Clinical pharmacological aspects of mitochondrial function in muscle

Diemen, M.P.J. van

CHAPTER I

INTRODUCTION

THE POWERHOUSES OF THE CELL

Mitochondria, the nifty little cell organelles also known as the powerhouses of the cell (Figure 1.1), have been gaining attention and prove to be much more than mere providers of over 90% of the cell's energy needs. The importance is clear, without mitochondria, we would die within 3 minutes. Mitochondria are widely believed to be of bacterial origin and incorporated into the eukaryotic cell through symbiosis around 1.5 billion years ago.[7] This led to a switch from glycolysis to oxidative phosphorylation, which is a far more efficient way to create adenosine triphosphate (ATP), and enabled the cell to perform more energy demanding tasks by oxidizing the major products of glucose: pyruvate, and nicotinamide-adenine-dinucleotide (NADH). Oxidative phosphorylation (OXPHOS) relies on the electron transport chain (ETC), a series of protein complexes (I, II, III and IV) that transfer electrons from complex to complex via redox reactions, with the sole purpose of transporting protons (H+) across the inner membrane of the mitochondria into the intermembrane space (Figure 1.2). The then created proton gradient pushes the protons back to the matrix, which drives the synthesis of ATP through the use of the ATP synthase channel (complex V). Mitochondria have their own, circular DNA, containing 37 genes: 13 for protein subunits of the complexes, 22 for mitochondrial TRNA (for the 20 standard amino acids, plus an extra gene for leucine and serine), and 2 for RRNA.[16] These genes are maternally inherited: paternal genes enter the egg via the sperm's mitochondria, but are marked with ubiquitin to select them for later destruction inside the embryo.[17] Mitochondria divide by binary fission, similar to bacterial cell division.

MITOCHONDRIAL (DYS)FUNCTION

So far, the clinical importance of mitochondria seems rather minimal. However, we now know that mitochondria are more involved in the cell's signaling pathways and survival than previously thought. Once thought to be mere powerhouses of the cell, mitochondria have been shown to play a role in many central cellular metabolic tasks, see Box 1.[18]

'An important source of damage to mitochondria is reactive oxygen species.'

Box 1: Several cellular functions of mitochondria

1. Signaling through mitochondrial reactive oxygen species
2. Regulation of the membrane potential
3. Apoptosis-programmed cell death
4. Calcium signaling (including calcium-evoked apoptosis)
5. Regulation of cellular metabolism
6. Certain heme synthesis reactions and
7. Steroid synthesis

It is therefore important to keep mitochondria in shape. This is achieved by the principle of 'use it or lose it' (when the energy needs of a cell are high, mitochondria grow and divide and when the energy use is low, mitochondria become inactive) and by repairing damaged mitochondria.[19,20] An important source of damage to mitochondria are reactive oxygen species (ROS), mostly across created through the oxidative metabolism inside the ETC.[21,22] As electrons pass through the ETC, a small fraction escape and prematurely react with molecular oxygen resulting in the production of superoxide. Damage to the mitochondrion results in a decreased efficiency of OXPHOS, thereby producing even more ROS and less ATP, which is termed mitochondrial dysfunction. The mitochondrial Theory of Aging hypothesizes that during an individual's life, oxidative stress damages DNA and thus impairs the ability to produce essential proteins over time.[23] mtDNA is especially vulnerable to oxidative stress, because it is located directly at the site of oxidative metabolism and is not protected by histones, which is the case in nuclear DNA.

'The result of mitophagy is that dysfunctional mitochondria are removed, keeping the mitochondria and their turnover healthy.'

Fortunately, ROS can be neutralized by so-called free-radical scavengers, including members of the superoxide dismutase (SOD) family, catalase, and glutathione peroxidase. However, when the ROS production exceeds the antioxidant capacity, oxidative damage is inflicted on mitochondrial components, causing mitochondrial dysfunction.[24] At this stage, dysfunctional mitochondria can be removed from the cell through mitophagy, a type of autophagy (see Figure 1.3). The term mitophagy was proposed by Lemasters *et al.* in 2005 to emphasize the selectiveness of this type of autophagy for dysfunctional mitochondria, previously assumed to be a

random process.[25] How mitophagy is initiated is not yet fully understood, but it is clear that signals from mitochondria that are beyond repair, trigger the outer membrane to be flagged for destruction. The result of mitophagy is that dysfunctional mitochondria are removed, keeping the mitochondria and their turnover healthy.[20]

MEASURING MITOCHONDRIAL FUNCTION

In order to determine mitochondrial (dys)function in a person, one must be able to measure it. The first quantitative method to determine mitochondrial function of isolated mitochondria was performed by Chance and Williams in 1955 by crudely measuring the oxygen disappearance rate (cellular respiration) *in vitro* as a measure of ATP production.[26] Nowadays, non-invasive *in vivo* techniques, such as phosphorous Magnetic Resonance Spectroscopy (31P-MRS, see Figure 1.4), are the new standard in measuring mitochondrial function. 31P-MRS estimates the ATP production rate in the calf muscles by measuring the phosphocreatine recovery rate after an in-scanner bout of exercise.[27] Other *in vivo* techniques include Near Infrared Spectroscopy (NIRS) and the recently developed protoporphyrin 9 triple state lifetime technique (PPIX-TSLT, see Figure 1.5). Both techniques measure the oxygen disappearance rate and with PPIX-TSLT this is measured inside the mitochondria. A big advantage of *in vivo* measurements is that the cellular environment of the mitochondria is left undamaged. Doing so, external factors that might influence mitochondrial function are considered, which makes this type of measurements more suitable for clinical studies.

'The importance of good mitochondrial function becomes clear when looking at what could go wrong if the powerhouses start to malfunction.'

Ex vivo measurements require tissue samples to be taken and are thus invasive by nature, but can additionally determine the function of each of the separate ETC complexes. This is especially useful when it is necessary to know where exactly within the ETC the dysfunction occurs. The most popular tissue for *ex vivo* measurements is muscle, which can be harvested with a relatively low burden for the patient by taking a biopsy. An array of parameters, including ETC complex function and abundance and gene expression, can then be measured in the laboratory using techniques such as western blot and Enzyme-Linked Immuno Sorbent Assay (ELISA). Another *ex vivo* method, that only requires a blood sample, is measuring the integrity of the mitochondrial membrane potential ($\Delta\Psi m$) in peripheral blood mononuclear cells (PBMCS). The $\Delta\Psi m$ reflects the general health of mitochondria and a special dye, attracted to the proton gradient within the inner-membrane space, is used in the measurement. Due to the location of the circulating PBMCS, this measurement gives an estimation of the systemic mitochondrial function. Lastly, the respirometry of cells in suspension has been extensively used, both *in vitro* as *ex vivo*. By adding substrates and inhibitors of the OXPHOS chain in the right order, the usage of oxygen and thus the activity of the individual complexes can be measured.[28] Popular commercial devices are the Oroboros (high-resolution method) and the SeaHorse (96-wells high-throughput method).[29-30]

CLINICAL IMPORTANCE: MITOCHONDRIAL DISEASES

The importance of good mitochondrial function becomes clear when looking at what could go wrong if the powerhouses start to malfunction. The first proof that a dysfunction of mitochondria could lead to clinical disease was only discovered in 1988 by Doug Wallace, as he described the pathophysiological role of dysfunctional mitochondria in Leber's hereditary optic neuropathy (LHON).[31] Wallace found that due to certain defects in mitochondrial DNA (mtDNA), mitochondria become dysfunctional, resulting in a disruption of ATP production and an increase in oxidative stress, to which retinal ganglion cells are highly sensitive. The cell-specific apoptosis that follows causes bilateral loss of central vision, leaving the patient partially or fully blind. Since the discovery, other diseases have been attributed to dysfunctional mitochondria, including Kearns-Sayre syndrome, Leigh syndrome, MELAS (Mitochondrial myopathy, Encephalomyopathy, Lactic Acidosis, Stroke-like symptoms) and MERRF (Myoclonic Epilepsy with Ragged Red Fibers').[16]

'Diagnosis of mitochondrial diseases is a challenging, costly and often an invasive process.'

Patients typically start showing symptoms from a young age and have an unfavorable prognosis. Mitochondrial diseases typically occur in tissues with a high energy demand, such as the brain or muscle and currently lack treatment. Diagnosis of

mitochondrial diseases is a challenging, costly and often an invasive process, that starts with a clinical suspicion and is confirmed by showing mitochondrial defects. A test panel was proposed by consensus, consisting of biochemical testing of blood, urine and spinal fluid, genetic testing and pathology and biochemical testing of tissue (typically a muscle biopsy).[32] Biochemical testing mainly focusses on lactate and pyruvate; due to a disturbed mitochondrial function, glycolysis will increase, thereby producing a higher level of lactate. Genetic testing, preferably in muscle tissue but also possible in blood, urine and buccal mucosa, identifies mutations in mtDNA and nDNA, that are known to correlate with mitochondrial disease. Histology of muscle tissue used to be the gold standard, but less needed nowadays with the availability of more sensitive molecular testing. However, the possibility of selectively measuring genetic and biochemical mitochondrial parameters in different tissues (i.e., muscle in exercise intolerance, heart in cardiomyopathy, and liver in liver disease) makes the use of invasive biopsy of tissues still relevant in this heterogenic group of diseases.

MITOCHONDRIAL DYSFUNCTION IN AGE-RELATED DISEASES

Although important for our understanding of mitochondrial function, mitochondrial diseases are luckily rare and the impact on society as a whole is therefore small. However, mitochondrial dysfunction has also been observed in many common, age-related diseases such as neurodegenerative disorders, type 2 diabetes mellitus and – affecting the musculoskeletal system – cardiovascular disease and sarcopenia (reviewed by Lane *et al.*).[33] The number of people over the age of 85 is expected to triple in America by 2050, drastically increasing the incidence and impact of such diseases (report by United Nations Department of Economic and Social Affairs, published in 2015). A point of discussion is whether the observed mitochondrial dysfunction is caused by age-related disease (i.e. exposure of mitochondria to chronic inflammation) or that the mitochondrial dysfunction itself drives the pathogenesis of age-related disease.

'The brain is most sensitive to a small decrease in energy supply.'

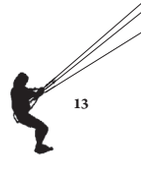
According to some, general bioenergetic decline is responsible for most age-related diseases, with mitochondria being the main actors.[34] Different tissues demand a different extent of energy and this explains tissue-specific symptoms. The brain is most sensitive to a small decrease in energy supply. Other high-energy demand tissues include the heart, muscle, kidney, and endocrine system, the organs commonly affected in metabolic and degenerative diseases. Individual genetic variations in mtDNA and nDNA, combined with environmental factors (changing energy resources, energy demands and toxins) cause mitochondrial dysfunction, which in turn raises oxidative stress, leading to a progressive bioenergetic decline. This decline may then lead to apoptosis of the high-energy demanding cells, resulting in disease.

Sarcopenia: illness through a sedentary lifestyle

One such an age-related disease is sarcopenia. Sarcopenia is derived from the Greek terms for 'flesh' (σάρξ; sarx) and 'poverty' (πενία; penia) and received its ICD-10 term only recently, giving it the official status of a disease. By age 85, approximately 20% of people meet criteria for sarcopenia (meaningful loss of muscle mass and strength).[35] Characterized both by loss of lean muscle mass and reduced skeletal muscle function, sarcopenia is a major contributor to loss of independence and frailty in the elderly.[36]

'Prevention of sarcopenia is currently managed by keeping elderly physically active.'

Studies in humans indicate that by the age of 70, there is a 25-30% reduction in the cross sectional area (CSA) of skeletal muscle and a decline in muscle strength by 30-40%.[37] Sarcopenia is a proven risk factor for falling in elderly, resulting often in hip fractures.[38] Recovery after hip fracture is notably difficult and 20% of people die within one year after the trauma. Prevention of sarcopenia is currently managed by keeping elderly physically active.[39] Although an increased level of ROS can be measured in muscle samples from elderly, mitochondrial function remains relatively preserved if these elderlies are physical active.[40] It has been suggested that the reported age-related reduction in ETC function (reduced mitochondrial complex I, II, III, and IV activity) is not related to the aging process per se, but rather due to other confounding factors, including physical inactivity.[41] Physical activity also protects mitochondria from oxidative stress. In a comparison study, active elderly had levels of oxidative stress similar to young subjects, whereas the anti-oxidant system in sedentary elderly was not intact.[42]



Mitophagy

An important mechanism through which mitochondrial dysfunction could occur is the failure of mitophagy. Removing dysfunctional mitochondria keeps the production of ROS in check, thereby preventing apoptosis to be triggered. There are multiple indications that mitophagy is reduced in aged muscle cells and happens through a decreased activity of mitofusin 2, a receptor for PINK- and PARKIN-targeted mitophagy.[43-45] Furthermore, exercise in sarcopenic mice leads to an increase in mitophagy and mitochondrial function.[46] Sarcopenia is just one example of age-related diseases in which mitochondrial dysfunction plays a role, but the mechanisms causing mitochondrial dysfunction greatly overlap.

MITOCHONDRIAL DYSFUNCTION AS A TARGET FOR PHARMACOLOGICAL TREATMENT

Since the discovery of the wide involvement of mitochondria in disease and the improved understanding of mitochondrial function in general, dysfunctional mitochondria have become a popular drug target for a wide variety of diseases. An increase in physical activity in aged mice and patients with mitochondrial disease strikingly showed an improvement in both mitochondrial biogenesis and OXPHOS capacity in muscle and other tissues, such as the brain.[47-52] Unfortunately, changing people's life style is known to be very challenging and, based on current child obesity statistics, will likely remain so in the foreseeable future. Multiple approaches to improve mitochondrial function have been studied, but only a handful have made it to clinical trials. Promising drugs have focused on increasing the anti-oxidant capacity, restoring OXPHOS capacity, improving mitochondrial biogenesis and inducing mitophagy.[53-56] Of the mitochondria-targeted anti-oxidative therapies, MitoQ and the SS-peptides have shown promising results in various animal models and have been taken furthest into human development. MitoQ is a mitochondria-targeted anti-oxidant and acts by protecting the inner membrane from lipid peroxidation.[57] So far, it has been studied in two phase 2 trials, with mostly negative results. In a study in patients with Parkinson's Disease it did not affect disease progression, but in a trial in patients with hepatitis C virus it did lead to a significant decrease in liver function enzymes.[55,58] SS-31, the lead compound of the SS-peptides (named after the creators Hazel Szeto and Peter W. Schiller and also known as elamipretide and Bendavia), prevents the peroxidation of cardiolipin, which organizes the complexes of the ETC, see Box 2.[53] However, there are no data on long-term benefits of elamipretide therapy (this will be discussed in more detail in Chapter 5 of this thesis).

Box 2: Efficacy of Bendavia (SS-31) in various animal models

1. Ischemia-reperfusion injury[3]
2. Parkinson's Disease[4]
3. Amyotrophic lateral sclerosis[5]
4. Alzheimer's Disease[8]
5. Muscle atrophy following hindlimb immobilization[9]
6. Insulin resistance as part of the metabolic syndrome[11]
7. Heart failure[12]

… and human clinical trials

8. Primary mitochondrial myopathy[13] *(increased distance in the 6-minute walk test)*
9. Heart failure[14] *(increased left ventricular ejection fraction)*
10. Renal artery stenosis[15] *(reduced ischemia-reperfusion damage after stent revascularization)*

QUESTION BASED DRUG DEVELOPMENT

The goal of drug development is to bring novel compounds to the market as an approved medication. This goal is far from easy to achieve and, on average, out of approximately every 5000 compounds, only one will eventually be approved by regulatory authorities.[59] After a compound is discovered to be potentially beneficial based on chemical properties, it is tested in pre-clinical studies. These studies are conducted in cell cultures and animals, with the main goal to assess the intended pharmacological efficacy of the compound on the one hand and its toxicity on the other. When screening for toxicity does not raise concerns, the compound proceeds to testing in humans: the clinical phase. Once the clinical phase has been reached, only 13.8% of drugs is estimated to get approved.[60] The main reasons for drugs with promising pre-clinical results to fail in the clinical phase is a lack of efficacy.[61,62] This also counts for mitochondria-targeted drugs.[63-66]

> *'Wasting resources by conducting trials in patients with ineffective therapies results in higher drug prices and bars patients from enrolling in other, more promising trials.'*

An apparent lack of efficacy can have different causes, with the main culprits being inadequate dose selection, absence of the intended pharmacology in humans and the inability of the compound to reach the site of action.[62] Question-based drug development is a step-wise method, focused on answering relevant questions to evaluate the value of a promising compound (see Box 3).[67] Selecting a dose to use in the clinical phase based on the results from the pre-clinical phase is always a risk: too high a dose may cause toxicity while too low a dose will not lead to measurable clinical effects. Often the investigated doses are derived from the no observed adverse effect level (NOAEL) in pre-clinical studies with an arbitrary safety factor to determine the maximum recommended starting dose (MRSD).[68] When basing the MRSD on the NOAEL, biological effects are not taken into account, resulting in uncertainty regarding efficacy in the early clinical phase.[69] Not having the intended pharmacology is another cause for drugs to fail in the clinical phase. The intended pharmacology is met when the compound binds to the right receptor on a cell, producing the desired effect. Hence, if the compound cannot bind to the intended receptor, there is no pharmacological effect. In pre-clinical testing it is therefore important to use (animal) models that express the right target receptor, enzyme or antigen, as witnessed with compound TGN1412, that due to a lack of the CD28 receptor in cynomolgus monkeys led to devastating toxicity in human volunteers during the phase 1 trial.[70] Before a compound can cause an effect, it must be able to reach the site of action. If the expected pharmacodynamic effect follows after drug administration, it is clear that the drug reached the site of action. But if no effect follows or one wants to know how much of the compound reaches the site in what time, the concentration must be measured at the level of the target tissue. The most direct way to do this is to isolate mitochondria and assay the drug in the lysate. Although it is possible to measure drug concentration directly inside tissue, e.g. in solid tumors and the lung, this is sometimes not possible without harming the patient.[71]

Box 3: Question-based drug development

1. Does the drug reach the site of action?
2. Is the on-target pharmacological effect present?
3. Are there off-target pharmacological effects?
4. Are there on-target pathophysiological effects?
5. What is the therapeutic window?
6. Are there off-target pathophysiological effects?

'A PoP model combines the benefits of lower variability of the pharmacodynamic outcome measure than present in patient studies with the greater ease and feasibility of performing non-therapeutic drug studies in healthy volunteers.'

A good example are drugs that target the central nervous system (CNS). To determine the penetration of a drug into the CNS, its concentration can be measured in the cerebrospinal fluid (CSF) as a proxy for the concentration in the brain. In the absence of drug in the CSF and effects on the CNS, the likely explanation is that the drug did not reach the site of action. When the drug can be measured in the CSF, but has no effects, this likely means that the compound does not work. With drugs that target mitochondria within cells of the CNS, this is further complicated. A measurable concentration in the CSF with negative effect on the CNS could mean that the compound has not been able to reach the mitochondria, or, alternatively, that the compound doesn't work. Summarized, the transition from the pre-clinical into the clinical phase has most chance of success when the right dose is chosen and when as much as possible is known about the efficacy of the compound before performing the phase 1 study. This is best done by basing the MRSD on the minimum anticipated biological effect level (MABEL).[72] Once in the clinical phase, the efficacy needs to be re-assessed in humans, because the efficacy in animal models can differ greatly from the efficacy in humans.[73] The question about efficacy is best answered early in the development process in order to make the go or no-go decision. Wasting resources by conducting trials in patients with ineffective therapies results in higher drug prices and bars patients from enrolling in other, more promising trials.[74]

PROOF-OF-PHARMACOLOGY MODELS IN HEALTHY HUMAN SUBJECTS

The full potential of question-based drug development can be achieved with intelligent study designs, one of which is the proof-of-pharmacology (POP) model. POP models use a challenge, pharmacological or non-pharmacological, in healthy subjects to mimic the pathophysiologically relevant mechanism.[67] Traditionally, phase 1 trials focus on safety and pharmacokinetics in healthy volunteers. During phase 2 trials, a small group of patients is enrolled in a randomized placebo-controlled study and efficacy are assessed using clinically relevant outcome measures. Assessing efficacy in patients is challenging, because variability in both

pharmacokinetics and pharmacodynamics are infamously high in patients, due to influences from co-morbidities and the disease itself.[67] Doing this in healthy volunteers is easier and safer. The limit in healthy volunteers, however, is that healthy, physiological function most often cannot be pharmacologically enhanced in healthy volunteers. When focusing on mitochondrial function targeted compounds, healthy volunteers typically have a normal, optimal, mitochondrial function, which cannot be expected to improve due to pharmacological intervention. A (pharmacological) challenge model in healthy volunteers can overcome this limit. A POP model combines the benefits of lower variability of the pharmacodynamic outcome measure than present in patient-studies with the greater ease and feasibility of performing non-therapeutic drug studies in healthy volunteers. In the case of drugs that are hypothesized to enhance mitochondrial function, the goal is to try to demonstrate a sometimes only small treatment effect in an often very heterogeneous population. If the level of mitochondrial dysfunction in the target population is highly variable, then demonstrating small improvements in a limited number of patients will not lead to statistically significant effects. Reducing the variability of the outcome measure in a population may allow for smaller – but still very clinically relevant – improvements to be demonstrated. Differences in mitochondrial function may therefore be difficult to detect in patients. Using a pharmacological challenge model in healthy volunteers could therefore be useful in early phase drug development. Additionally, in a POP model, the challenge effects must be reversible, because otherwise it will not be known whether a novel compound could be expected to reverse the challenge induced effects.

DRUG-INDUCED MITOCHONDRIAL DYSFUNCTION

Similar to the finding that dysfunctional mitochondria play a role in age-related diseases, it was observed that many commonly used drugs can cause a certain degree of mitochondrial dysfunction and the list is expanding. In fact, there are several cases of withdrawn medications that passed classic toxicology testing, but were found to be mitotoxic long after they were withdrawn from the market; blockbusters troglitazone and cerivastatin being the most famous ones.[75,76] There are multiple pathways, through which medications can be mitotoxic, see Box 4.[77] Pharmaceutical companies have started to actively screen newly discovered for mitochondrial dysfunction as part of the toxicology tests. At Pfizer for instance, focus on pre-clinical mitotoxicity has now been firmly established. However, *in vivo* monitoring of mitochondrial function will stay the most important assessment, which will need be performed during clinical trials.

Box 4: Examples of mitotoxic pathways by prescription medications

• **Statins**	inhibiting the biogenesis of co-enzyme Q10 and directly inhibiting complex III of the ETC[1]
• **Non-Steroidal Anti-Inflammatory Drugs (indomethacin and diclofenac)**	uncoupling of the proton gradient from ATP production to render oxidative phosphorylation inefficient[2]
• **Nucleoside reverse transcriptase inhibitors (antiretroviral medications)**	depletion of mtDNA, hampering transcription of essential mitochondrial components[6]
• **Metformin (anti-diabetic medication)**	inhibition of complex I of the ETC, inhibiting mitochondrial respiration[10]

AIM OF THIS THESIS

The aim of this thesis was to evaluate several clinical pharmacological perspectives of mitochondrial function within the musculoskeletal system.

Can physical activity influence mitochondrial function? Elderly with a sedentary lifestyle (i.e. pre-frail elderly) were compared to physically active elderly to evaluate the influence of mitochondrial function in the etiology of sarcopenia (**Chapter 2**). Further exploration on the association between musculoskeletal system and mitochondrial function were done in a population of total knee arthroplasty (TKA) patients (**Chapter 3**). Of this TKA population about 20% of patients are not satisfied with the postoperative outcome and even have less mobility than preoperative.[78-84] The latter might be related to muscle (i.e. mitochondrial) dysfunction. Currently it is known that sarcopenia and mitochondrial dysfunction occur in up to 44% of patients with osteoarthritis of the knee joint (Safdar *et al.*).[42]

Can we induce mitochondrial dysfunction in healthy volunteers in order to test mitochondrial enhancing drugs? In **Chapter 4**, the first human proof-of-pharmacology model for mitochondrial dysfunction was evaluated. A sub-clinical degree of

mitochondrial dysfunction was induced by simvastatin in a cohort of healthy volunteers, which was then reversed with simultaneous suppletion of ubiquinol (the reduced form of co-enzyme Q10).

Can we influence mitochondrial function in Huntington's Disease? Neuronal and muscular damage in Huntington's Disease (HD) is driven by mitochondrial dysfunction, due to an accumulation of misfolded huntingtin protein within cells.[85,86] In **Chapter 5,** a clinical trial was performed to evaluate the pharmacology of compound SBT-020, a novel mitochondria-targeted anti-oxidant, in a cohort with 24 HD patients. In **Chapter 6** we look at different associations between motor functioning, neurocognitive functioning and central/peripheral mitochondrial function, measured in the same group of patients.

How to clinically measure mitotoxicity of commonly described drugs? In **Chapter 7**, we discuss the importance of measuring oxygen consumption as a monitor of mitochondrial function *in vivo* to detect mitotoxicity (see Figures 1.1 to 1.5).

REFERENCES

1 Larsen S, Stride N, Hey-Mogensen M, et al. Simvastatin effects on skeletal muscle: relation to decreased mitochondrial function and glucose intolerance. Journal of the American College of Cardiology. 2013;61(1):44-53.

2 Moreno-Sanchez R, Bravo C, Vasquez C, Ayala G, Silveira LH, Martinez-Lavin M. Inhibition and uncoupling of oxidative phosphorylation by nonsteroidal anti-inflammatory drugs: study in mitochondria, submitochondrial particles, cells, and whole heart. Biochemical pharmacology. 1999;57(7):743-752.

3 Horton KL, Stewart KM, Fonseca SB, Guo Q, Kelley SO. Mitochondria-penetrating peptides. Chemistry & biology. 2008;15(4):375-382.

4 Yang L, Zhao K, Calingasan NY, Luo G, Szeto HH, Beal MF. Mitochondria targeted peptides protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. Antioxidants & redox signaling. 2009;11(9):2095-2104.

5 Petri S, Kiaei M, Damiano M, et al. Cell-permeable peptide antioxidants as a novel therapeutic approach in a mouse model of amyotrophic lateral sclerosis. Journal of neurochemistry. 2006;98(4):1141-1148.

6 Kakuda TN. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. Clin Ther. 2000;22(6):685-708.

7 Lang BF, Gray MW, Burger G. Mitochondrial genome evolution and the origin of eukaryotes. Annual review of genetics. 1999;33:351-397.

8 Manczak M, Mao P, Calkins MJ, et al. Mitochondria-targeted antioxidants protect against amyloid-beta toxicity in Alzheimer's disease neurons. Journal of Alzheimer's disease: JAD. 2010;20 Suppl 2:S609-631.

9 Min K, Smuder AJ, Kwon OS, Kavazis AN, Szeto HH, Powers SK. Mitochondrial-targeted antioxidants protect skeletal muscle against immobilization-induced muscle atrophy. Journal of applied physiology. 2011;111(5):1459-1466.

10 PIel S, Ehinger JK, Elmer E, Hansson MJ. Metformin induces lactate production in peripheral blood mononuclear cells and platelets through specific mitochondrial complex I inhibition. Acta physiologica (Oxford, England). 2015;213(1):171-180.

11 Anderson EJ, Lustig ME, Boyle KE, et al. Mitochondrial H_2O_2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. The Journal of clinical investigation. 2009;119(3):573-581.

12 Dai DF, Chen T, Szeto H, et al. Mitochondrial targeted antioxidant Peptide ameliorates hypertensive cardiomyopathy. Journal of the American College of Cardiology. 2011;58(1):73-82.

13 Karaa A, Haas R, Goldstein A, Vockley J, Weaver WD, Cohen BH. Randomized dose-escalation trial of elamipretide in adults with primary mitochondrial myopathy. Neurology. 2018;90(14):e1212-e1221.

14 Daubert MA, Yow E, Dunn G, et al. Novel Mitochondria-Targeting Peptide in Heart Failure Treatment: zA Randomized, Placebo-Controlled Trial of Elamipretide. Circulation Heart failure. 2017;10(12).

15 Saad A, Herrmann SMS, Eirin A, et al. Phase 2a Clinical Trial of Mitochondrial Protection (Elamipretide) During Stent Revascularization in Patients With Atherosclerotic Renal Artery Stenosis. Circulation Cardiovascular interventions. 2017;10(9).

16 DiMauro S, Schon EA. Mitochondrial respiratory-chain diseases. The New England journal of medicine. 2003;348(26):2656-2668.

17 Song WH, Yi YJ, Sutovsky M, Meyers S, Sutovsky P. Autophagy and ubiquitin-proteasome system contribute to sperm mitophagy after mammalian fertilization. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(36):E5261-5270.

18 McBride HM, Neuspiel M, Wasiak S. Mitochondria: more than just a powerhouse. Current biology: CB. 2006;16(14):R551-560.

19 Hawley JA, Lessard SJ. Mitochondrial function: use it or lose it. Diabetologia. 2007;50(4):699-702.

20 Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. Archives of biochemistry and biophysics. 2007;462(2):245-253.

21 Loschen G, Flohe L, Chance B. Respiratory chain linked H(2)O(2) production in pigeon heart mitochondria. FEBS letters. 1971;18(2):261-264.

22 Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochemical Journal. 1973;134(3):707-716.

23 Harman D. Aging: a theory based on free radical and radiation chemistry. Journal of gerontology. 1956;11(3):298-300.

24 Shokolenko I, Venediktova N, Bochkareva A, Wilson GL, Alexeyev MF. Oxidative stress induces degradation of mitochondrial DNA. Nucleic Acids Research. 2009;37(8):2539-2548.

25 Lemasters JJ. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. Rejuvenation research. 2005;8(1):3-5.

26 Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. The Journal of biological chemistry. 1955;217(1):383-393.

27 Lanza IR, Bhagra S, Nair KS, Port JD. Measurement of human skeletal muscle oxidative capacity by 31P-MR spectroscopy: a cross-validation with in vitro measurements. Journal of magnetic resonance imaging: JMRI. 2011;34(5):1143-1150.

28 Haller T, Ortner M, Gnaiger E. A respirometer for investigating oxidative cell metabolism: toward optimization of respiratory studies. Analytical biochemistry. 1994;218(2):338-342.

29 Lanza IR, Nair KS. Functional Assessment of Isolated Mitochondria In Vitro. Methods in enzymology. 2009;457:349-372.

30 Ferrick DA, Neilson A, Beeson C. Advances in measuring cellular bioenergetics using extracellular flux. Drug discovery today. 2008;13(5-6):268-274.

31 Wallace DC, Singh G, Lott MT, et al. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science (New York, NY). 1988;242(4884):1427-1430.

32 Parikh S, Goldstein A, Koenig MK, et al. Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. Genetics in medicine: official journal of the American College of Medical Genetics. 2015;17(9):689-701.

33 Lane RK, Hilsabeck T, Rea SL. The role of mitochondrial dysfunction in age-related diseases. Biochimica et biophysica acta. 2015;1847(11):1387-1400.

34 Wallace DC. A mitochondrial bioenergetic etiology of disease. The Journal of clinical investigation. 2013;123(4):1405-1412.

35 Dodds RM, Granic A, Davies K, Kirkwood TB, Jagger C, Sayer AA. Prevalence and incidence of sarcopenia in the very old: findings from the Newcastle 85+ Study. Journal of cachexia, sarcopenia and muscle. 2017;8(2):229-237.

36 Umanskaya A, Santulli G, Xie W, Andersson DC, Reiken SR, Marks AR. Genetically enhancing mitochondrial antioxidant activity improves muscle function in aging. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(42):15250-15255.

37 Porter MM, Vandervoort AA, Lexell J. Aging of human muscle: structure, function and adaptability. Scandinavian journal of medicine & science in sports. 1995;5(3):129-142.

38 Gonzalez-Montalvo JI, Alarcon T, Gotor P, et al. Prevalence of sarcopenia in acute hip fracture patients and its influence on short-term clinical outcome. Geriatrics & gerontology international. 2015.

39 Broskey NT, Greggio C, Boss A, et al. Skeletal muscle mitochondria in the elderly: effects of physical fitness and exercise training. The Journal of clinical endocrinology and metabolism. 2014:jc20133983.

40 Gianni P, Jan KJ, Douglas MJ, Stuart PM, Tarnopolsky MA. Oxidative stress and the mitochondrial theory of aging in human skeletal muscle. Experimental gerontology. 2004;39(9):1391-1400.

41 Barrientos A, Casademont J, Rotig A, et al. Absence of relationship between the level of electron transport chain activities and aging in human skeletal muscle. Biochemical and biophysical research communications. 1996;229(2):536-539.

42 Safdar A, Hamadeh MJ, Kaczor JJ, Raha S, Debeer J, Tarnopolsky MA. Aberrant mitochondrial homeostasis in the skeletal muscle of sedentary older adults. PloS one. 2010;5(5):e10778.

43 Garcia-Prat L, Martinez-Vicente M, Perdiguero E, et al. Autophagy maintains stemness by preventing senescence. Nature. 2016;529(7584):37-42.

44 Joseph AM, Adhihetty PJ, Wawrzyniak NR, et al. Dysregulation of mitochondrial quality control processes contribute to sarcopenia in a mouse model of premature aging. PloS one. 2013;8(7):e69327.

45 Sebastian D, Sorianello E, Segales J, et al. Mfn2 deficiency links age-related sarcopenia and impaired autophagy to activation of an adaptive mitophagy pathway. The EMBO Journal. 2016;35(15):1677-1693.

46 Kim YA, Kim YS, Oh SL, Kim HJ, Song W. Autophagic response to exercise training in skeletal muscle with age. Journal of physiology and biochemistry. 2013;69(4):697-705.

47 Safdar A, Little JP, Stokl AJ, Hettinga BP, Akhtar M, Tarnopolsky MA. Exercise increases mitochondrial PGC-1alpha content and promotes nuclear-mitochondrial cross-talk to coordinate mitochondrial biogenesis. The Journal of biological chemistry. 2011;286(12):10605-10617.

48 Safdar A, Bourgeois JM, Ogborn DI, et al. Endurance exercise rescues progeroid aging and induces systemic mitochondrial rejuvenation in mtDNA mutator mice. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(10):4135-4140.

49 Steiner JL, Murphy EA, McClellan JL, Carmichael MD, Davis JM. Exercise training increases mitochondrial biogenesis in the brain. Journal of applied physiology. 2011;111(4):1066-1071.

50 Russell AP. PGC-1alpha and exercise: important partners in combating insulin resistance. Current diabetes reviews. 2005;1(2):175-181.

51 Benton CR, Wright DC, Bonen A. PGC-1alpha-mediated regulation of gene expression and metabolism: implications for nutrition and exercise prescriptions. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2008;33(5):843-862.

52 Taivassalo T, Haller RG. Exercise and training in mitochondrial myopathies. Medicine and science in sports and exercise. 2005;37(12):2094-2101.

53 Birk AV, Liu S, Soong Y, et al. The mitochondrial-targeted compound SS-31 re-energizes ischemic mitochondria by interacting with cardiolipin. Journal of the American Society of Nephrology: JASN. 2013;24(8):1250-1261.

54 Keil U, Scherping I, Hauptmann S, Schuessel K, Eckert A, Müller WE. PIracetam improves mitochondrial dysfunction following oxidative stress. British journal of pharmacology. 2006;147(2):199-208.

55 Gane EJ, Weilert F, Orr DW, et al. The mitochondria-targeted anti-oxidant mitoquinone decreases liver damage in a phase II study of hepatitis C patients. Liver international: official journal of the International Association for the Study of the Liver. 2010;30(7):1019-1026.

56 Ryu D, Mouchiroud L, Andreux PA, et al. Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents. Nature medicine. 2016;22(8):879-888.

57 Siegel MP, Kruse SE, Percival JM, et al. Mitochondrial-targeted peptide rapidly improves mitochondrial energetics and skeletal muscle performance in aged mice. Aging cell. 2013;12(5):763-771.

58 Ghosh A, Chandran K, Kalivendi SV, et al. Neuroprotection by a mitochondria-targeted drug in a Parkinson's disease model. Free radical biology & medicine. 2010;49(11):1674-1684.

59 Pritchard JF, Jurima-Romet M, Reimer MLJ, Mortimer E, Rolfe B, Cayen MN. Making Better Drugs: Decision Gates in Non-Clinical Drug Development. Nature Reviews Drug Discovery. 2003;2:542.

60 Wong CH, Siah KW, Lo AW. Estimation of clinical trial success rates and related parameters. Biostatistics (Oxford, England). 2018.

61 Harrison RK. Phase II and phase III failures: 2013–2015. Nature Reviews Drug Discovery. 2016;15:817.

62 Sacks LV, Shamsuddin HH, Yasinskaya YI, Bouri K, Lanthier ML, Sherman RE. Scientific and regulatory reasons for delay and denial of fda approval of initial applications for new drugs, 2000-2012. JAMA: the journal of the American Medical Association. 2014;311(4):378-384.

63 Walters AM, Porter GA, Jr., Brookes PS. Mitochondria as a drug target in ischemic heart disease and cardiomyopathy. Circulation research. 2012;111(9):1222-1236.

64 Ishimoto Y, Inagi R. Mitochondria: a therapeutic target in acute kidney injury. Nephrology, dialysis, transplantation: official publication of the European Dialysis and Transplant Association-European Renal Association. 2016;31(7):1062-1069.

65 Liu J, Wang LN. Mitochondrial enhancement for neurodegenerative movement disorders: a systematic review of trials involving creatine, coenzyme Q10, idebenone and mitoquinone. CNS drugs. 2014;28(1):63-68.

66 Ienco EC, LoGerfo A, Carlesi C, et al. Oxidative stress treatment for clinical trials in neurodegenerative diseases. Journal of Alzheimer's disease: JAD. 2011;24 Suppl 2:111-126.

67 Cohen AF, Burggraaf J, van Gerven JM, Moerland M, Groeneveld GJ. The use of biomarkers in human pharmacology (Phase I) studies. Annual review of pharmacology and toxicology. 2015;55:55-74.

68 van den Bogert CA, Cohen AF, Leufkens HGM, van Gerven JMA. Pharmacological vs. classical approaches in the design of first in man clinical drug trials. British journal of clinical pharmacology. 2017;83(12):2807-2812.

69 van Gerven J, Cohen A. Integrating data from the Investigational Medicinal Product Dossier/investigator's brochure. A new tool for translational integration of pre-clinical effects. British journal of clinical pharmacology. 2018;84(7):1457-1466.

70 Pallardy M, Hunig T. Primate testing of TGN1412: right target, wrong cell. British journal of pharmacology. 2010;161(3):509-511.

71 ML Rizk, L Zou, RM Savic, KE Dooley. Importance of Drug Pharmacokinetics at the Site of Action. Clinical and Translational Science. 2017;10(3):133-142.

72 Agoram BM. Use of pharmacokinetic/ pharmacodynamic modelling for starting dose selection in first-in-human trials of high-risk biologics. British journal of clinical pharmacology. 2009;67(2):153-160.

73 Sena ES, van der Worp HB, Bath PM, Howells DW, Macleod MR. Publication bias in reports of animal stroke studies leads to major overstatement of efficacy. PLoS biology. 2010;8(3):e1000344.

74 Kimmelman J, Federico C. Consider drug efficacy before first-in-human trials. Nature. 2017;542(7639):25-27.

75 Tirmenstein MA, Hu CX, Gales TL, et al. Effects of troglitazone on HEPG2 viability and mitochondrial function. Toxicological sciences: an official journal of the Society of Toxicology. 2002;69(1):131-138.

76 Westwood FR, Bigley A, Randall K, Marsden AM, Scott RC. Statin-induced muscle necrosis in the rat: distribution, development, and fibre selectivity. Toxicologic pathology. 2005;33(2):246-257.

77 Vuda M, Kamath A. Drug induced mitochondrial dysfunction: Mechanisms and adverse clinical consequences. Mitochondrion. 2016;31:63-74.

78 Bourne RB, Chesworth BM, Davis AM, Mahomed NN, Charron KD. Patient satisfaction after total knee arthroplasty: who is satisfied and who is not? Clinical orthopaedics and related research. 2010;468(1):57-63.

79 Keurentjes JC, Blane D, Bartley M, Keurentjes JJ, Fiocco M, Nelissen RG. Socio-economic position has no effect on improvement in health-related quality of life and patient satisfaction in total hip and knee replacement: a cohort study. PloS one. 2013;8(3):e56785.

80 Dunbar MJ, Haddad FS. Patient satisfaction after total knee replacement: new inroads. The bone & joint journal. 2014;96-b(10):1285-1286.

81 Keurentjes JC, Fiocco M, So-Osman C, et al. Patients with severe radiographic osteoarthritis have a better prognosis in physical functioning after hip and knee replacement: a cohort-study. PloS one. 2013;8(4):e59500.

82 Keurentjes JC, Van Tol FR, Fiocco M, et al. Patient acceptable symptom states after totalhip or knee replacement at mid-term follow-up: Thresholds of the Oxford hip and knee scores. Bone & joint research. 2014;3(1):7-13.

83 Tilbury C, Haanstra TM, Verdegaal SHM, et al. Patients' pre-operative general and specific outcome expectations predict postoperative pain and function after total knee and total hip arthroplasties. Scandinavian journal of pain. 2018;18(3):457-466.

84 Tilbury C, Haanstra TM, Leichtenberg CS, et al. Unfulfilled Expectations After Total Hip and Knee Arthroplasty Surgery: There Is a Need for Better Preoperative Patient Information and Education. The Journal of arthroplasty. 2016;31(10):2139-2145.

85 Abramov AY, Berezhnov AV, Fedotova EI, Zinchenko VP, Dolgacheva LP. Interaction of misfolded proteins and mitochondria in neurodegenerative disorders. Biochemical Society transactions. 2017.

86 Jenkins BG, Koroshetz WJ, Beal MF, Rosen BR. Evidence for impairment of energy metabolism in vivo in Huntington's disease using localized 1H NMR spectroscopy. Neurology. 1993;43(12):2689-2695.

87 Sandoval H, Thiagarajan P, Dasgupta SK, et al. Essential role for Nix in autophagic maturation of erythroid cells. Nature. 2008;454(7201):232-235.

88 Liu L, Feng D, Chen G, et al. Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. Nature cell biology. 2012;14(2):177-185.

89 Murakawa T, Yamaguchi O, Hashimoto A, et al. Bcl-2-like protein 13 is a mammalian Atg32 homologue that mediates mitophagy and mitochondrial fragmentation. Nature communications. 2015;6:7527.

90 Strappazzon F, Nazio F, Corrado M, et al. AMBRA1 is able to induce mitophagy via LC3 binding, regardless of PARKIN and p62/SQSTM1. Cell death and differentiation. 2015;22(3):419-432.

91 Lazarou M, Sliter DA, Kane LA, et al. The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. Nature. 2015;524(7565):309-314.

92 Narendra DP, Jin SM, Tanaka A, et al. PINK1 is selectively stabilized on impaired mitochondria to activate PARKIN. PLoS biology. 2010;8(1):e1000298.

93 Kane LA, Lazarou M, Fogel AI, et al. PINK1 phosphorylates ubiquitin to activate PARKIN E3 ubiquitin ligase activity. The Journal of cell biology. 2014;205(2):143-153.

94 Koyano F, Okatsu K, Kosako H, et al. Ubiquitin is phosphorylated by PINK1 to activate PARKIN. Nature. 2014;510(7503):162-166.

95 Youle RJ, Narendra DP. Mechanisms of mitophagy. Nature reviews Molecular cell biology. 2011;12(1):9-14.

FIGURE 1 **Mitochondrion.** Schematic display of a mitochondrion. Work by Mariana 'LadyofHats' Ruiz Villareal and use of the work allowed under the Wikimedia Commons.

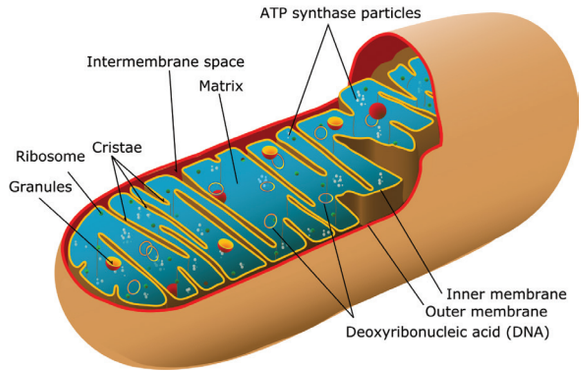


FIGURE 2 **Mitochondrial Electron Transport Chain.** Schematic display of the mitochondrial electron Transport chain (ETC). Electrons (e^-) are passed along the different complexes (I-IV) to build up a proton gradient in the mitochondrial intermembrane space. The protons then flow back by diffusion through the ATPase channel (complex V), energizing the formation of adenosine triphosphate (AT) from adenosine diphosphate (ADP) and inorganic phosphate (Pi). Work by OpenStax College and use of the work allowed under the Creative Commons Attribution 3.0 Unported license.

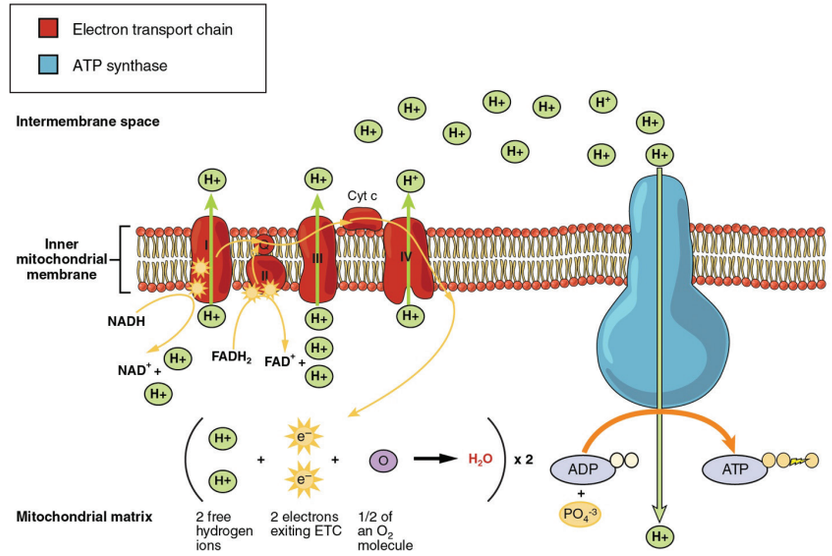


FIGURE 3 **Mitophagy.** The best-known mechanism for mitophagy is the PINK/PARKIN pathway, activated for instance in case of mitochondrial damage or the accumulation of misfolded protein.[17,87-91] PINK, PTEN-induced putative kinase 1, binds to the outer mitochondrial membrane, where it activates ubiquitin via phosphorylation, which in turn activates PARKIN, an E3 ubiquitin ligase.[92] PARKIN catalyzes ubiquitin transfer to the mitochondrial membrane and causes polyubiquitination. This leads to the formation of an autophagosome.[93,94] Fusion to a lysosome finally causes mitochondrial degradation.[91,95] ROS = reactive oxygen species, $\Delta\Psi m$ = mitochondrial membrane potential, Mfn2 = mitofusin 2, LC3 = microtubule associated light chain 3.

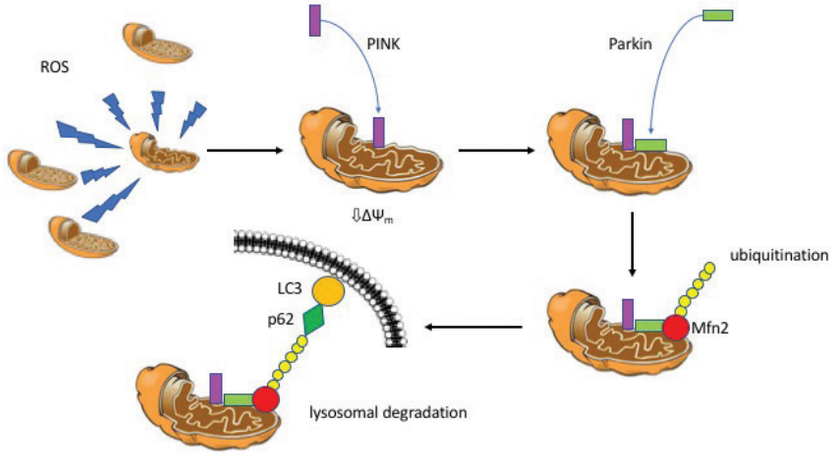
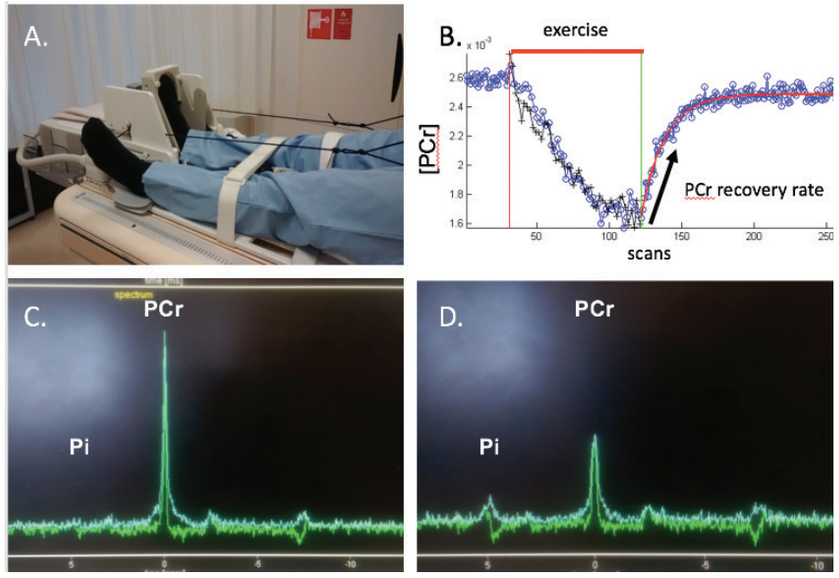


FIGURE 4 A-D **Phosphorous Magnetic Resonance Spectroscopy.** The subject is positioned on the MRI table with the right leg strapped on an MRI-compatible pedal ergometer (A). Using a surface coil to detect phosphorous metabolism, the concentration of phosphor-containing molecules can be measured. When the calf muscles are exercised, phosphocreatine (PCr) is broken down into inorganic phosphate (Pi) and creatine to supply energy to keep the adenosine triphosphate (ATP) concentration stable. This all takes place within the mitochondria. After the exercise, the PCr concentration is build up again in order to be ready for a next bout of exercise. The resulting PCr recovery rate constant reflects the mitochondrial function (B). This process can be monitored real time from rest (C) to end-of-exercise (D). Pictures are our own work.



PCr = phosphocreatine. Pi = inorganic phosphate. ATP = adenosine triphosphate.

FIGURE 5 A-D **Protoporphyrin IX Triple State Lifetime Technique.** The COMET device (A) is the latest development of the PPIX-TSLT technique to assess mitochondrial oxygen concentration and the mitochondrial oxygen consumption rate. The technique is based on the oxygen-dependent fluorescence of protoporphyrin IX. When an optode (B) emits laser light on protoporphyrin-loaded skin, PPIX reaches a triple state, which is emitted as fluorescence. The speed of emission is oxygen dependent and when pressure is applied to block microvasculature blood flow (C), the available oxygen is used by mitochondria. The resulting slope of the oxygen concentration curve reflects the mitochondrial oxygen consumption rate (D). Pictures by courtesy of Photonics Healthcare.