The role of mitochondrial metabolism in health and disease
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Chapter 1

Introduction
General Introduction

Mitochondria are intracellular organelles of eukaryotes, in which central metabolic processes are taking place. They are thought to have bacterial ancestry, most likely derived from an α-proteobacterium endosymbiont that was engulfed by an archaean host cell\textsuperscript{1,2}. Through time, much of the bacterial genome was eventually either lost or transferred to the nucleus, and now the modern mitochondria still contain remnants of this ancestral circular DNA with highly specialized and crucial functions. Their endosymbiotic origin also gives mitochondria their unique structure – two phospholipid bi-layers forming the sub-compartments of the mitochondrial matrix and the inter-membrane space. This complex architecture allows them to carry out a set of specialized biochemical reactions that fuel the cell with the required energy.

Two pathways that are crucial for energy homeostasis in mitochondria are the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation (OXPHOS) system. These are a series of chemical reactions that ultimately releases stored energy in the form of adenosine triphosphate (ATP). Additionally, its intermediate products can be further used in several biochemical pathways including for the biosynthesis of macromolecules. Most ATP is produced through the strictly aerobic OXPHOS system. This process, during which electrons are sequentially transferred from donors to acceptors, is carried out in all aerobic organisms, both prokaryotic and eukaryotic. In eukaryotes, it is carried out by five protein super-complexes, also known as OXPHOS complexes, that are embedded in the inner mitochondrial membrane (IMM) (Fig. 1). Four of these complexes comprise the electron transport chain (ETC) which creates an electrochemical gradient across the IMM necessary for the production of ATP from a fifth protein complex that functions as an ATP-synthase. The IMM forms extensive invaginations towards the mitochondrial matrix called cristae which increase the membrane surface. These membranes are enriched in the phospholipid cardiolipin (CL), which is present also in bacterial membranes. CL has a dimeric nature which is unique amongst other phospholipids and gives it a highly specific conical structure with particular physicochemical properties that facilitate membrane curvature and fusion\textsuperscript{3,4}. It is interacting with many mitochondrial proteins, including the OXPHOS complexes whereby it is also involved in their formation and stability serving as a “glue” in the IMM.

Apart from their pivotal role in cellular metabolism through bioenergetics and biosynthesis, mitochondria are involved in several other important biological functions including cellular signaling and apoptosis. Under physiological conditions, mitochondria communicate with the cell in cases of disturbed homeostasis, such as low oxygen levels through the release of reactive oxygen species (ROS), or
accumulation of proteins in the mitochondrial matrix through the activation of the mitochondrial unfolded protein response (UPR\textsuperscript{mt}). These mechanisms ultimately lead to adaptive gene expression and restored homeostasis. Under pathological conditions, mitochondria activate cellular pathways that lead either to mitophagy—the coordinated removal of dysfunctional mitochondria—or to cell death through the release of cytochrome c and the initiation of caspase activation\textsuperscript{5}.

The human mitochondrial genome has retained 13 genes encoding for subunits of the OXPHOS super-complexes (Fig. 1) and 24 genes coding for rRNAs and tRNAs, components of the mitochondrial translational machinery. Since the remaining ~80 subunits are encoded by the nuclear DNA (nDNA), a fine balance needs to be maintained in order to ensure proper mitochondrial function. Changes in mitochondrial metabolism, due to mutations in both parts of the genome that affect mitochondrial proteins, can have immense consequences at a cellular and organismal level, affecting different tissues, and with either beneficial (e.g. longer lifespan) or detrimental (development of disease) results.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{The oxidative phosphorylation (OXPHOS) complexes are embedded in the inner mitochondrial membrane (IMM) and consist of NADH dehydrogenase-CoQ oxidoreductase (Complex I), succinate dehydrogenase-CoQ oxidoreductase (Complex II), CoQ-cytochrome c oxidoreductase (Complex III), cytochrome c oxidase (Complex IV), and ATP synthase (Complex V). Complexes I, III, IV, and V are of mosaic nature, consisting of 13 core protein subunits that are mtDNA-encoded, and other subunits encoded in the nucleus. (Figure adapted from\textsuperscript{35})}
\end{figure}
Mitochondria in health and disease

Mitochondrial diseases can occur from mutations in either mitochondrial DNA (mtDNA) or nDNA, that often affect genes encoding OXPHOS subunits or components of the translational machinery. In the case of mtDNA mutations, the development and severity of the disease depends on the number of mtDNA molecules that carry the mutation, also termed heteroplasmy, and also the number of defective mitochondria that are maternally inherited as well as their distribution in the different tissues. Most commonly affected are high energy-demanding tissues, such as the brain and muscle, which require rapid ATP production through OXPHOS. For example, mutations in mitochondrial genes encoding core subunits of complex I cause LHON syndrome, which is characterized by optic neuropathy. Mutations on genes encoding specific subunits of complex I can also cause MELAS, which manifests with myopathy, encephalopathy, lactic acidosis and stroke-like episodes. Mutations in genes of the nDNA that encode OXPHOS subunits or regulatory factors of mtDNA maintenance and expression or mitochondrial biogenesis and homeostasis can also lead to the development of mitochondrial diseases. These diseases are inherited in a recessive or dominant manner, and they are usually rare. Amongst classical examples of mutations in nDNA leading to mitochondrial disease are mutations in genes encoding subunits of complex IV, that cause various symptoms, such as myopathy and encephalomyopathy.

Mitochondrial diseases present a broad spectrum of severity and tissue specificity. They may be relatively “mild”, or severe and even lethal. The nature of the molecular defect caused by the mutation can sometimes explain the degree of severity of the clinical symptoms, but not the heterogeneity in affected tissues, which may be affected by modifier genes. Tissues that are metabolically active are often affected, causing neurological, cardiac, skeletal muscle, gastrointestinal and liver manifestations, but also less expected systems such as the endocrine can be affected. This clinical heterogeneity is also reflected at the molecular level. Mutations in different genes can cause the same disease phenotype. On the other hand, mutations in a certain gene or group of genes with similar function can result in different clinical manifestations. For example, mutations in genes encoding mitochondrial tRNA synthetases can cause a variety of symptoms, ranging from cardiomyopathy and cerebral white matter abnormalities to hearing loss and ovarian failure.

Of particular focus in this thesis are two rare mitochondrial diseases that present both typical and atypical symptoms of mitochondrial dysfunction. In Perrault syndrome, the patients present with a relatively “mild” phenotype, that includes ovarian dysgenesis, a not so common phenotype of mitochondrial dysfunction, and hearing
loss, which is often encountered in mitochondrial diseases\textsuperscript{13}. Some of these patients also develop neurological symptoms, a feature commonly observed in OXPHOS deficiencies. Five different nuclear genes were previously found mutated in different cases of Perrault syndrome, including the genes $HSD17B4$, $HARS2$, $LARS2$, $CLPP$\textsuperscript{16} and $Twinkle$\textsuperscript{17}. $HSD17B4$ encodes a peroxisomal enzyme, $HARS2$ and $LARS2$ encode mitochondrial tRNA synthetases, $CLPP$ a protease of the mitochondrial matrix, and $Twinkle$ a mitochondrial helicase. The detailed mechanism through which these mutations cause mitochondrial dysfunction and affect fertility and hearing in the patients still remains unclear.

On the other hand, Barth syndrome (BTHS) patients develop a very severe phenotype early in life, which mainly involves skeletal and cardiac myopathy, the latter leading often to death\textsuperscript{18,19}. The muscle is a tissue with high energy demand and is often affected in patients with mitochondrial defects. BTHS patients do not present with brain-related signs and symptoms like many mitochondrial diseases, but they present some atypical symptoms that are not directly linked to mitochondria, such as neutropenia\textsuperscript{18-20}. BTHS is caused by mutations in the tafazzin gene, which encodes an acyl transferase involved in the remodeling of the signature phospholipid of mitochondria, cardiolipin (CL)\textsuperscript{3,21}. As a result, patients have less of the “mature” CL and accumulate a CL intermediate, monolysocardiolipin (MLCL)\textsuperscript{22}. At a cellular level, they present with abnormal mitochondria with fragmented cristae and disturbed OXPHOS complexes that are misassembled or malfunctioning. Similarly to Perrault syndrome, the exact pathophysiology of the disease has not been resolved.

**The conserved role of mitochondrial metabolism in aging**

Altered mitochondrial function due to mutations or nutritional and pharmacological interventions can change cellular and whole-organism physiology either towards the development of disease or towards an improved function. Apart from rare inherited mitochondrial diseases, there is a series of multi-factorial diseases in which mitochondrial metabolism plays a pivotal role, such as cancer, diabetes, and cardiovascular diseases. All these are tightly connected to the increased life expectancy in developed societies, since the risk of incidence increases with age, as well as to modern life-style which includes lack of exercise and high caloric intake. The role of mitochondria in aging and age-related disease has therefore become of central interest in recent years.

In the past decades, it has become evident that aging is not a passive process as was previously thought, but it is dictated by molecular pathways that are amenable to
intervention, whereby activation or blockage can lead to either shorter or extended lifespan. Some of these are central metabolic pathways, such as the nutrient sensing pathways of insulin/IGF-1, AMPK/FOXO and mTOR signaling. Specifically, in fruit flies and worms, a decrease of the insulin/IGF-1 signaling by introducing mutations in components of the pathway greatly extends lifespan\(^{23}\), while in mammals the outcome varies depending on the severity of the blockage\(^{24}\). Moreover, genetic and pharmacological interventions that block the mTOR pathway are extending lifespan in flies, worms and yeast\(^{25}\). The most consistently proven intervention that extends lifespan in a conserved cross-species fashion is caloric restriction (CR)\(^{26}\), and although the mechanism underlying CR-dependent lifespan extension is not fully elucidated, nutrient sensing pathways such as those mentioned above are at least partially involved\(^{27}\).

Mitochondrial dysfunction and activation of stress response pathways are consequences of aging but have also been proven to control aging. For example, aged mice present with lower expression levels of genes involved in mitochondrial processes such as oxidative phosphorylation and fatty acid oxidation and in mitochondrial biogenesis and stress response. This has an impact on their metabolic status that is overall changed towards a more obese phenotype compared to young mice\(^{28}\). Also, many common age-related diseases present with a mitochondrial phenotype, such as reduced oxidative capacity in muscles of type 2 diabetes patients, and disturbed mitochondrial proteostasis in Alzheimer’s disease\(^{7}\). On the other hand, interventions to improve mitochondrial function can directly affect longevity; in worms, mutations in OXPHOS genes or genes encoding elements of the mitochondrial ribosome extend lifespan through the activation of stress defense systems\(^{29}\).

The study of longevity networks can be done in mammalian systems but often involves other model organisms including the fruit fly, the nematode and budding yeast, since the basic structure of the molecular pathways is mostly conserved across species. The nematode \textit{Caenorhabditis elegans} has been of particular use in the discovery and understanding of molecular mechanisms underlying aging, especially because of its relatively short lifespan (approx. 20-30 days), its fully sequenced genome and the ease of genetic manipulation through RNAi-feeding\(^{30}\). Pathways involved in nutrient sensing, such as the insulin/IGF-1, sirtuins, AMPK/FOXO, and mTOR signaling, and those involved in mitochondrial dysfunction and stress responses have been studied in depth in \textit{C.elegans}\(^{30}\). It still remains unclear however how nutrition, mitochondrial function and aging are interconnected. To gain more insight to these processes it is vital to measure metabolic changes. The use of metabolomics in \textit{C. elegans} is hence a powerful tool to study aging. To address this aspect, some efforts
have been made in recent years through the application of gas chromatography-mass spectrometry (GC-MS)\textsuperscript{31,32} and nuclear magnetic resonance (NMR) spectroscopy\textsuperscript{33} to measure metabolites in worms. Application of such methods in a larger scale will eventually facilitate the clarification of the causes and effects of metabolic changes in aging.

**Introduction to thesis chapters**

The goal of the current PhD thesis was to elucidate how changes in mitochondrial function can lead to specific rare metabolic diseases, and introduce the nematode *C. elegans* as a plausible experimental model to unravel metabolic mechanisms related to metabolic disease and also genetic, pharmacological and diet interventions.

In **Chapter 2**, the effect of the antibiotic group of tetracyclines on mitochondrial function and the implications of their experimental use in inducible expression systems, especially in cancer research, is reviewed. Tetracyclines are widely used in the clinic for treatment of bacterial infections but these antibiotics are also a common tool in research to switch on and off gene expression in the so-called Tet-ON/Tet-OFF systems\textsuperscript{34}. What is largely overseen however is that these antibiotics target not only bacteria, but also mitochondria which are of bacterial origin. The chapter summarizes the findings of previous studies that show how tetracyclines affect mitochondrial function, gene expression and full-body physiology in organisms of different complexity.

In **Chapter 3**, a new mutation is described as the genetic cause of the rare inherited disorder Perrault syndrome in three unrelated women. Main symptoms of the disease include infertility and deafness, and most genes found mutated in previous cases are related to mitochondria, particularly to mitochondrial protein translation and homeostasis. This chapter describes the identification of a novel mutation in a gene that encodes the mitochondrial rRNA chaperone ERAL1 as the cause of Perrault syndrome via a combination of exome sequencing, bioinformatics and functional assays in patient material and in a worm model for the disease. Cells from these patients presented with decreased mitochondrial ribosomal assembly, and low respiration indicating mitochondrial dysfunction. Strikingly, defective mitochondrial respiration was also observed in ERAL1-deficient *C. elegans* which displayed impaired fertility, a typical symptom of Perrault syndrome.

**Chapter 4** is focused on the discovery of affected pathways that are either deregulated or activated in the rare mitochondrial disease Barth syndrome. This
inherited disorder is mainly characterized by cardiac and skeletal myopathy, neutropenia and abnormal mitochondria. It is caused by mutations in a gene controlling the remodeling of the mostly mitochondrial phospholipid cardiolipin and as a result patients present with a very specific biochemical profile that is used for early diagnosis. Although the genetic cause and biochemical defects of the disease have been identified, the pathophysiology has not been yet resolved. This chapter shows extensive proteome changes in cells from BTHS patients. Many mitochondrial protein complexes involved in metabolic processes are found disturbed, while others are increased in abundance, indicating possible compensatory responses to mitochondrial dysfunction. Alteration of key metabolic complexes is partly but not fully reflected in metabolic flux of patient fibroblasts.

In Chapter 5, the development of a new method for the accurate measurement of metabolites in the nematode *C. elegans* is described. Many metabolic pathways that affect aging have been studied in depth using this relatively short-lived model organism mainly through genetic manipulations, and a full metabolomic characterization in different conditions would shed more light into the genome x diet x aging interactions. This chapter describes the development and validation of a mass-spectrometry based method of measuring metabolites in worms, and the application of the method to detect metabolic changes in different life stages, genotypes and diets. The method allowed detection of three major metabolite classes, i.e. fatty acids, amino acids and phospholipids, and metabolic profiling of the different conditions revealed important changes in the metabolite classes.

Chapter 6 explores the potential of a *C. elegans* model for Barth syndrome through RNAi-mediated knockdown of the *C. elegans* homologue of *tafazzin*, which is *acl-3*. A multicellular organism such as *C. elegans* that is easy to culture and has well-defined tissues can be utilized as a means of genetic and drug screening for a rare mitochondrial disease. This chapter shows that the lipi dome of the *acl-3* knockdown worms shows the same biochemical defect as in patients, i.e. accumulation of MLCL species and reduction of ‘mature’ CL. RNA-seq analysis of these worms revealed widespread changes in gene expression, mostly in genes involved in metabolism.
## References


