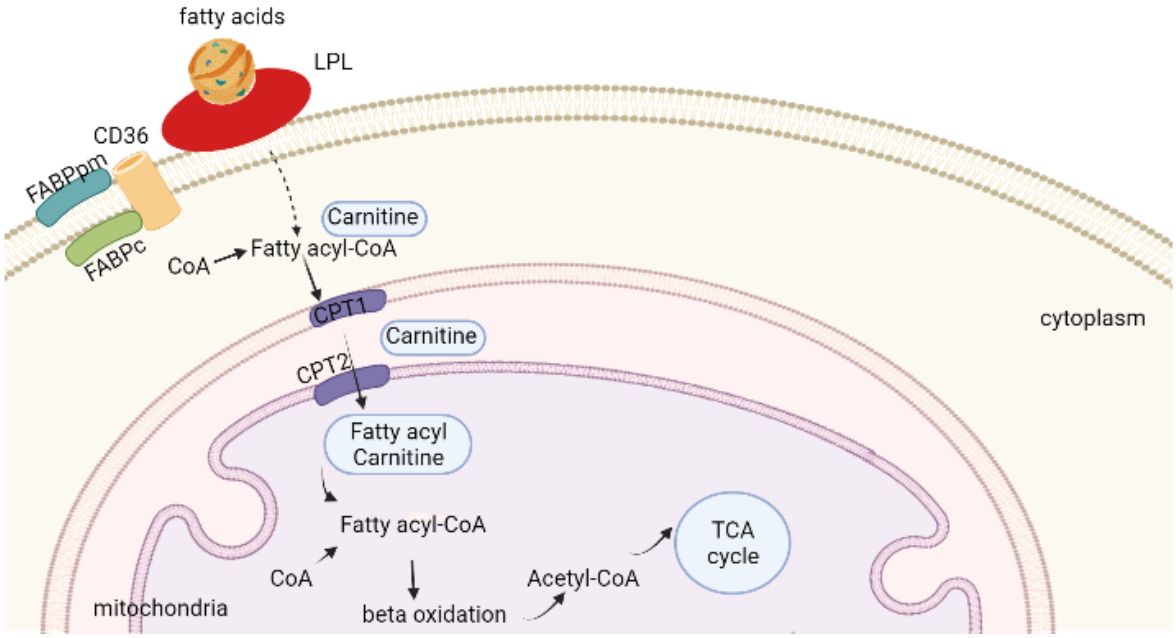

Figure 3 skeletal muscle lipid metaboism

2.5 CANCER CELL METABOLISM

Gene mutations can cause reprogramming of cellular metabolism and result in tumorigenesis [143]. The reprogramming results in high consumption of glucose and increased production of NADH and other nutrients for cell viability and the production of new biomass [144, 145]. The Warburg theory is based on the observation that cancer cells consume large amounts of glucose in glycolysis, and convert the surplus pyruvate to lactate, which is subsequently secreted into

the extracellular environment, instead of the more efficient oxidative phosphorylation [143]. This takes place in cancer cells also with fully functioning mitochondria. Despite a large interest and many studies, the mechanism of the Warburg effect is still unclear[146]. There are, however, several studies that try to explain the Warburg effect such as a more rapid ATP synthesis, more efficient biosynthetic pathways and importance for ROS mediated signal transduction[146]. In recent years many studies show that mitochondrial function is important for cancer cells. Mitochondrial DNA depletion reduces the chances of tumor generation in cancer cell lines *in vitro* and *in vivo* [147-149]. Lung cancer cells like A549 cells have powerful mitochondrial functions with high ability to balance the cellular redox state. Mitochondria benefits cancer cell survival in severe conditions, like hypoxia, lack of nutrition, and cancer treatment, so it plays a key role in tumorigenesis [150].

2.6 METFORMIN

2.6.1 Metformin in diabetes

Metformin has been widely used as a drug for treatment of type 2 diabetes for over 50 years. It is recommended as a first line medicine for newly diagnosed type 2 diabetes patients [151]. Its anti-diabetic effects are through lowering the hepatocellular gluconeogenesis [152]and through improving the utilization of glucose by peripheral tissues, such as adipose tissue and skeletal muscle [153].

2.6.2 Metformin action on mitochondrial complex I in cancer

In recent years, epidemiology has indicated that metformin slows down tumor growth and lowers the risk of tumorigenesis [154-158]. This has been confirmed by studies both *in vitro* and *in vivo*. Now it is generally acknowledged that metformin's activity on mitochondria is due to the pleiotropic effects of the drug[159]. The mechanisms of metformin effects on cancer cells are intensively studied but still not fully understood. Results from studies suggest that metformin has inhibitory effects specifically on mitochondrial complex I of the mitochondrial respiratory chain [156-158] and thereby lowers the level of NAD^+/NADH ratio [160]. There are studies that indicate that it may be the NAD^+ level that alters tumor growth, and thus the metformin action is to alter the level of NAD^+ through mitochondrial complex I.

Metformin inhibition of the mitochondrial respiratory chain complex I, the mitochondrial ATP synthesis, and oxygen consumption has been confirmed in several models, including mouse, rat, and human primary hepatocytes [156, 158, 161], liver cancer cell lines, and adrenocortical carcinoma derived cell lines [162-164], endothelial cells [165], pancreatic beta cells [166], neurons[164], peripheral blood mononuclear cells and platelets [167], and in cancer cells and cancer stem cells as well [157, 168-170].

The inhibition of mitochondrial complex I results in cellular ATP concentration decrease. This subsequently influences the ADP/ATP and AMP/ATP ratios, which actives the energy sensor AMPK [159].

2.7 THE SLC25A10 MITOCHONDRIAL CARRIER

SLC25A10 is a mitochondrial carrier located in the inner mitochondrial membrane, transporting malate and succinate out of mitochondrial, in exchange of phosphate and thiosulfate. SLC25A10 has recently been shown to be a key factor involved in lipid synthesis [171], supporting malate for the transport of citrate which is required for the synthesis of fatty acid[172]. Data from our lab showed that an A549 SLC25A10 knockdown cell line shifted the metabolism from glycolysis to oxidative phosphorylation and also increased the sensitivity of the cells to certain anti-cancer compounds [173].

3 RESEARCH AIMS

The general aim of the thesis is to gather knowledge on the connection between the nuclear encodes proteins and mitochondrial function, and to investigate nuclear-mitochondria interaction *in vivo* and *in vitro*.

- To study how the mitochondrial transporter SLC25A10 is regulated in the presence of metformin (**Paper I**).
- To construct and characterize a DGUOK complete knockout mouse model, with the aim to create a model for treatment strategies of DGUOK deficiency, as well as for other disorders with mtDNA depletion (**Paper II**).
- To investigate the molecular mechanisms that enabled the extended survival of the DGUOK complete knockout mice (**Paper III**).
- To construct and characterize a cardiac and skeletal muscle specific knockout of TK2, since muscle tissue is primarily affected with mtDNA deficiency in humans with TK2 disorders (**Paper IV**).
- To construct and characterize a liver specific TK2 knockout mice model for investigations of liver mtDNA deficiency and its effects on the pathology and life expectancy of the affected mice (**Paper V**).

4 MATERIALS AND METHODS

4.1 MATERIALS AND REAGENTS

DNA extraction kit (Qiagen, DNeasy Blood & Tissue Kit 69506), RNeasy Kit (Qiagen, 74106), RNeasy Fibrous Tissue Mini Kit (50) (Qiagen, 74704), Absolute quantification PCR with KAPA PROBE Fast Universal Kit (Sigma: KK4702), cDNA synthesis kit (Applied Biosystems, Life Technologies Corporation 4368814), KAPA SYBR® Fast qPCR Master Mix (2X) Universal (Kapa Biosystems, Merck KGaA, KM4602), lactate assay kit (Sigma, MAK064-1KT), Cell Proliferation Kit II (XTT) (Sigma: 11465015001), MitoSOX™ Red (Thermofisher, M36008).

Rabbit polyclonal to ACE2 antibody (Abcam Inc, Ab15348), mouse monoclonal to MTCO1 (Abcam Inc, ab14705), anti CD68 (marker of macrophages) antibody (Life Technologies, MA5-16363) from Thermo Fisher Scientific, anti CD31 (platelet endothelial cell adhesion molecule) antibody (Abcam Inc, ab28364) from Abcam, anti VDAC antibody (Santa Cruz Biotechnology, sc390996), donkey anti rabbit with HRP (Santa Cruz Biotechnology, Inc. sc-2313), Dako Polyclonal Rabbit anti-Mouse Immunoglobulins/HRP (Santa Clara, CA, P0260). Dry ice from Karolinska hospital, isopentane(2-methylbutane) from Sigma-Aldrich, 277258 CAS:78-78-4, cryostat embedding solution from Sakura Finetek Tissue, Tek 4583. rabbit anti-ADFP antibody (ab78920), anti-Ki-67 (Abcam; ab92742), Mouse anti-beta actin (Santa Cruz) or rabbit-anti-VADC (Abcam).

Dulbecco's Modified Eagle's medium (DMEM) (Life Tech, REF 31966), 10% FBS (Gibco, ref 10270), 1000 U/ml penicillin and 1000 µg/mL streptomycin (Thermo Fisher, ref 15140). Fetal bovine serum (FBS, REF: F0392), Metformin (1, 1-dimethylbiguanide hydrochloride) (Sigma, PHR1084), N-acetylcystein (NAC, negative control) (Sigma, A7250), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, negative control) (Sigma, 238813-1G), 4β-phorbol 12-myristate 13-acetate (PMA, positive control) (Santa Cruz, sc-3576B), Rotenone (Sigma, R8875); PKM2 inhibitor compound 3 k (Sigma, AMBH303C6BA3); DASA-58 (Sigma, SML2853); Serine (Sigma, S4500), Methanol, HPLC-grade was obtained from Fischer Scientific, Chloroform, Suprasolv for GC was obtained from Merck, Stable isotopes internal standards: L-proline-13C5, alpha-ketoglutarate-13C4, myristic acid-13C3, cholesterol-D7 were obtained from Cil. Succinic acid-D4, salicylic acid-D6, L-glutamic acid-13C5,15 N, putrescine-D4, hexadecanoic acid-13C4, D-glucose-13C6, D-sucrose-13C12 were obtained from Sigma.

4.2 METHODS

The methods for the experiments in lab used in this thesis are well established methods. Cell culture techniques were done in a P1 lab, DNA (excellent quality) extraction, RNA extraction,

and cDNA synthesis followed the protocol from the kit. Hotshot method DNA extraction, and polymerase chain reaction (PCR) were used for genotyping. Western blot was used for protein expression studies, and real time PCR (qPCR) for mtDNA copy number determination and gene expression profiling, serum biochemistry analysis, are all described in detail in papers I, II, III, IV, and V. Histopathology and immunohistochemistry analysis of mouse tissues using microscopy, confocal microscopy, and electron microscopy was performed in collaboration with the Division of Pathology, Department of Laboratory Medicine, Karolinska Institute.

4.2.1 Transgene constructs

4.2.1.1 DGUOK KO mice construct

To generate DGUOK KO mice, 129S6/SvEvTac mice DNA were used as a vector to replace the DGUOK (NM 013764.2) gene in 129/SVJ mice embryonic stem cells. A clone (RPCI22-274F23) from bacterial artificial chromosome library that contained exon II and part of exon III of the *Dguok* gene was identified. Those *Dguok* genes were subcloned into a pBluescript plasmid vector with extra designed two complementary oligonucleotides including multiple restriction enzyme sites, Neo cassette and two LoxP sites. The targeting vector was electroporated into 129/SVJ embryonic stem cells, continued with gentamicin selection and southern blot screening. For heterozygous DGUOK knockout mice, the exon II and neomycin cassette were simultaneously deleted after the new generation born, by breeding *Dguok*+/Neo-loxP mice with transgenic mice expressing Cre recombinase. The *Dguok* $^{+/-}$ knockout mice were intercrossed to generate homozygous *Dguok* $^{-/-}$ mice.

4.2.1.2 Cardiac and skeletal muscle specificTK2 knockout mice construct

With homologous recombination, original TK2 exon V were replaced with exon V additionally two loxp sites, two flp sites, and a neo cassette, were inserted in the mouse stem cells to generate TK2 conditional knockout mice. $TK2^{loxP}/TK2^{loxP}$ mice with neo cassette depletion were mated to heterozygous transgenic mice (+/Ckmm-cre). Double heterozygous (+/TK2 ^{loxP}, +/Ckmm-cre) mice were backcrossed with TK2 ^{loxP}/TK2 ^{loxP} strain to generate tissue specific knockout mice ($TK2^{-/-}$, +/Ckmm-cre).

4.2.1.3 Liver specific TK2 knockout mice construct

TK2 conditional knockout mice were generated by using homologous recombination with replaced modified TK2 exon V (with additionally two loxp sites, two flp sites, and a neo cassette). $TK2^{loxP}/TK2^{loxP}$ mice with a deleted neo cassette were mated to heterozygous transgenic mice (+/liver-cre) to generate double heterozygous mice. Double heterozygous (+/TK2 ^{loxP}, +/liver-cre) mice were cross breeding with $TK2^{loxP}/TK2^{loxP}$ strain to generate the liver tissue specific knockout mice ($TK2^{-/-}$, +/liver-cre).

4.2.1.4 Ethical consideration

All studies in the thesis were honest reported data, results, methods and procedures, and publication status. No studies involved any human or patient's data. In paper I a commercial

cancer cell line was the only study object. Paper II to V used mice model as the study objects. All animal experiments were approved and performed following the guidelines of the local ethical committee (B101–15) and (6487-2021).

4.2.1.5 Determination of fat volume with CT scan and muscle strength

PET CT scan was used to analyze the 16 weeks old, sacrificed mice. The Analyze 12.0 software from AnalyzeDirect was used to determine the 3D volumes of fat, skeletal muscle, and heart.

The control group and cardiac and skeletal muscle Tk2 knockout mice muscle strength was measured, with 5 mice included in each group. The animals were tested for muscle strength for a maximum of 2 minutes and then let them free. The mice were tested every week between 4 to 16 weeks of age and were sacrificed when they showed low body weights or other conditions according to the ethical permit.

4.2.1.6 Gene expression determined by microarray

The experiment was performed with Mouse GENE 2.2-ST Array and the data use analyzed by the Qlucore Omics Explorer version 3.2. Two-group comparison was performed using unpaired student's t-test and cluster analysis (k-means) with false discovery rate (FDR) adjusted at $P(q) < 0.05$. Gene set enrichment analysis (GSEA) was performed in STRING version 10.

4.2.1.7 Statistical analysis

For Paper I, delta Ct analysis was used to analyze the gene expression data, all conditions were normalized to A549 control cell line. For the Paper II, III, IV, and V, each individual mouse was measured as a biological replicate. For the real-time PCR, two or three technical replicates were performed for each biological replicate. Body weights, mtDNA copy number and fat volume were analyzed with unpaired two-tailed t-tests. The Mann-Whitney U-test was used to compare the non-parametric gene expression data. Significant level was set to $p < 0.05$.

5 RESULTS AND DISCUSSION

In **study I**, cell growth experiment results showed that wild type A549 cells consumed nutrition faster in the medium and reached the growth plateau earlier than the siSLC25A10 cells. The proliferation marker Ki-67 was investigated in 4.5, 1.0, and 0.1g/L glucose concentration in presence of 5 mM metformin. The results showed that proliferation was inhibited mostly in 0.1g/L glucose, indicating that Ki-67 gene expression was dependent on glucose in the presence of metformin. The siSLC25A10 gene expression and protein level were tested in cells grown in 4.5, 1.0 or 0.1g/L glucose and 5mM metformin as well, showing an inhibitory effect of SLC25A10 expression especially in 0.1 g/L glucose medium.

Expression levels of the metabolic enzymes pyruvate dehydrogenase (PDH), lactate dehydrogenase (LDH), and glutamate dehydrogenase (GDH) were investigated 24 h after addition of metformin. Compared to the control A549 cells, PDHA, but not PDHB, was significantly augmented in the siSLC25A10 cell line, with and without metformin. GDH was not significantly changed compared to the untreated cells in both cell lines, although the average level in siSLC25A10 cell lines was higher, with and without metformin. Both LDHA and LDHB increased their expression in siSLC25A10 cells. The high expression of PDH and LDH in siSLC25A10 cells, indicated a fast use of glucose in glycolysis. The expression of GOT1 increased in the siSLC25A10 cells, whereas it decreased in control A549 cells. This suggested that in siSLC25A10 cells, grown in 0.1g/L glucose with metformin, aspartate was converted to oxaloacetate and thereafter goes back to mitochondria to generate ATP, with the final purpose to keep the cell surviving. The significant increase of the cyclin-dependent kinase inhibitor (CDKN1A) p21 gene in the siSLC25A10 cells suggested an arrest of the cell cycle following the decrease of SLC25A10 expression in the presence of metformin. Metformin stimulated mitochondrial reactive oxygen species (ROS) production at low glucose concentration but not at high glucose concentrations. These results indicated that siSLC25A10 cells could be more dependent on mitochondrial function than the control cells, both with and without metformin. The BioGRID database shows that the SLC25A10 protein is deeply interactive with the mitochondrial NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex, 4 (NDUFA4) gene, which is part of mitochondrial complex I. This may contribute to the results that siSLC25A10 cells were more affected by metformin, since metformin is generally accepted to target complex I [174]. However, the mechanisms om metformin is still not clearly elucidated.

In **study II** and **study III**, a model of DGUOK knockout mice was generated to study the molecular mechanism of DGUOK deficiency and to explore novel treatment strategies. The Dguok complete knockout mice ($Dguok^{-/-}$) were born normal, but the body weight began to drop at week 6. Multiple tissues showed a mtDNA copy number decrease, with liver as the most severely affected organ among the tissues investigated. The mtDNA related cytochrome c oxidase protein was decreased, while the nuclear encoded protein succinate dehydrogenase complex subunit A was unaffected. The subcutaneous fat layer in $Dguok^{-/-}$ mice was not visible by eye, suggesting an altered lipid metabolism. The $Dguok^{-/-}$ mice, especially the

female mice could survive for one year. This phenomenon was studied by using transcriptomics, proteomics and metabolomics followed by *in vitro* assays, aiming to illustrate the mechanism of this extended survival. We found that serine synthesis and increased folate cycle supported the early stage of the Dguok$^{-/-}$ mice. The TCA cycle and the urea cycle were highly functioning to fuel the body. Two pyruvate kinase genes, PKLR and PKM, showed increased expression, suggesting that this was to support the TCA cycle. The mechanism of how the DGUOK knockout mice could survive long indicated the strong compensation ability in liver. The invisible adipose tissue also supported the mechanism that the DGUOK knockout mice has used the energy to maintain living activity instead of storing the energy as fat in body.

In **study IV** and **study V**, we have generated a cardiac and skeletal muscle specific TK2 knockout mice, and a liver specific TK2 knockout mice. The mtDNA copy number was specifically decreased in the targeted tissue. The mTK2 KO mice body weights decreased and survived about 16 weeks, but the livTK2 KO mice showed normal body weight, and could survive for more than one and a half years. The body weight loss of mTK2 KO mice was mainly due to loss of adipose tissue and a decrease of muscle volume. We further found an altered lipid metabolism in mTK2 KO mice and histopathology results showed damage in the cardiac and skeletal muscle tissue. In the livTK2 KO mice, only the hepatocytes mtDNA copy number dropped and no other symptoms were observed. The comparison of these two knockout mice indicated the heart and skeletal muscle relying on the mitochondrion function very much. Only liver mtDNA deficiency could be compensated and do not show any symptoms. The mTK2 KO mice showed dilated heart and with ACE2 highly expressed in cardiomyocytes. Since ACE2 is the SARS-CoV-2 receptor, fragile patients with heart failure may be even more susceptible to infection with SARS-Cov-2 if the receptor of the virus is up regulated. If mitochondrial dysfunction contributes to poor prognosis of corona virus infection has not yet been clarified.

6 CONCLUSIONS

The nuclear encoded genes SLC25A10, TK2 and DGUOK support an essential function for mitochondria in energy production. The metformin treatment can downregulate the SLC25A10 expression especially in siSLC25A10 cell. The knockdown of SLC25A10 together with metformin treatment, can almost stop the cell cycle and arrested the cell growth, suggesting a deficiency of energy. This phenomenon can be rescued by a high glucose condition, indicating that SLC25A10 and metformin treatment played an important role for mitochondria to efficiently produce energy.

The complete DGUOK knockout in mice significantly changed the lipid metabolism and caused a loss of body weight. Long survival mechanism illustrated that somehow the body could compensate the energy crisis. In contrast, all the cardiac and skeletal muscle TK2 knockout mice only survived 16 weeks, indicating the TK2 deficiency can not be compensated and caused lethal damage to the mice. The fast loss of adipose tissue may be one reason for the short life of cardiac and skeletal muscle TK2 knockout mice. In conclusion the DGUOK caused mtDNA deficiency which could be partly compensated, but cardiac and skeletal muscle TK2 knockout mice caused mtDNA deficiency that could not be compensated by other enzymes. The liver TK2 knockout mice also decreased the mtDNA copy number, but did not reach the threshold, and thus the mice lived as normal as the control mice during the conditions used.

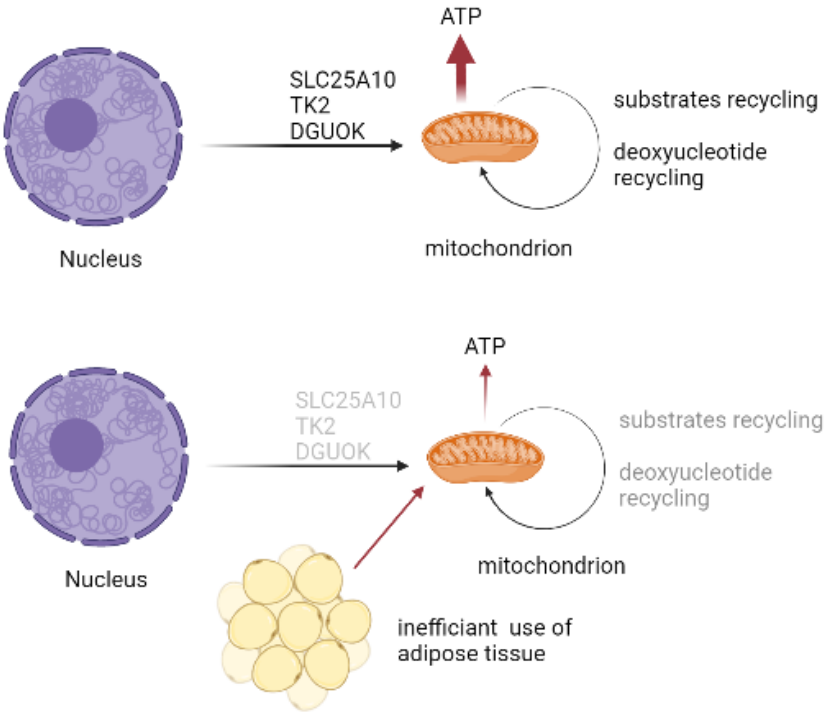

Figure 5. Nuclear communication with mitochondria

7 POINTS OF PERSPECTIVE

The SLC25A10 is involved in mitochondrial metabolism and lipid synthesis, and this transporter is highly expressed in cancer tissue. It should be of interest to find an antagonist against SLC25A10 to slow tumor growth, or even kill the tumor, possibly combined with other compounds like metformin. Metformin is believed to target mitochondrial complex I, but the mechanism is still not clear. It is worth to investigate the full mechanism of action of metformin.

Our group has studied three models of TK2 complete or tissue specific knockout mice. The results indicate that the TK2 activity in heart and skeletal muscle is vital for sufficient mtDNA synthesis to keep the mice alive. The administration of deoxynucleosides, effects of exercise, and high energy food intake are among future plans to investigate. The aim would be to compensate the deficiency of TK2 and prolong the lifespan of the mice.

The complete DGUOK knockout mice have been observed to survive at least one year. However, we had indications of a possible gender difference, since the male mice did not survive as long as the female mice. This will be further investigated. Preliminary data from an ongoing intervention with high fat diet show a prolonged life span of complete DGUOK knockout mice.

8 ACKNOWLEDGEMENTS

First of all, I would like to thank **Anna Karlsson**, my main supervisor, for offering me the opportunity to do research in your group. Your guidence and supporting really help me to become confident and believe in myself could be an independent scientist. You have been always training me towards a full developed person, from preparing seminars to writing manuscript and thesis. You always respect my opinions and ideas on the research, and have a very good communication with me all these years. You have been a wonderful supervisor, thank you!

I would like to thank my co-supervisors Doctor **Sophie Curbo** and Doctor **Xiaoshan Zhou**, for the valuable scientific input and intellegent disscussion in every seminar. Thank you Sophie for every time helping me when I have met troubles in misunderstanding the Swedish language and the beginning time of culture shock when I just start my PhD. You are a so nice and kind person, giving me many good suggestions in both research and life.

Thank you Xiaoshan, for the guidance of research and kind communications whenever I met troubles in the research. And I am specially so touched, you help me finished the animal experiments when I am in materal leave.

Raoul Kuiper, thank you for all these years collaboration. You have exllent experience in histology analysis and gave me good suggestions in my research.

Thank you **Shuba Krishna**. You have a really good introduction of the animal work, the QPCR machine etc. Also you are very talent in communication with people, making me feel relax in the lab.

Thank you **Vasily Aleshin**. We have discussed the experiment and everything. You are very good friends. And you have a talent of cooking muschrooms!

Thank you **Fan Han** and **Chao Wang**, for the friendly support when I am in the tough time. Thank you **Yumei Diao** and **Ning Liang**. Those two years, I have met such a big trouble, but it is so nice that you two always support me and helping me. Thank you **Fen Yang** and **Jianjian Gao** for visiting me and helping me.

Thank you **Liyan, Jingyi, Xi Chen, Bai** for the happy time in lab. It is so good to have you here and disscuss the research, work, and life.

Thank you **Lydia**, for the years we together of refilling liquid nitrogen and recycling in division.

The whole division of clinical microbiology, thank you for the nice time, especially **Shambu, Rekha, Emon, Katie, Robert, Sara, Maike, Giota, Angela, Lisa**.

I would also like to thank my former supervisor **Xin Guo**. You have supported me very well during eight years in University.

I would like to Thank my family, my mum **Lizhen**, my husband **Chungang**, and my son **Yiqi**. I love you so much. My son Yiqi, I can feel you are struggling to learn and to talk, for the half a year, from typical autism to becoming aware of yourself, aware of other people. I am so proud of you. Thank you coming to my life.

Thank you **Jassica, Annita**, and **Kiki**, from Entitan preschool. You helped me to take care of my son and helped my son to involved in more social activity with other children. I am so touched for your effort.

9 REFERENCES

1. Zhou, X., et al., *Progressive loss of mitochondrial DNA in thymidine kinase 2-deficient mice.* Hum Mol Genet, 2008. **17**(15): p. 2329-35.
2. Zhou, X., et al., *Thymidine kinase 2 deficiency-induced mtDNA depletion in mouse liver leads to defect beta-oxidation.* PLoS One, 2013. **8**(3): p. e58843.
3. Stephen W. Schaffer, M.-S.S., *Mitochondria*, in *The Dynamic Organelle*, S.W. Schaffer, Suleiman, M. Saadeh (Eds.), Editor. 2007, Springer.
4. Lane, N. and W. Martin, *The energetics of genome complexity.* Nature, 2010. **467**(7318): p. 929-34.
5. Gray, M.W., *Mitochondrial evolution.* Cold Spring Harb Perspect Biol, 2012. **4**(9): p. a011403.
6. Cole, L.W., *The Evolution of Per-cell Organelle Number.* Front Cell Dev Biol, 2016. **4**: p. 85.
7. Nicholls, D.G. and S. Chalmers, *The integration of mitochondrial calcium transport and storage.* J Bioenerg Biomembr, 2004. **36**(4): p. 277-81.
8. Nunnari, J. and A. Suomalainen, *Mitochondria: in sickness and in health.* Cell, 2012. **148**(6): p. 1145-59.
9. Neupert, W. and J.M. Herrmann, *Translocation of proteins into mitochondria.* Annu Rev Biochem, 2007. **76**: p. 723-49.
10. Schmidt, O., N. Pfanner, and C. Meisinger, *Mitochondrial protein import: from proteomics to functional mechanisms.* Nat Rev Mol Cell Biol, 2010. **11**(9): p. 655-67.
11. Friedman, J.R. and J. Nunnari, *Mitochondrial form and function.* Nature, 2014. **505**(7483): p. 335-43.
12. Veltri, K.L., M. Espiritu, and G. Singh, *Distinct genomic copy number in mitochondria of different mammalian organs.* J Cell Physiol, 1990. **143**(1): p. 160-4.
13. Suomalainen, A. and P. Isohanni, *Mitochondrial DNA depletion syndromes--many genes, common mechanisms.* Neuromuscul Disord, 2010. **20**(7): p. 429-37.
14. Gandhi, V.V. and D.C. Samuels, *A review comparing deoxyribonucleoside triphosphate (dNTP) concentrations in the mitochondrial and cytoplasmic compartments of normal and transformed cells.* Nucleosides Nucleotides Nucleic Acids, 2011. **30**(5): p. 317-39.
15. Sun, R., *Studies of enzymes in mitochondrial DNA precursor synthesis.* Sveriges lantbruksuniv., Acta Universitatis Agriculturae Sueciae, 2013.
16. Johansson, R., et al., *Structural Mechanism of Allosteric Activity Regulation in a Ribonucleotide Reductase with Double ATP Cones.* Structure, 2016. **24**(6): p. 906-917.
17. Brown, N.C., et al., *Ribonucleoside diphosphate reductase. Purification of the two subunits, proteins B1 and B2.* Eur J Biochem, 1969. **9**(4): p. 561-73.

18. Mann, G.J., et al., *Ribonucleotide reductase M1 subunit in cellular proliferation, quiescence, and differentiation.* Cancer Res, 1988. **48**(18): p. 5151-6.

19. Eriksson, S., et al., *Cell cycle-dependent regulation of mammalian ribonucleotide reductase. The S phase-correlated increase in subunit M2 is regulated by de novo protein synthesis.* J Biol Chem, 1984. **259**(19): p. 11695-700.

20. Engstrom, Y., et al., *Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits.* J Biol Chem, 1985. **260**(16): p. 9114-6.

21. Elledge, S.J., Z. Zhou, and J.B. Allen, *Ribonucleotide reductase: regulation, regulation, regulation.* Trends Biochem Sci, 1992. **17**(3): p. 119-23.

22. Hakansson, P., A. Hofer, and L. Thelander, *Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells.* J Biol Chem, 2006. **281**(12): p. 7834-41.

23. Tanaka, H., et al., *A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage.* Nature, 2000. **404**(6773): p. 42-9.

24. Guittet, O., et al., *Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells.* J Biol Chem, 2001. **276**(44): p. 40647-51.

25. Pontarin, G., et al., *Mammalian ribonucleotide reductase subunit p53R2 is required for mitochondrial DNA replication and DNA repair in quiescent cells.* Proc Natl Acad Sci U S A, 2012. **109**(33): p. 13302-7.

26. Tran, P., et al., *De novo dNTP production is essential for normal postnatal murine heart development.* J Biol Chem, 2019. **294**(44): p. 15889-15897.

27. Zhu, C.Y., M. Johansson, and A. Karlsson, *Incorporation of nucleoside analogs into nuclear or mitochondrial DNA is determined by the intracellular phosphorylation site.* Journal of Biological Chemistry, 2000. **275**(35): p. 26727-26731.

28. Kocabas, N.A., et al., *Gemcitabine pharmacogenomics: deoxycytidine kinase and cytidylate kinase gene resequencing and functional genomics.* Drug Metab Dispos, 2008. **36**(9): p. 1951-9.

29. Johansson, M. and A. Karlsson, *Cloning of the cDNA and chromosome localization of the gene for human thymidine kinase 2.* J Biol Chem, 1997. **272**(13): p. 8454-8.

30. Johansson, M., et al., *Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of Drosophila melanogaster.* Journal of Biological Chemistry, 1999. **274**(34): p. 23814-23819.

31. Johansson, M., et al., *Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of Drosophila melanogaster.* J Biol Chem, 1999. **274**(34): p. 23814-9.

32. Munch-Petersen, B., et al., *Functional expression of a multisubstrate deoxyribonucleoside kinase from Drosophila melanogaster and its C-terminal deletion mutants.* J Biol Chem, 2000. **275**(9): p. 6673-9.

33. Knecht, W., B. Munch-Petersen, and J. Piskur, *Identification of residues involved in the specificity and regulation of the highly efficient multisubstrate*

deoxyribonucleoside kinase from Drosophila melanogaster. J Mol Biol, 2000. **301**(4): p. 827-37.

34. Sun, R. and L. Wang, *Thymidine kinase 2 enzyme kinetics elucidate the mechanism of thymidine-induced mitochondrial DNA depletion.* Biochemistry, 2014. **53**(39): p. 6142-50.

35. Mazurova, S., et al., *Thymidine kinase 2 and alanyl-tRNA synthetase 2 deficiencies cause lethal mitochondrial cardiomyopathy: case reports and review of the literature.* Cardiol Young, 2017. **27**(5): p. 936-944.

36. Saada, A., et al., *Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy.* Nat Genet, 2001. **29**(3): p. 342-4.

37. Mancuso, M., et al., *Mitochondrial myopathy of childhood associated with mitochondrial DNA depletion and a homozygous mutation (T77M) in the TK2 gene.* Arch Neurol, 2003. **60**(7): p. 1007-9.

38. Oskoui, M., et al., *Clinical spectrum of mitochondrial DNA depletion due to mutations in the thymidine kinase 2 gene.* Arch Neurol, 2006. **63**(8): p. 1122-6.

39. Blakely, E., et al., *Novel mutations in the TK2 gene associated with fatal mitochondrial DNA depletion myopathy.* Neuromuscul Disord, 2008. **18**(7): p. 557-60.

40. Gotz, A., et al., *Thymidine kinase 2 defects can cause multi-tissue mtDNA depletion syndrome.* Brain, 2008. **131**(Pt 11): p. 2841-50.

41. Behin, A., et al., *Adult cases of mitochondrial DNA depletion due to TK2 defect: an expanding spectrum.* Neurology, 2012. **78**(9): p. 644-8.

42. Alston, C.L., et al., *Late-onset respiratory failure due to TK2 mutations causing multiple mtDNA deletions.* Neurology, 2013. **81**(23): p. 2051-3.

43. Akman, H.O., et al., *Thymidine kinase 2 (H126N) knockin mice show the essential role of balanced deoxynucleotide pools for mitochondrial DNA maintenance.* Hum Mol Genet, 2008. **17**(16): p. 2433-40.

44. Krishnan, S., et al., *Transgene Expression of Drosophila melanogaster Nucleoside Kinase Reverses Mitochondrial Thymidine Kinase 2 Deficiency.* Journal of Biological Chemistry, 2013. **288**(7): p. 5072-5079.

45. Krishnan, S., et al., *Long Term Expression of Drosophila melanogaster Nucleoside Kinase in Thymidine Kinase 2-deficient Mice with No Lethal Effects Caused by Nucleotide Pool Imbalances.* Journal of Biological Chemistry, 2014. **289**(47).

46. Johansson, M., et al., *Localization of the human deoxyguanosine kinase gene (DGUOK) to chromosome 2p13.* Genomics, 1996. **38**(3): p. 450-1.

47. Salviati, L., et al., *Mitochondrial DNA depletion and dGK gene mutations.* Ann Neurol, 2002. **52**(3): p. 311-7.

48. Labarthe, F., et al., *Clinical, biochemical and morphological features of hepatocerebral syndrome with mitochondrial DNA depletion due to deoxyguanosine kinase deficiency.* J Hepatol, 2005. **43**(2): p. 333-41.

49. Nobre, S., et al., *Neonatal liver failure due to deoxyguanosine kinase deficiency.* BMJ Case Rep, 2012. **2012**.

50. Grabhorn, E., et al., *Long-term outcomes after liver transplantation for deoxyguanosine kinase deficiency: a single-center experience and a review of the literature.* Liver Transpl, 2014. **20**(4): p. 464-72.

51. Mancuso, M., et al., *New DGK gene mutations in the hepatocerebral form of mitochondrial DNA depletion syndrome.* Arch Neurol, 2005. **62**(5): p. 745-7.

52. Zeviani, M., et al., *An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region.* Nature, 1989. **339**(6222): p. 309-11.

53. Moraes, C.T., et al., *mtDNA depletion with variable tissue expression: a novel genetic abnormality in mitochondrial diseases.* Am J Hum Genet, 1991. **48**(3): p. 492-501.

54. Mandel, H., et al., *The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA.* Nat Genet, 2001. **29**(3): p. 337-41.

55. Spinazzola, A., et al., *MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion.* Nat Genet, 2006. **38**(5): p. 570-5.

56. Van Goethem, G., et al., *Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions.* Nat Genet, 2001. **28**(3): p. 211-2.

57. Lonnqvist, T., et al., *Recessive twinkle mutations cause severe epileptic encephalopathy.* Brain, 2009. **132**(Pt 6): p. 1553-62.

58. Elpeleg, O., et al., *Deficiency of the ADP-forming succinyl-CoA synthase activity is associated with encephalomyopathy and mitochondrial DNA depletion.* Am J Hum Genet, 2005. **76**(6): p. 1081-6.

59. Ostergaard, E., et al., *Deficiency of the alpha subunit of succinate-coenzyme A ligase causes fatal infantile lactic acidosis with mitochondrial DNA depletion.* Am J Hum Genet, 2007. **81**(2): p. 383-7.

60. Bourdon, A., et al., *Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion.* Nat Genet, 2007. **39**(6): p. 776-80.

61. Nishino, I., A. Spinazzola, and M. Hirano, *Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder.* Science, 1999. **283**(5402): p. 689-92.

62. Kornblum, C., et al., *Loss-of-function mutations in MGME1 impair mtDNA replication and cause multisystemic mitochondrial disease.* Nat Genet, 2013. **45**(2): p. 214-9.

63. Reyes, A., et al., *RNASEH1 Mutations Impair mtDNA Replication and Cause Adult-Onset Mitochondrial Encephalomyopathy.* Am J Hum Genet, 2015. **97**(1): p. 186-93.

64. Stiles, A.R., et al., *Mutations in TFAM, encoding mitochondrial transcription factor A, cause neonatal liver failure associated with mtDNA depletion.* Mol Genet Metab, 2016. **119**(1-2): p. 91-9.

65. Jurkute, N., et al., *SSBP1 mutations in dominant optic atrophy with variable retinal degeneration.* Ann Neurol, 2019. **86**(3): p. 368-383.

66. Bonora, E., et al., *Biallelic variants in LIG3 cause a novel mitochondrial neurogastrointestinal encephalomyopathy.* Brain, 2021. **144**(5): p. 1451-1466.

67. Rouzier, C., et al., *The MFN2 gene is responsible for mitochondrial DNA instability and optic atrophy 'plus' phenotype.* Brain, 2012. **135**(Pt 1): p. 23-34.

68. Nasca, A., et al., *Recessive mutations in MSTO1 cause mitochondrial dynamics impairment, leading to myopathy and ataxia.* Hum Mutat, 2017. **38**(8): p. 970-977.

69. Russell, B.E., et al., *Expanding and Underscoring the Hepato-Encephalopathic Phenotype of QIL1/MIC13.* Hepatology, 2019. **70**(3): p. 1066-1070.

70. Boczonadi, V., et al., *Mitochondrial oxodicarboxylate carrier deficiency is associated with mitochondrial DNA depletion and spinal muscular atrophy-like disease.* Genet Med, 2018. **20**(10): p. 1224-1235.

71. Punzi, G., et al., *SLC25A10 biallelic mutations in intractable epileptic encephalopathy with complex I deficiency.* Hum Mol Genet, 2018. **27**(3): p. 499-504.

72. Calvo, S.E., et al., *Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing.* Sci Transl Med, 2012. **4**(118): p. 118ra10.

73. Bonnen, P.E., et al., *Mutations in FBXL4 cause mitochondrial encephalopathy and a disorder of mitochondrial DNA maintenance.* Am J Hum Genet, 2013. **93**(3): p. 471-81.

74. Besse, A., et al., *The GABA transaminase, ABAT, is essential for mitochondrial nucleoside metabolism.* Cell Metab, 2015. **21**(3): p. 417-27.

75. Garone, C., et al., *Defective mitochondrial rRNA methyltransferase MRM2 causes MELAS-like clinical syndrome.* Hum Mol Genet, 2017. **26**(21): p. 4257-4266.

76. Hoppeler, H. and M. Fluck, *Plasticity of skeletal muscle mitochondria: structure and function.* Med Sci Sports Exerc, 2003. **35**(1): p. 95-104.

77. Schirrmacher, V., *Mitochondria at Work: New Insights into Regulation and Dysregulation of Cellular Energy Supply and Metabolism.* Biomedicines, 2020. **8**(11).

78. Kharitonenkov, A., et al., *FGF-21 as a novel metabolic regulator.* J Clin Invest, 2005. **115**(6): p. 1627-35.

79. Nishimura, T., et al., *Identification of a novel FGF, FGF-21, preferentially expressed in the liver.* Biochim Biophys Acta, 2000. **1492**(1): p. 203-6.

80. Luo, S., et al., *Biparental Inheritance of Mitochondrial DNA in Humans.* Proc Natl Acad Sci U S A, 2018. **115**(51): p. 13039-13044.

81. Alcolado, J., *Intrauterine environment and later disease development: infertility treatment and the risk of diabetes in offspring.* Med Hypotheses, 2006. **66**(6): p. 1133-6.

82. Chocron, E.S., E. Munkacsy, and A.M. Pickering, *Cause or casualty: The role of mitochondrial DNA in aging and age-associated disease.* Biochim Biophys Acta Mol Basis Dis, 2019. **1865**(2): p. 285-297.

83. Niemi, A.K., et al., *Mitochondrial DNA polymorphisms associated with longevity in a Finnish population.* Hum Genet, 2003. **112**(1): p. 29-33.

84. Niemi, A.K., et al., *A combination of three common inherited mitochondrial DNA polymorphisms promotes longevity in Finnish and Japanese subjects.* Eur J Hum Genet, 2005. **13**(2): p. 166-70.

85. Phadnis, N., R.A. Sia, and E.A. Sia, *Analysis of repeat-mediated deletions in the mitochondrial genome of Saccharomyces cerevisiae.* Genetics, 2005. **171**(4): p. 1549-59.

86. Cao, Z., et al., *Mitochondrial DNA deletion mutations are concomitant with ragged red regions of individual, aged muscle fibers: analysis by laser-capture microdissection.* Nucleic Acids Res, 2001. **29**(21): p. 4502-8.

87. Yui, R. and E.T. Matsuura, *Detection of deletions flanked by short direct repeats in mitochondrial DNA of aging Drosophila.* Mutat Res, 2006. **594**(1-2): p. 155-61.

88. Tanhauser, S.M. and P.J. Laipis, *Multiple deletions are detectable in mitochondrial DNA of aging mice.* J Biol Chem, 1995. **270**(42): p. 24769-75.

89. Samuels, D.C., *Mitochondrial DNA repeats constrain the life span of mammals.* Trends Genet, 2004. **20**(5): p. 226-9.

90. Bratic, A. and N.G. Larsson, *The role of mitochondria in aging.* J Clin Invest, 2013. **123**(3): p. 951-7.

91. Zhang, R., et al., *Independent impacts of aging on mitochondrial DNA quantity and quality in humans.* BMC Genomics, 2017. **18**(1): p. 890.

92. Wachsmuth, M., et al., *Age-Related and Heteroplasmy-Related Variation in Human mtDNA Copy Number.* PLoS Genet, 2016. **12**(3): p. e1005939.

93. Mengel-From, J., et al., *Mitochondrial DNA copy number in peripheral blood cells declines with age and is associated with general health among elderly.* Hum Genet, 2014. **133**(9): p. 1149-59.

94. Lee, J.W., et al., *Mitochondrial DNA copy number in peripheral blood is associated with cognitive function in apparently healthy elderly women.* Clin Chim Acta, 2010. **411**(7-8): p. 592-6.

95. Donoghue, M., et al., *A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9.* Circ Res, 2000. **87**(5): p. E1-9.

96. Tipnis, S.R., et al., *A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase.* J Biol Chem, 2000. **275**(43): p. 33238-43.

97. Vickers, C., et al., *Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase.* J Biol Chem, 2002. **277**(17): p. 14838-43.

98. Kowalczuk, S., et al., *A protein complex in the brush-border membrane explains a Hartnup disorder allele.* FASEB J, 2008. **22**(8): p. 2880-7.

99. Hashimoto, T., et al., *ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation.* Nature, 2012. **487**(7408): p. 477-81.

100. Li, W., et al., *Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus.* Nature, 2003. **426**(6965): p. 450-4.

101. Fraga-Silva, R.A., et al., *An orally active formulation of angiotensin-(1-7) produces an antithrombotic effect.* Clinics (Sao Paulo), 2011. **66**(5): p. 837-41.

102. Marques, F.D., et al., *An oral formulation of angiotensin-(1-7) produces cardioprotective effects in infarcted and isoproterenol-treated rats.* Hypertension, 2011. **57**(3): p. 477-83.

103. Marques, F.D., et al., *Beneficial effects of long-term administration of an oral formulation of Angiotensin-(1-7) in infarcted rats.* Int J Hypertens, 2012. **2012**: p. 795452.

104. Bertagnolli, M., et al., *An orally active angiotensin-(1-7) inclusion compound and exercise training produce similar cardiovascular effects in spontaneously hypertensive rats.* Peptides, 2014. **51**: p. 65-73.

105. Crackower, M.A., et al., *Angiotensin-converting enzyme 2 is an essential regulator of heart function.* Nature, 2002. **417**(6891): p. 822-8.

106. Santos, R.A.S., et al., *The ACE2/Angiotensin-(1-7)/MAS Axis of the Renin-Angiotensin System: Focus on Angiotensin-(1-7).* Physiol Rev, 2018. **98**(1): p. 505-553.

107. Niu, M.J., et al., *Loss of angiotensin-converting enzyme 2 leads to impaired glucose homeostasis in mice.* Endocrine, 2008. **34**(1-3): p. 56-61.

108. Donoghue, M., et al., *Heart block, ventricular tachycardia, and sudden death in ACE2 transgenic mice with downregulated connexins.* J Mol Cell Cardiol, 2003. **35**(9): p. 1043-53.

109. Nadarajah, R., et al., *Podocyte-specific overexpression of human angiotensin-converting enzyme 2 attenuates diabetic nephropathy in mice.* Kidney Int, 2012. **82**(3): p. 292-303.

110. Xia, H., et al., *ACE2-mediated reduction of oxidative stress in the central nervous system is associated with improvement of autonomic function.* PLoS One, 2011. **6**(7): p. e22682.

111. Xiao, L., et al., *Brain-selective overexpression of angiotensin-converting enzyme 2 attenuates sympathetic nerve activity and enhances baroreflex function in chronic heart failure.* Hypertension, 2011. **58**(6): p. 1057-65.

112. Chen, J., et al., *Neuronal over-expression of ACE2 protects brain from ischemia-induced damage.* Neuropharmacology, 2014. **79**: p. 550-8.

113. Zheng, J.L., et al., *Angiotensin converting enzyme 2/Ang-(1-7)/mas axis protects brain from ischemic injury with a tendency of age-dependence.* CNS Neurosci Ther, 2014. **20**(5): p. 452-9.

114. A.Griffin, B., *Lipid metabolism*, in *Surgery (Oxford)*, P. Lamb, Editor. June 2013, ELSEVIER. p. 267-272.

115. Feingold, K.R., *Introduction to Lipids and Lipoproteins*, in *Endotext*, K.R. Feingold, et al., Editors. 2000: South Dartmouth (MA).

116. Corvilain, B., *[Lipoprotein metabolism].* Rev Med Brux, 1997. **18**(1): p. 3-9.

117. Ekstedt, M., et al., *Long-term follow-up of patients with NAFLD and elevated liver enzymes.* Hepatology, 2006. **44**(4): p. 865-73.

118. Rui, L., *Energy metabolism in the liver.* Compr Physiol, 2014. **4**(1): p. 177-97.

119. Alves-Bezerra, M. and D.E. Cohen, *Triglyceride Metabolism in the Liver.* Compr Physiol, 2017. **8**(1): p. 1-8.

120. Lambert, J.E. and E.J. Parks, *Postprandial metabolism of meal triglyceride in humans.* Biochim Biophys Acta, 2012. **1821**(5): p. 721-6.

121. Berlanga, A., et al., *Molecular pathways in non-alcoholic fatty liver disease.* Clin Exp Gastroenterol, 2014. **7**: p. 221-39.

122. Nguyen, P., et al., *Liver lipid metabolism.* J Anim Physiol Anim Nutr (Berl), 2008. **92**(3): p. 272-83.

123. Hamilton, D.J., *Mechanisms of disease: is mitochondrial function altered in heart failure?* Methodist Debakey Cardiovasc J, 2013. **9**(1): p. 44-8.

124. Weiss, R.G., G. Gerstenblith, and P.A. Bottomley, *ATP flux through creatine kinase in the normal, stressed, and failing human heart.* Proc Natl Acad Sci U S A, 2005. **102**(3): p. 808-13.

125. Neubauer, S., *The failing heart--an engine out of fuel.* N Engl J Med, 2007. **356**(11): p. 1140-51.

126. Neely, J.R. and H.E. Morgan, *Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle.* Annu Rev Physiol, 1974. **36**: p. 413-59.

127. Lopaschuk, G.D., et al., *Myocardial fatty acid metabolism in health and disease.* Physiol Rev, 2010. **90**(1): p. 207-58.

128. An, D. and B. Rodrigues, *Role of changes in cardiac metabolism in development of diabetic cardiomyopathy.* Am J Physiol Heart Circ Physiol, 2006. **291**(4): p. H1489-506.

129. Yagyu, H., et al., *Lipoprotein lipase (LpL) on the surface of cardiomyocytes increases lipid uptake and produces a cardiomyopathy.* J Clin Invest, 2003. **111**(3): p. 419-26.

130. Morales, P.E., J.L. Bucarey, and A. Espinosa, *Muscle Lipid Metabolism: Role of Lipid Droplets and Perilipins.* J Diabetes Res, 2017. **2017**: p. 1789395.

131. Fritzen, A.M., A.M. Lundsgaard, and B. Kiens, *Tuning fatty acid oxidation in skeletal muscle with dietary fat and exercise.* Nat Rev Endocrinol, 2020. **16**(12): p. 683-696.

132. Dube, J.J., et al., *Effects of acute lipid overload on skeletal muscle insulin resistance, metabolic flexibility, and mitochondrial performance.* Am J Physiol Endocrinol Metab, 2014. **307**(12): p. E1117-24.

133. Ukropcova, B., et al., *Dynamic changes in fat oxidation in human primary myocytes mirror metabolic characteristics of the donor.* J Clin Invest, 2005. **115**(7): p. 1934-41.

134. McFarlan, J.T., et al., *In vivo, fatty acid translocase (CD36) critically regulates skeletal muscle fuel selection, exercise performance, and training-induced adaptation of fatty acid oxidation.* J Biol Chem, 2012. **287**(28): p. 23502-16.

135. Kazantzis, M. and A. Stahl, *Fatty acid transport proteins, implications in physiology and disease.* Biochim Biophys Acta, 2012. **1821**(5): p. 852-7.

136. Hotamisligil, G.S. and D.A. Bernlohr, *Metabolic functions of FABPs--mechanisms and therapeutic implications.* Nat Rev Endocrinol, 2015. **11**(10): p. 592-605.

137. Kiens, B., et al., *Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender.* J Appl Physiol (1985), 2004. **97**(4): p. 1209-18.

138. Tunstall, R.J., et al., *Exercise training increases lipid metabolism gene expression in human skeletal muscle.* Am J Physiol Endocrinol Metab, 2002. **283**(1): p. E66-72.

139. Talanian, J.L., et al., *Exercise training increases sarcolemmal and mitochondrial fatty acid transport proteins in human skeletal muscle.* Am J Physiol Endocrinol Metab, 2010. **299**(2): p. E180-8.

140. Bonen, A., et al., *Extremely rapid increase in fatty acid transport and intramyocellular lipid accumulation but markedly delayed insulin resistance after high fat feeding in rats.* Diabetologia, 2015. **58**(10): p. 2381-91.

141. Kanaley, J.A., et al., *Relationship between plasma free fatty acid, intramyocellular triglycerides and long-chain acylcarnitines in resting humans.* J Physiol, 2009. **587**(Pt 24): p. 5939-50.

142. Turnbull, P.C., et al., *Increases in skeletal muscle ATGL and its inhibitor G0S2 following 8 weeks of endurance training in metabolically different rat skeletal muscles.* Am J Physiol Regul Integr Comp Physiol, 2016. **310**(2): p. R125-33.

143. Pavlova, N.N. and C.B. Thompson, *The Emerging Hallmarks of Cancer Metabolism.* Cell Metab, 2016. **23**(1): p. 27-47.

144. Schiliro, C. and B.L. Firestein, *Mechanisms of Metabolic Reprogramming in Cancer Cells Supporting Enhanced Growth and Proliferation.* Cells, 2021. **10**(5).

145. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation.* Science, 2009. **324**(5930): p. 1029-33.

146. Liberti, M.V. and J.W. Locasale, *The Warburg Effect: How Does it Benefit Cancer Cells?* Trends Biochem Sci, 2016. **41**(3): p. 211-218.

147. Cavalli, L.R., M. VarellaGarcia, and B.C. Liang, *Diminished tumorigenic phenotype after depletion of mitochondrial DNA.* Cell Growth & Differentiation, 1997. **8**(11): p. 1189-1198.

148. Morais, R., et al., *Tumor-Forming Ability in Athymic Nude-Mice of Human Cell-Lines Devoid of Mitochondrial-DNA.* Cancer Research, 1994. **54**(14): p. 3889-3896.

149. Tan, A.S., et al., *Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA.* Cell Metab, 2015. **21**(1): p. 81-94.

150. Zong, W.X., J.D. Rabinowitz, and E. White, *Mitochondria and Cancer.* Mol Cell, 2016. **61**(5): p. 667-76.

151. American Diabetes, A., *Standards of medical care in diabetes--2014.* Diabetes Care, 2014. **37 Suppl 1**: p. S14-80.

152. Madiraju, A.K., et al., *Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase.* Nature, 2014. **510**(7506): p. 542-6.

153. Natali, A. and E. Ferrannini, *Effects of metformin and thiazolidinediones on suppression of hepatic glucose production and stimulation of glucose uptake in type 2 diabetes: a systematic review.* Diabetologia, 2006. **49**(3): p. 434-41.

154. Griss, T., et al., *Metformin Antagonizes Cancer Cell Proliferation by Suppressing Mitochondrial-Dependent Biosynthesis.* PLoS Biol, 2015. **13**(12): p. e1002309.

155. Ota, S., et al., *Metformin suppresses glucose-6-phosphatase expression by a complex I inhibition and AMPK activation-independent mechanism.* Biochem Biophys Res Commun, 2009. **388**(2): p. 311-6.

156. Owen, M.R., E. Doran, and A.P. Halestrap, *Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain.* Biochem J, 2000. **348 Pt 3**: p. 607-14.

157. Wheaton, W.W., et al., *Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis.* Elife, 2014. **3**: p. e02242.

158. El-Mir, M.Y., et al., *Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I.* J Biol Chem, 2000. **275**(1): p. 223-8.

159. Foretz, M., et al., *Metformin: from mechanisms of action to therapies.* Cell Metab, 2014. **20**(6): p. 953-66.

160. Gui, D.Y., et al., *Environment Dictates Dependence on Mitochondrial Complex I for NAD+ and Aspartate Production and Determines Cancer Cell Sensitivity to Metformin.* Cell Metab, 2016. **24**(5): p. 716-727.

161. Stephenne, X., et al., *Metformin activates AMP-activated protein kinase in primary human hepatocytes by decreasing cellular energy status.* Diabetologia, 2011. **54**(12): p. 3101-10.

162. Guigas, B., et al., *Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological in vitro study.* Biochem J, 2004. **382**(Pt 3): p. 877-84.

163. Hirsch, A., et al., *Metformin inhibits human androgen production by regulating steroidogenic enzymes HSD3B2 and CYP17A1 and complex I activity of the respiratory chain.* Endocrinology, 2012. **153**(9): p. 4354-66.

164. Kim, K.H., et al., *Metformin-induced inhibition of the mitochondrial respiratory chain increases FGF21 expression via ATF4 activation.* Biochem Biophys Res Commun, 2013. **440**(1): p. 76-81.

165. Detaille, D., et al., *Metformin prevents high-glucose-induced endothelial cell death through a mitochondrial permeability transition-dependent process.* Diabetes, 2005. **54**(7): p. 2179-87.

166. Hinke, S.A., et al., *Methyl succinate antagonises biguanide-induced AMPK-activation and death of pancreatic beta-cells through restoration of mitochondrial electron transfer.* Br J Pharmacol, 2007. **150**(8): p. 1031-43.

167. Piel, S., et al., *Metformin induces lactate production in peripheral blood mononuclear cells and platelets through specific mitochondrial complex I inhibition.* Acta Physiol (Oxf), 2015. **213**(1): p. 171-80.

168. Bridges, H.R., et al., *Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria.* Biochem J, 2014. **462**(3): p. 475-87.

169. Janzer, A., et al., *Metformin and phenformin deplete tricarboxylic acid cycle and glycolytic intermediates during cell transformation and NTPs in cancer stem cells.* Proc Natl Acad Sci U S A, 2014. **111**(29): p. 10574-9.

170. Scotland, S., et al., *Mitochondrial energetic and AKT status mediate metabolic effects and apoptosis of metformin in human leukemic cells.* Leukemia, 2013. **27**(11): p. 2129-38.

171. Kulyte, A., et al., *Global transcriptome profiling identifies KLF15 and SLC25A10 as modifiers of adipocytes insulin sensitivity in obese women.* PLoS One, 2017. **12**(6): p. e0178485.

172. Mizuarai, S., et al., *Identification of dicarboxylate carrier Slc25a10 as malate transporter in de novo fatty acid synthesis.* J Biol Chem, 2005. **280**(37): p. 32434-41.

173. Zhou, X., et al., *The mitochondrial carrier SLC25A10 regulates cancer cell growth.* Oncotarget, 2015. **6**(11): p. 9271-83.

174. Fontaine, E., *Metformin-Induced Mitochondrial Complex I Inhibition: Facts, Uncertainties, and Consequences.* Front Endocrinol (Lausanne), 2018. **9**: p. 753.