MARCUS VAN DIEMEN

CLINICAL PHARMACOLOGICAL ASPECTS OF MITOCHONDRIAL FUNCTION IN MUSCLE

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'Watch your thoughts, for they become words.
Watch your words, for they become actions.
Watch your actions, for they become habits.
Watch your habits, for they become your character.
Watch your character, for it will make your destiny '

Margaret Thatcher

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PROMOTORES

Prof. dr. A.F. Cohen
Prof. dr. R.G.H.H Nelissen

CO-PROMOTOR

Prof.dr. G.J. Groeneveld

DESIGN
Caroline de Lint, Voorburg (caro@delint.nl)

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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.'

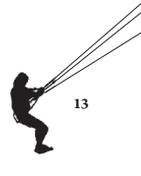
According to some, general bioenergetic decline is responsible for most age-related diseases, with mitochondria being the main actors.[34] Different tissues demand a different extent of energy and this explains tissue-specific symptoms. The brain is most sensitive to a small decrease in energy supply. 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.

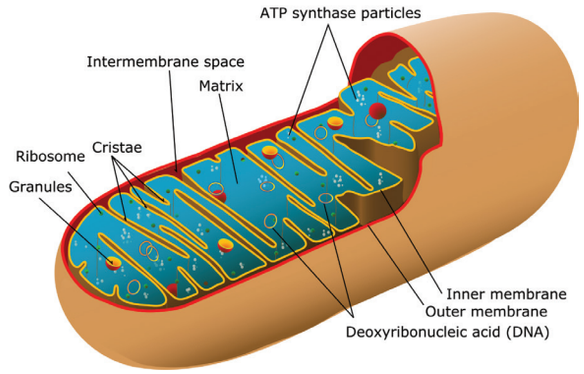


FIGURE 2 **Mitochondrial Electron Transport Chain.** Schematic display of the mitochondrial electron Transport chain (ETC). Electrons (e^-) are passed along the different complexes (I-IV) to build up a proton gradient in the mitochondrial intermembrane space. The protons then flow back by diffusion through 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.

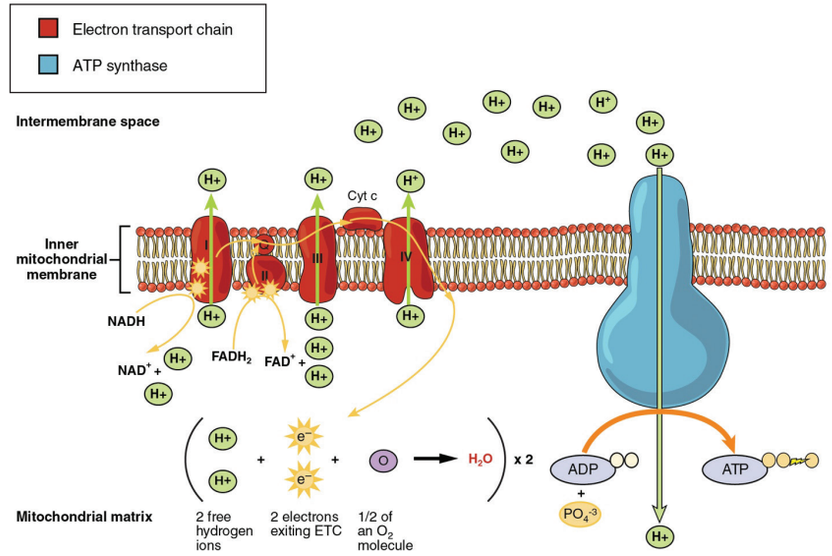


FIGURE 3 **Mitophagy.** The best-known mechanism for mitophagy is the PINK/PARKIN pathway, activated for instance in case of mitochondrial damage or the accumulation of misfolded protein.[17,87-91] PINK, PTEN-induced putative kinase 1, binds to the outer mitochondrial membrane, where it activates ubiquitin 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.

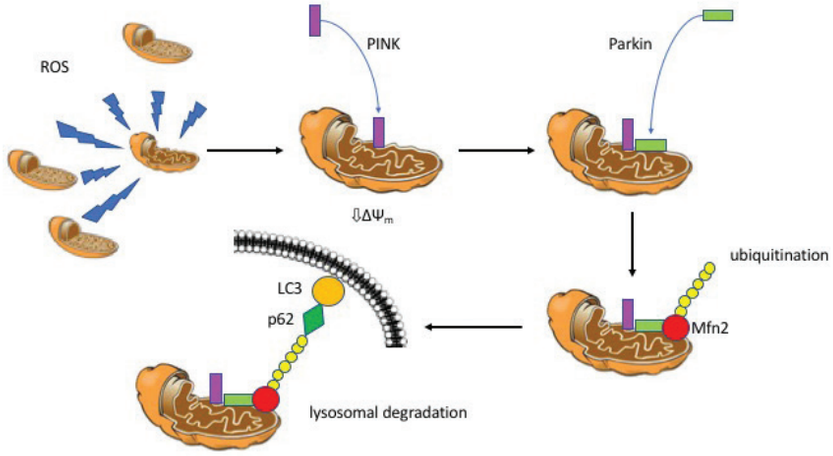
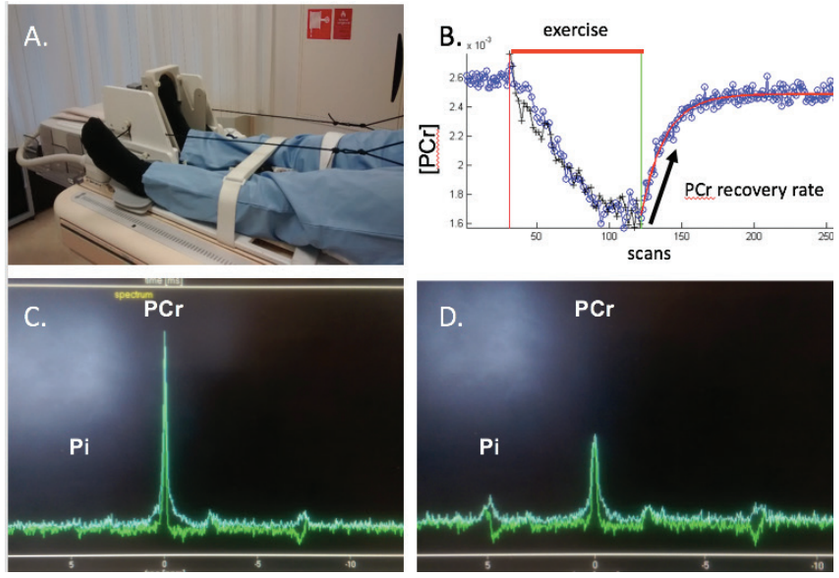
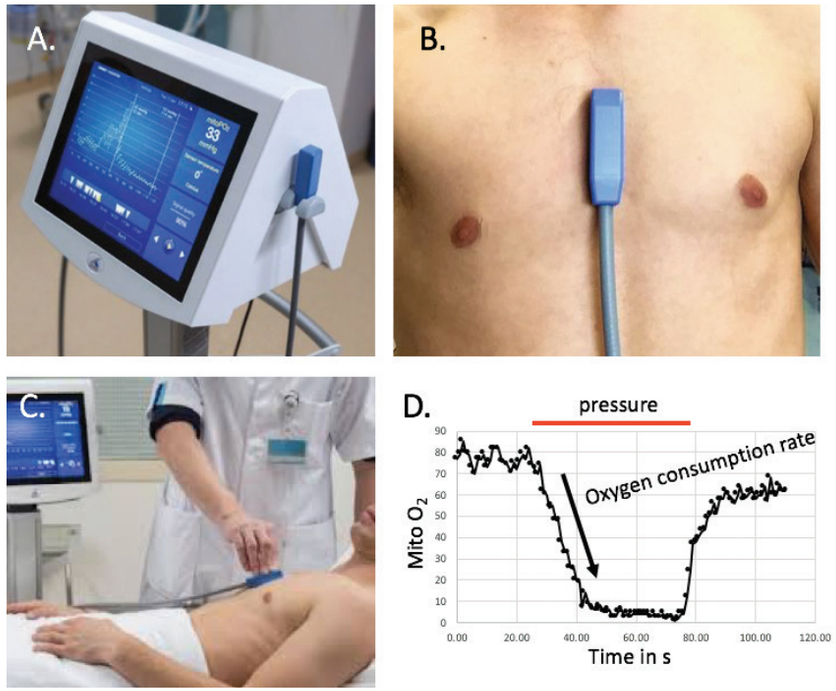


FIGURE 4 A-D **Phosphorous Magnetic Resonance Spectroscopy.** The subject is positioned on the MRI table with the right leg strapped on an MRI-compatible pedal ergometer (A). Using a surface coil to detect phosphorous metabolism, the concentration of phosphor-containing molecules can be measured. When 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.



CHAPTER II

MITOCHONDRIAL FUNCTION IS IMPAIRED IN THE SKELETAL MUSCLE OF PRE-FRAIL ELDERLY

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Pénélope A. Andreux[1,4*] ⋮ Marcus P.J. van Diemen[2,4] ⋮ Maxime R. Heezen[2] ⋮ Johan Auwerx[3] ⋮ Chris Rinsch[1] ⋮ Geert Jan Groeneveld[2,5] ⋮ Anurag Singh[1,5] ⋮⋮ *1. Amazentis SA, Lausanne, Switzerland ⋮ 2. Center for Human Drug Research, Leiden, The Netherlands ⋮ 3. Laboratory for Integrative and Systems Physiology, Lausanne, Switzerland ⋮ 4. Joint first-authors 5. Joint senior authors ⋮ *Corresponding author*

ABSTRACT Aging is accompanied by a gradual decline in both muscle mass and strength over time, which can eventually lead to pathologies, such as frailty and sarcopenia. While these two conditions are well characterized, further investigation of the early biological signs present in pre-frail elderly is still needed to help identify strategies for preventative therapeutic intervention. The goal of the present clinical study was to evaluate the level of mitochondrial (dys)function in a well-defined population of pre-frail elderly (>60 years of age). Pre-frail elderly were compared with an age-matched population of active elderly. Muscle mitochondrial function was assessed in vivo using phosphorus magnetic resonance spectroscopy (31P-MRS) and a comprehensive set of biological biomarkers were measured ex vivo in vastus lateralis muscle biopsies. In pre-frail subjects, phosphocreatine recovery was impaired and mitochondrial respiratory complex protein and activity levels were significantly lower when compared with active elderly. Analysis of microarray data showed that mitochondrial genes were also significantly down-regulated in muscle of pre-frail compared to active elderly. These results show that mitochondrial impairment is a hallmark of pre-frailty development and the onset of decline in muscle function in the elderly.

INTRODUCTION

Population based studies have demonstrated that approximately half of the elderly population displays early signs of either muscle mass or function decline[1]. If left unaddressed, the muscle health of elderly can deteriorate further, manifesting as frailty and sarcopenia, two geriatric conditions that have a tremendous health economic impact.[2] Today, one way of diagnosing frailty in the clinic is to use Fried's criteria, which consists of five components: unintentional weight loss, general fatigue, decline in muscle strength, slow gait speed and low physical activity.[3] Elderly exhibiting three or more of these criteria are classified as frail, while those experiencing one or two Fried's criteria are classified as pre-frail.[3]

Sarcopenia is diagnosed based on the gradual loss of muscle mass leading to a decline in muscle strength, with muscle atrophy being the hallmark feature in elderly with sarcopenia.[4] As life expectancy increases in society with 1 out of every 5 people in the world expected to be elderly (>60 years) by 2050, maintaining muscle health will be key to ensure an independent and unassisted lifestyle in the elderly population.[5] Further understanding of the biological causes driving age-related muscle decline is therefore needed.

From a broader perspective, most aging cells progressively lose their capacity to maintain their optimal function, ultimately leading to organ specific symptoms and the development of systemic age-related diseases. This time-dependent phenomenon is currently thought to result from the accumulation of damage both at the DNA and protein levels.[6] One of the most sensitive organelles to these changes is the mitochondrion. Research in a wide range of preclinical models has shown that mitochondrial bioenergetics, defined as the capacity of the mitochondria to respond to the energetic and metabolic demand of the cell, is declining with age.[7,8] Furthermore with aging there is a reduction in mitochondrial turnover due to lower biogenesis and damaged mitochondria being less effectively cleared by mitophagy.[6,9] Previous clinical studies have reported associations with low mitochondrial abundance and impairment in mitochondrial function in sedentary elderly when compared to either young or age matched active elderly subjects.[10,11] Some evidence also points to decreased mitochondrial function in frail elderly immobilized due to hip fracture and in clinically diagnosed sarcopenia.[12,13] However, to our knowledge, the level of mitochondrial (dys)function in pre-frail elderly (i.e. the beginning phase of the symptomatic decline in muscle and physical performance associated with aging) has previously not been well characterized.

In the current clinical study, age-matched pre-frail elderly were compared to active elderly with respect to the status of their muscle mitochondrial function *in vivo* using phosphorus magnetic resonance spectroscopy (31P-MRS) and a comprehensive set of biological biomarkers, measured *ex vivo* in *vastus lateralis* muscle biopsies. The results of this investigation demonstrate a striking association of pre-frailty status in elderly with mitochondrial impairment in skeletal muscle and provide a strong rationale for employing interventions that improve mitochondrial function to either reverse the pre-frailty phenotype or delay the progression to full onset frailty syndrome.

RESULTS

Demographics

In total, 11 pre-frail (6 males and 5 females) and 11 active (6 males and 5 females) subjects between the ages of 61 to 80 years old participated in this study. One pre-frail male subject was excluded from the final study analyses, due to a lack of compliance to study dietary restrictions between visits on Day 1 and Day 14 (Figure 2.2). However, because he had already been collected for muscle biopsy on Day 1, he was included for the microarray genomic analysis only. In the end, data from 10 pre-frail (5 males and 5 females) and 11 active (6 males and 5 females) subjects were included for all analysis except gene expression via microarray. Pre-frail and active subjects were matched on age (70.2±5.8 *vs* 70.0±6.7 yrs) and body mass index (BMI) (25.7±4.2 *vs* 24.6±3.9 kg/m^2) (Table 2.1). Subjects were all of Caucasian descent, except for one active subject, who was of Afro-Dutch descent.

Muscle function and physical performance measures

As described in the methods section (inclusion/exclusion criteria), subjects were selected as pre-frail when they fulfilled at least one to two out of the three following Fried criteria, i.e. muscle weakness (low muscle strength), slow gait speed and low physical activity.[4]

All subjects analyzed in the pre-frail population in the end met at least two of the Fried frailty criteria. Pre-frail subjects had low physical activity with a mean energy expenditure of 392 MET minutes per week, which corresponds to less than 20 minutes of walking per day. In comparison, the mean energy expenditure in the active elderly group was 6,508 MET minutes per week (p=<0.0001 when

compared to pre-frail group), which corresponds to 1 hour of vigorous exercise per day. There was a slight trend, though not statistically different, in the decline of skeletal muscle mass index in elderly (10.9 kg/m^2 in pre-frail *vs* 12.1 kg/m^2 in active elderly, p=0.21), highlighting, at least in these populations, that differences in muscle performance were not strictly linked to muscle mass, but were driven through other changes in the muscle biology.

Upon inclusion in the study, four additional tests were performed at day 1 and day 14: 1) SPPB, 2) handgrip strength by Jamar dynamometry, 3) quadriceps strength, 4) postural stability and 5) *in vivo* evaluation of mitochondrial function using phosphorus magnetic resonance spectroscopy (31P-MRS) (Table 2.2). All these tests were very reproducible from one visit to another (data not shown). Therefore, the average value between the two visits per subject was used to compute statistical significance. No differences were observed in the total SPPB score when comparing the two groups (total mean SPPB score of 10 in pre-frail subjects *vs* 11 in active elderly, p=0.58; Table 2.3). There was no difference in the balance test performed during the SPPB (total score of 3 compared to 3.18 in the active elderly, p=0.33, Table 2.3), as well as the postural stability test (383 compared to 392 in the active elderly, p=0.88, Table 2.3). However, when looking at the other individual SPPB domain tests, there were significant differences in the pre-frail group for the duration sit-to-stand transfer test: 13.24 seconds compared to 9.77 seconds in the active group (p=0.03) and for the gait-speed: 0.99 m/s as compared to 1.40 m/s in the active group (p=0.0088). Pre-frail elderly clearly exhibited low muscle strength, with a handgrip strength of 22.3 kg *vs* 38.8 kg in the active group (p=0.0002) and a quadriceps strength of 141 Newton *vs* 223 Newton in the active group (p=0.0002) (Table 2.3). Altogether, these results show that the screening parameters were consistent and led to a clear selection of two distinct populations.

Non-invasive measurement of mitochondrial function in muscle

Mitochondrial function was first assessed in calf muscle using 31P-MRS by measuring phosphocreatine (PCR) recovery rate (Table 2.2). This parameter corresponds to the rate at which creatine is re-phosphorylated after exercise and is a function of the rate at which mitochondria produce ATP.[14] PCR recovery rate over both visits was found to be significantly longer in pre-frail subjects than in active subjects (40.82 seconds in pre-frail elderly *vs* 29.53 in active elderly; p = 0.0064, Figure 2.2A), indicating a lower rate of ATP production in the muscle in pre-frail subjects. These results with 31P-MRS were reproducible during the two visits on Day 1 and 14 of the study.

Measurement of mitochondrial respiratory complexes activity in muscle biopsies

Mitochondrial function was evaluated in muscle biopsies collected at day 1 of the study (Table 2.2), by measuring complexes I, IV and V abundance and complexes I, II and IV activity. Active subjects presented both higher abundance of complexes I, IV and V and higher enzymatic activity of complex I, II and IV than measured in pre-frail subjects (Figure 2.2B-C). These results agree with the PCR recovery rate data and indicate that mitochondria in the muscle of active subjects are both more abundant and/or more efficient than those found in pre-frail subjects. Mitochondrial abundance was estimated by measuring the mitochondrial DNA (mtDNA) over nuclear DNA (nuDNA) ratio. There was a trend to have higher mtDNA/nuDNA ratio in active subjects compared to pre-frail subjects, which however was not significant (p=0.11) (Figure 2.2D).

Transcriptomics analysis of skeletal muscle biopsies

Gene expression in skeletal muscle was compared using Affymetrix HTA2.0 microarray. Data were analyzed running a Gene Set Enrichment type of Analysis (GSEA), which tells which biological processes are up or down-regulated at the scale of entire gene-sets, rather than gene by gene.[15] Results were filtered by selecting genesets up- or down-regulated with a false discovery rate (FDR) equal or less than 0.1. In total, there were 307 genesets significantly differentially expressed between the two groups, with 298 downregulated genesets and 9 upregulated genesets in pre-frail compared to active subjects (Table 2.S1). Downregulated genesets in pre-frail subjects were related to RNA processing and translation, histone H4 acetyltransferase activity, proteasome, fatty acid oxidation, amino acid metabolism, and a majority to mitochondrion and the respiratory chain (Figure 2.3). In fact, the top 10 downregulated genesets in pre-frail subjects were all related to mitochondrion (Table 2.4). Finally, the genes that were present in these genesets were compared to the top 100 of the most differentially expressed genes between the pre-fail and the active subjects. A short list of eight genes was selected and tested by QPCR, including solute carrier family 25 member 20 (SLC25A20),

acyl-CoA synthetase long chain family member 1 (ACSL1), carnitine palmitoyltransferase 1B (CPT1B), fatty acid binding protein 3 (FABP3), coenzyme Q_3, methyltransferase (COQ_3), lactate dehydrogenase B (LDHB), creatine kinase, mitochondrial 2 (CKMT2), and cytochrome c oxidase copper chaperone (COX17). Amongst these, genes encoding proteins related to fatty acid oxidation (CPT1B and FABP3), Coenzyme Q10 synthesis (COQ_3), anaerobic glycolysis (LDHB), and creatine phosphorylation (CKMT2) had a significantly lower level of expression in the pre-frail samples (Figure 2.4).

DISCUSSION

Pre-frailty is the stepping stone to geriatric disorders that restrict mobility such as the frailty syndrome and sarcopenia, and intervening at this stage by targeting key biological pathways may have important implications in either reversing pre-frailty or halting the subsequent development of frailty. The goal of our clinical study was to characterize a pre-frail elderly population with respect to their mitochondrial function status using a comprehensive set of *ex vivo* and *in vivo* methods. The results reported in this manuscript demonstrate a striking association of the development of pre-frailty with a decline in skeletal muscle mitochondrial function.

When comparing with the existing literature, a comprehensive evaluation of mitochondrial function status in a well-characterized and clinically relevant population such as the pre-frail elderly is generally lacking. Our study is differentiated from these published studies on three aspects: a) a careful clinical selection of a pre-frail Dutch population using the very well defined Fried frailty criteria in the clinic; all pre-frail subjects met at least 2 criteria of low muscle strength and low physical activity; b) a comprehensive evaluation of mitochondrial function status using both *in vivo* (31P-MRS) and *ex vivo* on muscle biopsy samples (protein and genomic expression) methods, and c) comparing the pre-frail to an age-matched, physically very active group of elderly subjects with robust muscle strength. This last point was key in order to stratify the two studied elderly populations based on muscle strength and physical activity levels with the chosen number of participants. One of the limitations of the current study is that having a third group of frail elderly (3 or more Fried criteria) would perhaps have made the current study even more robust. We elected to focus on the pre-frail subjects only, also as we felt it was most relevant to understand the biology linked to the onset of the decline in mobility during aging.

Only a few studies have employed similar techniques to carefully assess muscle mitochondrial function, though inconsistent inclusion criteria have been employed to differentiate 'low' *vs* 'high'-functioning elderly, and the early pre-frail stage has not been a focus of these investigations. Elderly population differ depending on these inclusion criteria, focused on either aerobic endurance capacity (VO_2 peak), physical performance scores (SPPB), self-reported physical activity levels, muscle strength or mass. Overall, 'low'-functioning elderly present a lower mitochondrial function, compared to 'high'-functioning subjects, when the two populations are separated by VO_2 peak, [14,16] self-reported habits of exercise[11,17] and muscle strength.[18] On the contrary, there is no difference in muscle mitochondrial function when populations have similar levels of physical activity, but are distinguished based on muscle mass or SPPB score.[13,19] This reinforces the hypothesis that lower mitochondrial function is tightly coupled to low physical capacity and low muscle strength in elderly.

Decrease in mitochondrial capacity in the muscle can be explained by a diminution in the number of mitochondria, their volume and/or their energetic yield. In the present study, pre-frail subjects had significantly less abundant respiratory complexes I, IV and V, lower activity of complexes I, II and IV, and trend for a lower mtDNA/nuDNA. This was accompanied by a striking lower transcription level of mitochondrial gene sets in muscle. Likewise, lower abundance of complex I, IV and V, a lower mitochondrial volume density as determined by electron microscopy, and a lower mitochondrial function was also reported in muscle of sedentary *vs* active elderly.[11,17] With regard to the microarray data, to the best of our knowledge, we were not able to find a similar study where muscle transcriptome was compared in pre-frail *vs* active elderly. Only two studies reported a significant increase in the transcript levels of mitochondrial genes in muscle of elderly either after a 3-month of exercise training at 80% of maximal or a 6-month resistance-training program on muscle transcript data of 14 healthy elderly.[20,21] A common signature that is redundant between these studies and ours is the upregulation of fatty acid oxidation related genes, such as FABP3 and CPT1B, in active elderly or after an exercise intervention compared to sedentary elderly.[21] Likewise, induction of these fatty acid oxidation related genes was also described in studies where transcripts in muscle of young subjects were compared before and after exercise, or between sedentary and athletic subjects.[22-25] Today, there are limited interventions for the management of age-related muscle decline and associated geriatric conditions such as pre-frailty, frailty and sarcopenia. Pharmacologically, most interventions have focused on improvements in muscle mass either via

selective androgen receptor modulation,[26,27] testosterone replacement therapy in men[28] or with blocking antibodies targeting myostatin inhibition to improve muscle function.[29] The limitations in these approaches lie in the side effects of the different molecules or their route of administration, and the fact that they are rather used at late stages of muscle function decline such as frailty or sarcopenia. When prevention is still possible, optimal nutrition, especially a high protein diet along with resistance and aerobic exercise protocols is recommended to slow down the progression of these conditions.[30] However, adherence to exercise regimens and the quality of protein intake often dictates the outcome of these interventions in elderly. A complementary approach could integrate the current innovations in nutrition to help optimize mitochondrial function. In fact, several ingredients derived from food are being investigated to alleviate the burden of conditions associated with mitochondrial dysfunction, including L-carnitine,[31-33] epicatechin[34] (contained in green tea) and nicotinamide riboside[35-38] (present in beer and dairy products). Another interesting candidate is the newly discovered inducer of mitophagy, urolithin A, a gut metabolite derived from foods containing ellagitannins, such as pomegranate, red berries and nuts.[39] In fact, urolithin A was demonstrated to improve muscle and mitochondrial function in both young rats and aged mice.[40] These results are consistent with the hypothesis that a decrease in autophagy and mitophagy plays a key role in the decline of mitochondrial and muscle function observed during aging.[41] In conclusion, results reported in this manuscript highlight the need to intervene early in the decline of muscle function and call for an integration of exercise, dietary interventional strategies and pharmaceutical approaches to boost mitochondrial function to manage muscle health in the elderly.

MATERIALS AND METHODS

Trial design

The study was conducted as a single center, observational and case-control, clinical study. It was approved by an independent human ethics committee Stichting Bebo (Assen, the Netherlands) and conducted in accordance with the principles of the Helsinki Declaration. Informed consent was obtained from all the subjects. Adverse events and concomitant medications were continuously registered throughout the entire study period. The study was registered in a clinical trial registry as NCT02472340 (www.clinicaltrials.gov).

In- and exclusion criteria

General inclusion criteria included an age of 61 years or higher; body mass index 15-35 m^2/kg. Additional inclusion criteria for pre-frail subjects were derived from the Fried frailty criteria.[3] Subjects were considered pre-frail when fulfilling one to two of the following three criteria: either a walking speed < 0.8 m/s in the 4-m walking test, or a low muscle mass (SMI for males < 10.75 kg/m^2, for females < 6.75 kg/m^2) or a low muscle strength (handgrip strength of < 30 kg for males and < 20 kg for females); and sedentary behavior, which was defined as having an activity category of 1 (< 600 MET (metabolic equivalent unit) – minutes per week). Additional inclusion criteria for active subjects included an activity level of category 2 or 3 (≥ 600 MET – minutes per week); normal gait speed (a walking ≥ 0.8 m/s in the 4-m walking test); normal skeletal muscle mass (SMI for males ≥ 10.75 kg/m^2, for females ≥ 6.75 kg/m^2); normal muscle strength (handgrip strength of ≥ 30 kg for males and ≥ 20 kg for females).

General exclusion criteria included any contraindication to have an MRI scan; diabetes mellitus, any underlying chronic disease and/or lower extremity peripheral vascular disease; a history (within 3 months of screening) of alcohol consumption exceeding 2 standard drinks per day on average; inability to refrain from smoking more than half a pack of cigarettes (or similar for other tobacco products) per day during the course of the study; alcohol consumption within 48 hours of the study visits; unwillingness or inability to have a muscle biopsy performed; a history of allergy to lidocaine; and participation in a clinical trial within 90 days of screening or more than 4 times in the previous year. Participants were also advised to refrain from consuming muscle health promoting supplements two weeks prior to study enrollment. Additional exclusion criteria for pre-frail subjects included ruling out cachexia i.e. unintentional weight loss ≤5% of usual body weight during the last 6 months; anorexia; and anorexia-related symptoms.

Study schedule

Subjects were screened for eligibility up to 45 days before study enrollment. The tests used to identify eligible subjects included International Physical Activity Questionnaire (IPAQ), body composition by bioelectrical impedance analysis, Short-Physical Performance Battery Test (SPPB) and handgrip strength by Jamar dynamometry (Table 2.2). From the SPPB, only the walking speed was used as inclusion criteria.

Upon study enrollment, subjects were tested at day 1 and day 14 for 1) SPPB, 2) handgrip strength by Jamar dynamometry, 3) quadriceps strength, 4) postural stability and 5) *in vivo* evaluation of mitochondrial function using phosphorus magnetic resonance spectroscopy (31P-MRS) (Table 2.2). Muscle tissue collection and blood sampling for *ex vivo* biological markers of mitochondrial function were only performed at the day 1 visit to limit the burden on the subjects. The muscle biopsy procedure was overall well tolerated. These biopsies were used to assess mitochondrial function *ex vivo*, by measuring mitochondrial respiratory complexes activity and abundance, and mtDNA/nuDNA ratio. Subjects returned to the clinical research unit for the removal of the suture and inspection of the wound from the muscle biopsy procedure on day 7. Subjects were contacted by telephone for follow-up 7 to 10 days after the day 14 visit.

Physical performance measurements

International Physical Activity Questionnaire (IPAQ)

The Dutch version of the IPAQ was used to estimate an individual's level of physical activity in the domains of household and yard work activities, occupational activity, self-powered transport, and leisure-time physical activity as well as sedentary activity. The long version was used to gain a more detailed insight in the level of activity. An additional question asked about the pace of walking and cycling. The questionnaire and its translation into the Dutch language has been validated by direct comparison to activity measurements using an accelerometer.[42] The questionnaire was only taken during screening.

Bioelectrical impedance analysis

Bioelectrical impedance analysis has been found to correlate well to Dual Energy X-ray Absorptiometry (DEXA) and to Magnetic Resonance Imaging (MRI) in estimating muscle mass and body composition measurements and is therefore a validated part of the diagnostic work-up in elderly populations.[43-45] Using the InBody720 body composition analyzer (Biospace Co., Ltd., Korea), the Skeletal Muscle Mass index (SMI) was derived as part of the screening. The SMI (in kg/m^2) was calculated by dividing the lean muscle mass (in kg) by the square body height (in m^2). The SMI was only performed during screening.

Short Physical Performance Battery (SPPB)

The short physical performance battery (SPPB) has been described as a group of measures that combines the results of the gait speed, chair stand and balance tests.[46] It has been used as a predictive tool for possible disability and can aid in the monitoring of function in older people.[47] The scores ranged from 0 (worst performance) to 12 (best performance). We tested the ability to maintain standing balance during 10 seconds in three different positions: side-by-side, semi-tandem and tandem stance. A score of one was attributed to each of the position if the balance was maintained successfully. We also measured walking speed during the 4-meter walking test and the timed sit-to-stand transfer test. The test instructions have been described by Guralnik *et al.*[46]

Grip Strength

Grip strength was measured using the Jamar Plus dynamometer device (Patterson Medical, Nottinghamshire, United Kingdom). Each subject was positioned in a straight-backed chair with both feet placed flat on the floor. Grip strength (in kg) was determined in the dominant hand. Subjects were instructed to keep an upright posture, with the elbow flexed at 90° and the forearm and wrist in neutral position. The subject was verbally motivated to provide maximum grip force. Measurements were performed three times per occasion by the same investigator and the highest force was used for further analysis.

Quadriceps Strength

Maximal voluntary strength testing of the right quadriceps muscle was assessed using a handheld dynamometer (CITEC, type CT 3001, C.I.T. Technics, Haren, The Netherlands). Such devices have previously been utilized and validated for lower limb muscle strength testing[48] and correlate well with other methods such as isokinetic strength testing to assess lower limb muscle strength.[49] The subjects were laying on an examining table in prone position with the right knee flexed to a 90° angle with the dynamometer placed against the instep. Maximal voluntary strength in the quadriceps was exerted by extending the knee joint with the investigator keeping the position of the dynamometer fixed. Subjects were verbally motivated to apply maximal voluntary eccentric force.[50] Measurements were performed three times per occasion by the same investigator and the highest force was used for further analysis.

Postural stability

The sway of a subject was assessed in a single, horizontal plane. A string was attached to the subject's belt to measure postural stability with closed eyes to exclude the influence of vision on postural control.[51] The total amount of sway (in mm) was measured over a time period of 2 minutes.

In vivo mitochondrial function measurements

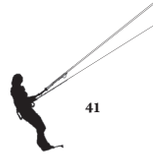
31 phosphorus – Magnetic Resonance Spectroscopy (31P-MRS)

31P-MRS scanning to determine mitochondrial function *in vivo* has been widely employed in characterizing the mitochondrial function status in multiple disease populations.[13,52-55] Dynamic 31P-MRS was performed on a 7-tesla human MRI scanner (Phillips, Best, The Netherlands) on the right posterior calf using a custom-built 8×6 cm^{31} P-surface coil during exercise on a specially-designed MRI-compatible pedal. Subjects were tested in the morning in the fed state. The pedal was designed to allow the study subjects to perform isometric plantar flexion exercise by pressing against a foot pedal while lying in the supine position. The right foot was strapped firmly to the exercise device using non-elastic Velcro straps proximal to the base of the fifth digit with the right knee supported. The subject's lower extremity was secured to the MRI table with straps across the mid-thigh and mid-lower leg in order to isolate usage of the posterior calf muscles. Subjects were instructed to sub-maximally flex the right foot in order to contract the calf muscle and thus to decrease the phosphocreatine levels, which could be monitored real time by the investigator. Muscle force was built up over the exercising period up to maximal voluntary contraction. The subjects performed plantar flexions for a maximum of 3 minutes with rest intervals between (2.5 seconds exercise, 1.5 seconds rest). The scanning protocol consisted of localizer sequences and the acquisition of a field map for shimming purposes using a custom-built outer partial volume coil tuned to the proton frequency. Thereafter,[31] P MRS data were acquired before, during and after exercise with a time resolution of 1 second. Peak integrals of the inorganic phosphate (PI) and PCR signals were obtained using the JMRUI software package (version 5.0, JMRUI Consortium). The frequency difference between PCR and PI was used to calculate tissue pH. Scans with an end-of-exercise pH of < 6.8 were excluded from the analysis, as determination of the PCR recovery rate in this situation is unreliable.[56] In such a case, subjects were rescanned once with a minimal time of 15 minutes in between scans. Mitochondrial function was determined by plotting the PCR peak integrated area against the time during exercise recovery.[57] Recovery curves were fitted to a mono-exponential function to determine the PCR recovery rate (τPCR) using a custom made MatLab script (version 2012b). τPCR recovery rates were compared between the occasions. Outliers were manually removed using the MatLab script.

Ex vivo biomarkers for mitochondrial function

Muscle biopsy procedure

Muscle biopsy were performed around noon, after a 5-hours fasting period following breakfast. Tissue was collected from the *vastus lateralis* muscle of the right leg using a 4.5 mm Bergström muscle biopsy needle (Maastricht Instruments, Maastricht, The Netherlands). The subject was placed in a semi-supine position with the knees supported and slightly flexed. The lateral side of the leg was palpated to determine the location of biopsy, 10 cm proximal of the upper pole of the patella on a line between the patella and the anterior superior iliac spine. After disinfecting the skin, the skin and muscle fascia were locally anaesthetized with a 5 mL lidocaine 5% solution. More lidocaine (up to 10 mL) was administered when the anaesthetic effect was not sufficient. During all procedures it was ensured that the lidocaine did not infiltrate the muscle. A sterile cloth with a hole was placed on the leg, keeping the biopsy site exposed. A small incision of 5 mm was made in the skin and the muscle fascia was incised minimally, just wide enough for the biopsy needle to pass through. The biopsy needle was introduced via the skin and fascia into the muscle. A vacuum was applied on the needle using a sterile 20ml syringe to increase the muscle yield.[58] Immediately after collection the samples were weighed to determine if more tissue was required. After collecting the required amount of muscle tissue, the wound was closed with a single non-absorbable skin suture and pressure was applied by an elastic bandage. Subjects were instructed not to perform strenuous physical activity with the right leg for two days. The suture was removed after 7 days at the clinical research unit. Tissue collected for DNA quantification and protein analysis was snap frozen in liquid nitrogen within 30 minutes of collection and stored at -80°C. All the analysis performed on muscle biopsies were done in a blinded fashion by independent laboratory personnel and only the initials of the subject and date of collection of muscle biopsy samples were listed on the samples.



Preparation of protein lysates from muscle biopsies

Frozen tissue samples were embedded in Cryoembedding Medium (Medite, Germany) and cut into 20 µm sections using a CryoStar NX70 cryostat (Fisher Scientific, Schwerte). For protein analysis by ELISA, tissue slices were incubated on ice for 30-60 min in 1xLM lysis buffer from NADH dehydrogenase (Complex I) Human SimpleStep® ELISA kit (Abcam, ab178011). Buffers were added in complete mini Protease Inhibitor Cocktail (Roche, Germany), HALT Protease inhibitor (Fisher Scientific, Germany) and Phosphatase inhibitor cocktails 2 and 3 (Sigma, Germany). Lysates were vortexed before centrifugation for 10 min at 13,000 x g to precipitate tissue debris. Supernatants were aliquoted and stored at -80°C until analysis. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Sigma, Germany).

Quantification of mitochondrial respiratory complexes abundance and activity by ELISA in muscle biopsies

Protein lysates were analyzed for complex I abundance using the NADH dehydrogenase Human SimpleStep® ELISA kit (Abcam, ab178011), for complex IV abundance using the cytochrome c oxidase Human SimpleStep® ELISA kit (Abcam, ab179880) and for complex V using the ATP synthase Human Profiling ELISA kit (Abcam, ab124539). Mitochondrial respiratory complex activity was determined using the Complex I enzyme activity microplate assay kit (Abcam, ab109721), the Complex II enzyme activity microplate assay kit (Abcam, ab109908) and the Complex IV human enzyme activity microplate assay kit (Abcam, ab109909). Protein abundance or activity were determined using the linear range of a standard curve made with HELA cells lysates (complexes I and IV abundance), with HEPG2 cells lysate (complex V abundance) or bovine heart mitochondrial lysate (BHM) (Abcam, ab110338) (complexes I, II and IV activity). All assays were performed following the manufacturer's instructions. In addition, all assays were validated to ensure that the signal was in the linear range of the detection system.

DNA extraction

Muscle samples were incubated overnight in 360 µl of buffer ATL and 40 µl Proteinase (Qiagen, USA) at 55°C in a thermomixer set at 300 rpm. Cell debris were removed by centrifugation and 200 µl of clear lysates were placed in the QIAsymphony SP workstation (Qiagen, USA). DNA was extracted with the QIAsymphony DNA Mini kit (Qiagen cat# 937236) following manufacturer's procedures.

Quantitative PCR analysis

Quantitative PCR was performed on the Fluidigm Biomark system following the Fluidigm Specific Target Amplification Quick Reference (Fluidigm, USA). Samples were loaded as technical triplicates. The real-time PCR data were analyzed using the Linear Derivative baseline correction and User (detector) Ct threshold method on the latest version of the Fluidigm BioMark software (ver. 4.1.3). Quantification of mitochondrial DNA (mtDNA) was performed using two customized Taqman assays targeted against a nuclear DNA sequence (RNA18SN1) and a conserved region of mtDNA (MTND1).[59] Relative mtDNA copy number was determined comparing MTND1 to RNA18SN1 signal. All quantifications were determined using the 2-ΔΔCT method and the mean Ct of the technical triplicates.

RNA extraction and cDNA synthesis

Muscle samples were homogenized in 800 µl of buffer RLT plus (Qiagen, USA) plus 2 steel balls using a Tissue Lyser (Qiagen, USA). Cell debris were removed by centrifugation and clear lysates were placed in the QIAsymphony SP workstation (Qiagen, USA). RNA was extracted with the QIAsymphony RNA kit (Qiagen cat# 931636) following manufacturer's procedures.

RNA was quantified and checked for purity on a Nanodrop-8000. RNA integrity was controlled using RNA 6000 Nano LabChip kit (Agilent technologies cat# 5065-4476) on an Agilent Bioanalyzer (Agilent technologies).

Analysis of gene expression by microarray

2 ng of total RNA was run on a GeneChip® Human Transcriptome Array 2.0 (Affymetrix) after CDNA synthesis following manufacturer's instructions. The array was read on a GeneChip 3000 Scanner (Affymetrix). The data were normalized with the SST-RMA method (SST = Signal Space Transformation; RMA = Robust Multi-array Average).

GSEA method

All the analysis were done using the R statistical programming language program and Bioconductor R libraries.[60-61] The genes were ordered in a ranked list according to the magnitude and direction of their differential expression between the pre-frail and active groups using the limma package. Genesets used for the GSEA analysis were taken from the Human Gene Ontology (GO) categories (http://www.geneontology.org). Genesets were considered significantly up- or downregulated with a false discovery rate (FDR) ≤ 0.1. Network representation of the significantly downregulated genesets in pre-frail subjects was based on the similarity coefficient (i.e. number of genes in common between two genesets) and was done using Cytoscape 3.4.0.

Statistical analysis

This is an exploratory study and as such the subject number was based on previously published studies conducted in sedentary elderly.[11] To establish whether significant effects could be detected on the repeatedly measured pharmacodynamic parameters, each parameter was analyzed with a mixed model analysis of covariance (ANCOVA) with treatment, time, sex and the interactions as fixed factors and subject as random factor and the (average) baseline measurement as covariate. Pre-frail and active subjects were matched in pairs based on age, gender and physical activity status. The Kenward-Roger approximation was used to estimate denominator degrees of freedom and model parameters are estimated using the restricted maximum likelihood method. For the physical performance tests, statistical significance was computed on the screening values for the skeletal muscle index and for the level of physical activity assessed by IPAQ. For all the other parameters that were measured at day 1 and day 14, the statistical significance was determined between the two groups on the averaged values between day1 and day 14. The general group effect and specific contrasts were reported with the estimated difference and the 95% confidence interval, the Least Squares Means (LSM) estimates and the p-value. All calculations were performed using SAS for windows v9.4 (SAS Institute Inc., Cary, North Carolina, United States).

Data availability

The genomic dataset generated during the current study are not publicly available due to intellectual property purposes but are available from the corresponding author on reasonable request.

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REFERENCES

1 Fernandez-Garrido J, Ruiz-Ros V, Buigues C, Navarro-Martinez R, Cauli O. Clinical features of prefrail older individuals and emerging peripheral biomarkers: a systematic review. *Archives of gerontology and geriatrics.* 2014;59(1):7-17.

2 Manton KG, Gu X. Changes in the prevalence of chronic disability in the United States black and nonblack population above age 65 from 1982 to 1999. *Proceedings of the National Academy of Sciences of the United States of America.* 2001;98(11):6354-6359.

3 Fried LP, Tangen CM, Walston J, et al. Frailty in older adults: evidence for a phenotype. *The journals of gerontology Series A, Biological sciences and medical sciences.* 2001;56(3):M146-156.

4 Cruz-Jentoft AJ, Baeyens JP, Bauer JM, et al. Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age and ageing.* 2010;39(4):412-423.

5 Werner C. The Older Population: 2010. In: U.S. Department of Commerce EaSA, ed2011.

6 Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell.* 2013;153(6):1194-1217.

7 Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. *Science.* 2011;333(6046):1109-1112.

8 Houtkooper RH, Williams RW, Auwerx J. Metabolic networks of longevity. *Cell.* 2010;142(1):9-14.

9 Kauppila TES, Kauppila JHK, Larsson NG. Mammalian Mitochondria and Aging: An Update. *Cell Metab.* 2017;25(1):57-71.

10 Short KR, Bigelow ML, Kahl J, et al. Decline in skeletal muscle mitochondrial function with aging in humans. *Proceedings of the National Academy of Sciences of the United States of America.* 2005;102(15):5618-5623.

11 Broskey NT, Boss A, Fares EJ, et al. Exercise efficiency relates with mitochondrial content and function in older adults. *Physiological reports.* 2015;3(6).

12 Marzetti E, Calvani R, Lorenzi M, et al. Association between myocyte quality control signaling and sarcopenia in old hip-fractured patients: Results from the Sarcopenia in HIp FracTure (SHIFT) exploratory study. *Experimental gerontology.* 2016;80:1-5.

13 Waters DL, Mullins PG, Qualls CR, Raj DS, Gasparovic C, Baumgartner RN. Mitochondrial function in physically active elders with sarcopenia. *Mechanisms of ageing and development.* 2009;130(5):315-319.

14 Santanasto AJ, Glynn NW, Jubrias SA, et al. Skeletal Muscle Mitochondrial Function and Fatigability in Older Adults. *The journals of gerontology Series A, Biological sciences and medical sciences.* 2015;70(11):1379-1385.

15 Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America.* 2005;102(43):15545-15550.

16 Coen PM, Jubrias SA, Distefano G, et al. Skeletal muscle mitochondrial energetics are associated with maximal aerobic capacity and walking speed in older adults. *The journals of gerontology Series A, Biological sciences and medical sciences.* 2013;68(4):447-455.

17 Broskey NT, Greggio C, Boss A, et al. Skeletal muscle mitochondria in the elderly: effects of physical fitness and exercise training. *J Clin Endocrinol Metab.* 2014;99(5):1852-1861.

18 Zane AC, Reiter DA, Shardell M, et al. Muscle strength mediates the relationship between mitochondrial energetics and walking performance. *Aging cell.* 2017;16(3):461-468.

19 Joseph AM, Adhihetty PJ, Buford TW, et al. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. *Aging cell.* 2012;11(5):801-809.

20 Radom-Aizik S, Hayek S, Shahar I, Rechavi G, Kaminski N, Ben-Dov I. Effects of aerobic training on gene expression in skeletal muscle of elderly men. *Medicine and science in sports and exercise.* 2005;37(10):1680-1696.

21 Melov S, Tarnopolsky MA, Beckman K, Felkey K, Hubbard A. Resistance exercise reverses aging in human skeletal muscle. *PloS one.* 2007;2(5):e465.

22 Leick L, Plomgaard P, Gronlokke L, Al-Abaiji F, Wojtaszewski JF, Pilegaard H. Endurance exercise induces MRNA expression of oxidative enzymes in human skeletal muscle late in recovery. *Scand J Med Sci Sports.* 2010;20(4):593-599.

23 Pilegaard H, Ordway GA, Saltin B, Neufer PD. Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol Endocrinol Metab.* 2000;279(4):E806-814.

24 Schmitt B, Fluck M, Decombaz J, et al. Transcriptional adaptations of lipid metabolism in tibialis anterior muscle of endurance-trained athletes. *Physiol Genomics.* 2003;15(2):148-157.

25 Lammers G, Poelkens F, van Duijnhoven NT, et al. Expression of genes involved in fatty acid transport and insulin signaling is altered by physical inactivity and exercise training in human skeletal muscle. *Am J Physiol Endocrinol Metab.* 2012;303(10):E1245-1251.

26 Dalton JT, Barnette KG, Bohl CE, et al. The selective androgen receptor modulator GTx-024 (enobosarm) improves lean body mass and physical function in healthy elderly men and postmenopausal women: results of a double-blind, placebo-controlled phase II trial. *J Cachexia Sarcopenia Muscle.* 2011;2(3):153-161.

27 Papanicolaou DA, Ather SN, Zhu H, et al. A phase IIA randomized, placebo-controlled clinical trial to study the efficacy and safety of the selective androgen receptor modulator (SARM), MK-0773 in female participants with sarcopenia. *The journal of nutrition, health & aging.* 2013;17(6):533-543.

28 Storer TW, Basaria S, Traustadottir T, et al. Effects of Testosterone Supplementation for 3 Years on Muscle Performance and Physical Function in Older Men. *J Clin Endocrinol Metab.* 2017;102(2):583-593.

29 Rooks D, Praestgaard J, Hariry S, et al. Treatment of Sarcopenia with Bimagrumab: Results from a Phase II, Randomized, Controlled, Proof-of-Concept Study. *J Am Geriatr Soc.* 2017;65(9):1988-1995.

30 Yanai H. Nutrition for Sarcopenia. *Journal of Clinical Medicine Research.* 2015;7(12):926-931.

31 Evans M, Guthrie N, Pezzullo J, Sanli T, Fielding RA, Bellamine A. Efficacy of a novel formulation of L-Carnitine, creatine, and leucine on lean body mass and functional muscle strength in healthy older adults: a randomized, double-blind placebo-controlled study. *Nutr Metab (Lond).* 2017;14:7.

32 Badrasawi M, Shahar S, Zahara AM, Nor Fadilah R, Singh DK. Efficacy of L-carnitine supplementation on frailty status and its biomarkers, nutritional status, and physical and cognitive function among prefrail older adults: a double-blind, randomized, placebo-controlled clinical trial. *Clin Interv Aging.* 2016;11:1675-1686.

33 Gimenes AC, Bravo DM, Napolis LM, et al. Effect of L-carnitine on exercise performance in patients with mitochondrial myopathy. *Braz J Med Biol Res.* 2015;48(4):354-362.

34 Ramirez-Sanchez I, Taub PR, Ciaraldi TP, et al. (–). Epicatechin rich cocoa mediated modulation of oxidative stress regulators in skeletal muscle of heart failure and type 2 diabetes patients. *Int J Cardiol.* 2013;168(4):3982-3990.

35 Ryu D, Zhang H, Ropelle ER, et al. NAD+ repletion improves muscle function in muscular dystrophy and counters global PARylation. *Sci Transl Med.* 2016;8(361):361ra139.

36 Canto C, Houtkooper RH, Pirinen E, et al. The NAD(+) precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell Metab.* 2012;15(6):838-847.

37 Gong B, Pan Y, Vempati P, et al. Nicotinamide riboside restores cognition through an upregulation of proliferator-activated receptor-gamma coactivator 1alpha regulated beta-secretase 1 degradation and mitochondrial gene expression in Alzheimer's mouse models. *Neurobiol Aging.* 2013;34(6):1581-1588.

38 Khan NA, Auranen M, Paetau I, et al. Effective treatment of mitochondrial myopathy by nicotinamide riboside, a vitamin B3. *EMBO Mol Med.* 2014;6(6):721-731.

39 Tomas-Barberan FA, Gonzalez-Sarrias A, Garcia-Villalba R, et al. Urolithins, the rescue of 'old' metabolites to understand a 'new' concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. *Mol Nutr Food Res.* 2017;61(1).

40 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.

41 Marzetti E, Csiszar A, Dutta D, Balagopal G, Calvani R, Leeuwenburgh C. Role of mitochondrial dysfunction and altered autophagy in cardiovascular aging and disease: from mechanisms to therapeutics. *American journal of physiology Heart and circulatory physiology.* 2013;305(4):H459-476.

42 Craig CL, Marshall AL, Sjostrom M, et al. International physical activity questionnaire: 12-country reliability and validity. *Medicine and science in sports and exercise.* 2003;35(8):1381-1395.

43 Janssen I, Heymsfield SB, Baumgartner RN, Ross R. Estimation of skeletal muscle mass by bioelectrical impedance analysis. *Journal of applied physiology.* 2000;89(2):465-471.

44 Steihaug OM, Gjesdal CG, Bogen B, Ranhoff AH. Identifying Low Muscle Mass in Patients with Hip Fracture: Validation of Biolectrical Impedance Analysis and Anthropometry Compared to Dual Energy X-ray Absorptiometry. *The journal of nutrition, health & aging.* 2016;20(7):685-690.

45 Pietilainen KH, Kaye S, Karmi A, Suojanen L, Rissanen A, Virtanen KA. Agreement of bioelectrical impedance with dual-energy X-ray absorptiometry and MRI to estimate changes in body fat, skeletal muscle and visceral fat during a 12-month weight loss intervention. *The British journal of nutrition.* 2013;109(10):1910-1916.

46 Guralnik JM, Simonsick EM, Ferrucci L, et al. A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission. *Journal of gerontology.* 1994;49(2):M85-94.

47 Guralnik JM, Ferrucci L, Pieper CF, et al. Lower extremity function and subsequent disability: consistency across studies, predictive models, and value of gait speed alone compared with the short physical performance battery. *The journals of gerontology Series A, Biological sciences and medical sciences.* 2000;55(4):M221-231.

48 Mentiplay BF, Perraton LG, Bower KJ, et al. Assessment of Lower Limb Muscle Strength and Power Using Hand-Held and Fixed Dynamometry: A Reliability and Validity Study. *PloS one.* 2015;10(10):e0140822.

49 Whiteley R, Jacobsen P, Prior S, Skazalski C, Otten R, Johnson A. Correlation of isokinetic and novel hand-held dynamometry measures of knee flexion and extension strength testing. *Journal of science and medicine in sport.* 2012;15(5):444-450.

50 Koblbauer IF, Lambrecht Y, van der Hulst ML, et al. Reliability of maximal isometric knee strength testing with modified hand-held dynamometry in patients awaiting total knee arthroplasty: useful in research and individual patient settings? A reliability study. *BMC musculoskeletal disorders.* 2011;12:249.

51 Wright BM. A simple mechanical ataxia-meter. *The Journal of physiology.* 1971;218 Suppl:27p-28p.

52 Wu JS, Buettner C, Smithline H, Ngo LH, Greenman RL. Evaluation of skeletal muscle during calf exercise by 31-phosphorus magnetic resonance spectroscopy in patients on statin medications. *Muscle & nerve.* 2011;43(1):76-81.

53 Pipinos, II, Shepard AD, Anagnostopoulos PV, Katsamouris A, Boska MD. Phosphorus 31 nuclear magnetic resonance spectroscopy suggests a mitochondrial defect in claudicating skeletal muscle. *Journal of vascular surgery.* 2000;31(5):944-952.

54 Nachbauer W, Boesch S, Schneider R, et al. Bioenergetics of the calf muscle in Friedreich ataxia patients measured by 31P-MRS before and after treatment with recombinant human erythropoietin. *PloS one.* 2013;8(7):e69229.

55 Di Marzo L, Miccheli A, Sapienza P, et al. 31Phosphorus magnetic resonance spectroscopy to evaluate medical therapy efficacy in peripheral arterial disease. A pilot study. *Panminerva medica.* 1999;41(4):283-290.

56 van den Broek NM, De Feyter HM, de Graaf L, Nicolay K, Prompers JJ. Intersubject differences in the effect of acidosis on phosphocreatine recovery kinetics in muscle after exercise are due to differences in proton efflux rates. *American journal of physiology Cell physiology.* 2007;293(1):C228-237.

57 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.

58 Tarnopolsky MA, Pearce E, Smith K, Lach B. Suction-modified Bergstrom muscle biopsy technique: experience with 13,500 procedures. *Muscle & nerve.* 2011;43(5):717-725.

59 Spendiff S, Reza M, Murphy JL, et al. Mitochondrial DNA deletions in muscle satellite cells: implications for therapies. *Human molecular genetics.* 2013;22(23):4739-4747.

60 Team RC. R: A Language and Environment for Statistical Computing. 2015; https://www.r-project.org/.

61 Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43(7):e47.

TABLE 1 Subject demographics.

Demographics	Pre-frail (n = 10)	Active (n = 11)
Age, mean (SD), range, in years	70.2 (5.8), 61-80	70.0 (6.7), 63-78
Sex, n (%) Male Female	5 (50) 5 (50)	6 (55) 5 (45)
Race, n (%) Caucasian Afro Dutch	10 (100) 0 (0)	10 (91) 1 (9)
Body Mass Index, mean (SD), range, in kg/m^2	25.7 (4.2), 17.8-33.2	24.6 (3.9), 20.1-32.0

TABLE 2 Study schedule.

Test	Screening	Visit 1 (Day 1)	Visit 2 (Day 14)
International Physical Activity Questionnaire (IPAQ)	✳		
Body composition by bioelectrical impedance analysis	✳		
Short Physical Performance Battery (SPPB) test	✳	✳	✳
Handgrip strength by Jamar dynamometry	✳	✳	✳
Quadriceps strength		✳	✳
Postural stability		✳	✳
31P-Magnetic Resonance Spectroscopy (31P-MRS)		✳	✳
Muscle biopsy (vastus lateralis)		✳	

TABLE 3 Subject physical performance characteristics. Subject physical performance characteristics. All values are given as mean (SD), range. *: p value <0.05 when comparing group means (between pre-frail and active groups).

Parameters measured at screening		
Physical activity in MET minutes per week	392* (109.2), 262.0-579.0	6508 (5258.5), 2555-19344
Skeletal Muscle Index in kg/m^2	10.90 (2.70), 4.94-13.61	12.10 (2.50), 8.82-16.00
PARAMETERS MEASURED DURING THE STUDY (AVERAGE BETWEEN DAY 1 AND DAY 14)		
Handgrip strength in kg	22.3* (5.7), 12.4-31.2	38.8 (10.3), 23.9-52.3
Quadriceps strength in Newton meters	141* (55.97), 61-220	223 (51.93), 133-302
SHORT PHYSICAL PERFORMANCE BATTERY		
• Total score	10 (1.70), 7-12	11 (1.81), 8-12
• Gait speed in meters per second	0.99* (0.22), 0.46 – 1.19	1.40 (0.20), 1.13 – 1.76
• Duration sit to stand transfer in seconds	13.24* (6.32), 7.66-24.0	9.77 (2.70), 5.75-15.78
• Balance test	3 (0), 3-3	3.18 (0.60), 3-5
Postural stability in mm sway	383 (386.85), 202.5-488.0	392 (413.37), 196.3-970.1

TABLE 4 Sarcopenic genes. Top 10 down-regulated pathways in elderly pre-frail.

Geneset Name	**ES**	**NES**	**FDR**
Go_cellular_respiration	-0.7709094	-3.813652	<1×10-6
Go_mitochondrial_membrane_part	-0.7268397	-3.695907	<1×10-6
Go_mitochondrial_protein_complex	-0.7401110	-3.666778	<1×10-6
Go_oxidative_phosphorylation	-0.7954150	-3.560389	<1×10-6
Go_inner_mitochondrial_membrane_protein_complex	-0.7571530	-3.549975	<1×10-6
Go_organelle_inner_membrane	-0.6057178	-3.547871	<1×10-6
Go_respiratory_chain	-0.7788278	-3.544574	<1×10-6
Go_electron_transport_chain	-0.7635362	-3.467508	<1×10-6
Go_aerobic_respiration	-0.8388420	-3.463427	<1×10-6
Go_mitochondrial_part	-0.5713046	-3.439396	<1×10-6

ES=enrichment score; NES=normalized enrichment score; FDR=false discovery rate.

FIGURE 1 Subject allocation. Flow diagram of the subject enrollment and analysis. Subjects were screened for eligibility up to 45 days before study enrollment. Out of the 38 elderly subjects assessed, 22 were included into the study of which 11 were pre-frail and 11 were active elderly. 21 subjects completed the study and were included in the final analysis. One pre-frail subjects who was non-compliant with the study protocol dietary restriction between the visits on Day 1 and Day 14 was excluded from all analysis except the genomic expression via microarray (*as muscle biopsy had already been collected on Day 1), and was considered a drop-out.

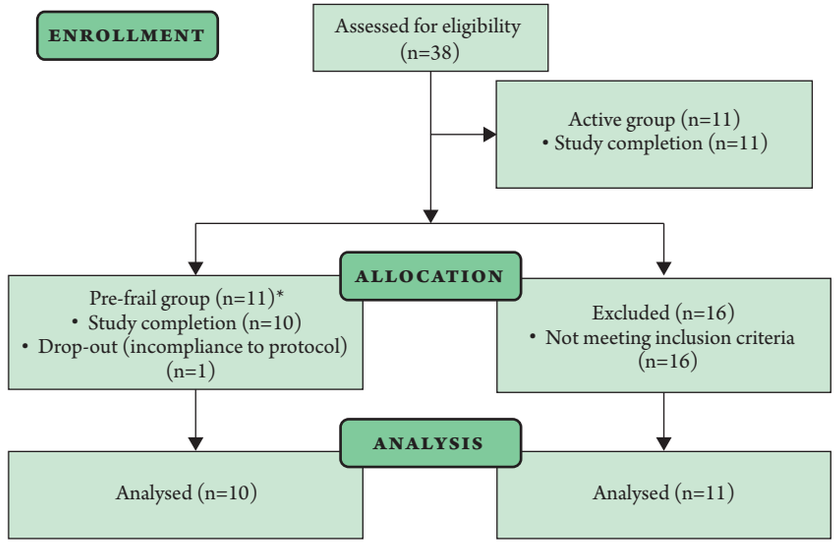
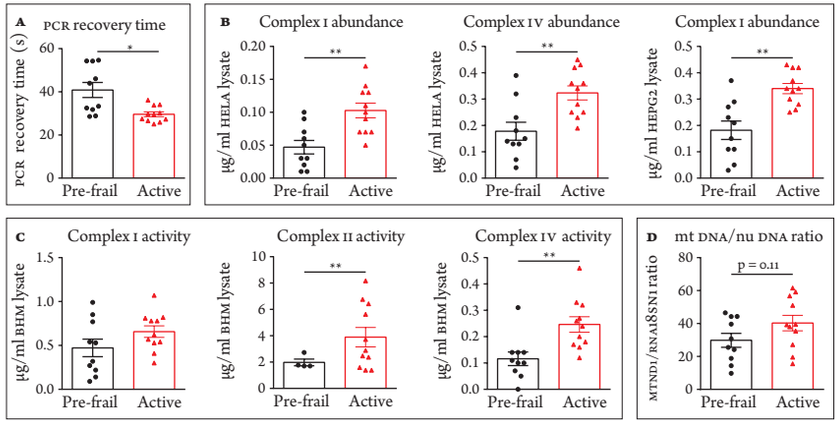


FIGURE 2 A-D **Mitochondrial function.** Mitochondrial function is lower in vastus lateralis of pre-frail subjects. (A) Phosphorus Magnetic Resonance Spectroscopy (31P-MRS) was used to measure PCr recovery time, a marker of mitochondrial function, in the right posterior calf muscle. (B) Mitochondrial respiratory complexes I, IV and V abundance in vastus lateralis. C) Mitochondrial respiratory complexes I, II and IV activity in vastus lateralis. (D) Relative abundance of mitochondrial DNA (mtDNA) over nuclear DNA (nuDNA) measured by qPCR. The ratio was calculated by comparing the relative abundance of mitochondrial encoded NADH dehydrogenase 1 (MTND1) to the nuclear encoded gene RNA 18S ribosomal N1 (RNA18SN1).



*Data represent mean ± SEM. *p<0.05 after a two-tailed Student t test*

FIGURE 3 **Geneset downregulation.** Network representing the genesets downregulated in pre-frail subjects, organized by geneset similarity. Every node is a geneset that is significantly downregulated in pre-frail subjects. The size of the node is dependent on the size of the geneset. The nodes are connected depending on geneset similarity, i.e. on the number of genes that they have in common. The more opaque nodes correspond to the highest normalized enrichment score. Several functions were found more represented than others and are highlighted in color (Mitochondrion; Mitochondrial ribosome and translation; RNA processing and translation; Fatty acid oxidation; Amino acid metabolism; Histone H4 acetyltransferase activity; Proteasome). This figure shows that the Mitochondrion functional group is the most represented and the most downregulated in the prefrail muscle.

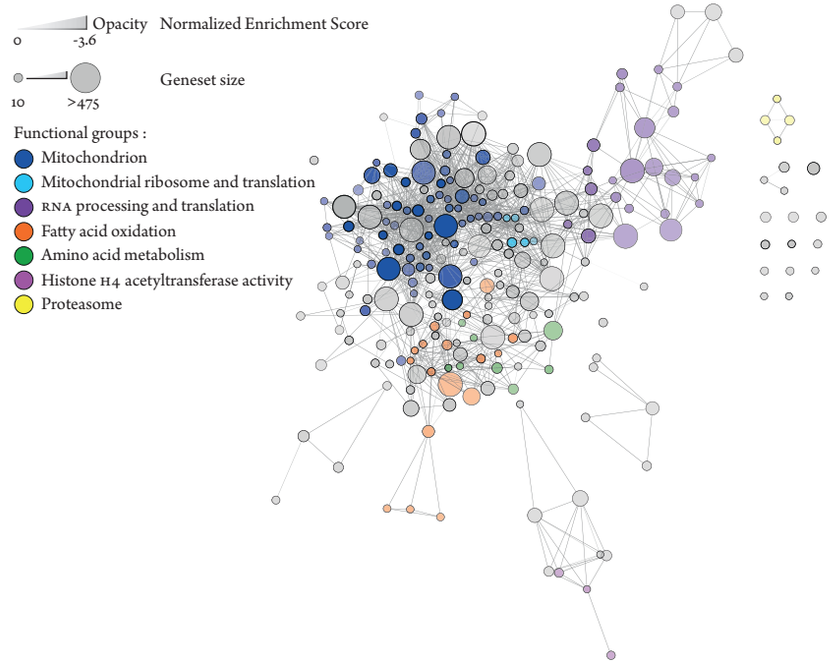
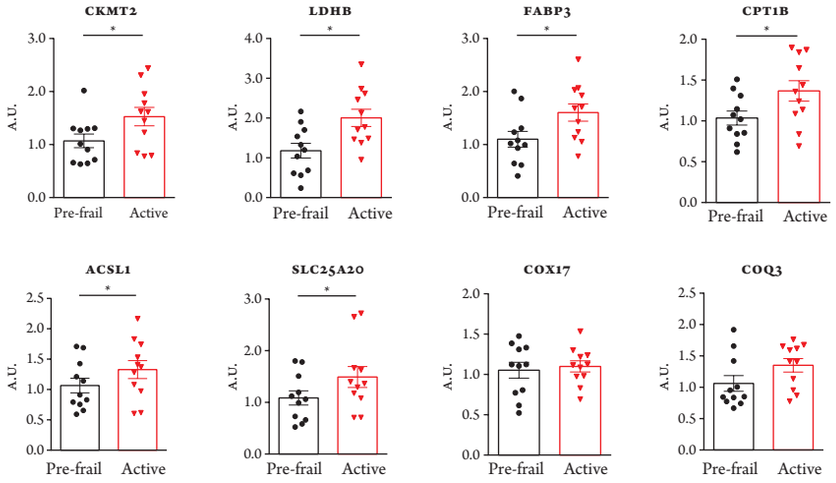


FIGURE 4 **Geneset expression.** Gene expression in muscle biopsies measured by qPCR. Expression of genes, as measured by qPCR, that were in the top 100 of the most differentially expressed genes between pre-frail and active elderly, and in the genesets significantly downregulated in pre-frail subjects.



*Solute carrier family 25 member 20, SLC25A20; acyl-CoA synthetase long chain family member 1, ACSL1; carnitine palmitoyltransferase 1B, CPT1B; fatty acid binding protein 3, FABP3; coenzyme Q3 methyltransferase, COQ3; lactate dehydrogenase B, LDHB; creatine kinase, mitochondrial 2, CKMT2; and cytochrome c oxidase copper chaperone, COX17. Bargraphs represent mean±SEM. *P<0.05 after a two-tailed Student t test.*

CHAPTER III

MITOCHONDRIAL FUNCTION, GRIP STRENGTH AND ACTIVITY ARE RELATED TO RECOVERY AFTER A TOTAL KNEE ARTHROPLASTY

Submitted to Journal of Orthopedic Research

Marcus P.J. van Diemen[1,2] ⋮ Dimitrios Ziagkos[1] ⋮ Matthijs D. Kruizinga[1] ⋮ Menno R. Bénard[3] ⋮ Philip Lambrechtse[1] Joris A.J. Jansen[3] ⋮ Barbara A.M. Snoeker[4] ⋮ Maaike G.J. Gademan[2,5] ⋮ Adam F. Cohen[1 6] ⋮ Rob G.H.H. Nelissen[2] Geert Jan Groeneveld[1,7] ⋮⋮⋮ *1 Centre for Human Drug Research, Leiden, NL ⋮ 2 Department of Orthopedics, Leiden University Medical Center, Leiden, NL ⋮ 3 Department of Orthopedics, Alrijne Hospital, Leiden, NL ⋮ 4 Department of Clinical Epidemiology, Amsterdam Medical Centre, Amsterdam, NL⋮ 5 Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, NL ⋮ 6 Department of Nephrology, Leiden University Medical Center, Leiden, NL ⋮ 7 Department of Anesthesiology, Leiden University Medical Center, Leiden, NL*

ABSTRACT Low muscle quality and a sedentary lifestyle are indicators for a slow recovery after a total knee arthroplasty (TKA). Mitochondrial function is an important part of muscle quality and a key driver of sarcopenia. However, it is not known whether it relates to recovery. In this pilot study, we monitored activity after TKA using a wrist mounted activity tracker and assessed the relation of mitochondrial function on the rate of recovery after TKA. Additionally, we compared the increase in activity as a way to measure recovery to traditional outcome measures.

Patients were studied 2 weeks before TKA and up to 6 months after. Activity was monitored continuously. Baseline mitochondrial function (citrate synthase and complex 1-5 of the electron transport chain) was determined on muscle tissue taken during TKA. Traditional outcome measures (knee osteoarthritis observation score (KOOS), timed up-and-go (TUG) completion time, grip and quadriceps strength) were performed 2 weeks before, 6 weeks after and 6 months after TKA.

Using a multivariate regression model with various clinical baseline parameters, the following were significantly related to recovery: complex 5 abundance, grip strength and activity (regression weights 0.13, 0.02 and 2.89, respectively). During recovery, activity correlated to the KOOS-ADL score (r=0.55, p=0.009) and TUG completion time (r=-0.61, p=0.001).

Mitochondrial function seems to be related to recovery, but so are activity and grip strength, all indicators of sarcopenia. Using activity trackers before and after TKA might give the surgeon valuable information on the expected recovery and the opportunity to intervene if recovery is low.

INTRODUCTION

The total knee arthroplasty (TKA) procedure is the main treatment of end-stage osteoarthritis (OA) affecting the knee joint and one of the most successful orthopedic interventions to date. Worldwide, approximately 1.5 million TKAs are performed annually. In the Netherlands alone, 25.569 were performed in 2018, an increase of almost 40% compared to 2010.[1] The procedure has a 10-year survival of more than 90% and in most cases, patients return to their level of physical activity in 6 months' time.[2-3] However, despite advances in technology and patient care, an estimated 20-25% of procedures have unsatisfactory results, with 16-30% dissatisfaction in functional outcome.[4-6] Many factors have been proposed to predict outcome, including sarcopenia, the age-related decline in muscle quality.[7] It is estimated that up to 44% of TKA patients suffer from sarcopenia.[8] Sarcopenia is hypothesized to be driven partly by dysfunctional mitochondria, which in OA patients is induced by a sedentary lifestyle.[9-12] Muscle cells rely heavily on mitochondria to fulfill their high energy demand and are easily affected by a disturbance in mitochondrial function.[13] Recovery after a TKA procedure depends, amongst other factors, on muscle function, but it is currently not known if mitochondrial function can predict or relates to recovery after a TKA.[3] Traditionally, recovery after orthopedic procedures is evaluated with patient reported outcome measures (PROMS) and functional tests, such as the timed up-and-go test (TUG) or the measurement of quadriceps strength, at a very limited number of outpatient visits.[3] Although important, PROMS are subjective and functional tests, especially when only performed during the very limited number of outpatient visits, do not reflect the patient's activity outside the hospital.[14] With modern technology increasingly being a part of our lives, wearables, such as activity trackers, have become mainstream and widely used.[15] Measuring daily activity objectively and continuously during a period of time, without visiting the outpatient clinic, will not only monitor functional recovery in patients after orthopedic procedures, but will also give leads to be addressed if discrepancies exist with PROM data, which is often the case.[16] Moreover, it will allow the treating surgeon to monitor the recovery online and offer the possibility to adjust rehabilitation if deemed necessary. However, it is not known if activity trackers can accurately track recovery after a TKA and how activity correlates to PROM and other functional measurements.

The primary aim of this study was to assess the relation of mitochondrial function at baseline on the recovery after TKA. The secondary aim was to use an activity tracker to measure that recovery and to evaluate the association between the activity tracker and traditional outcome measures (PROM, TUG, grip strength and quadriceps strength) during the recovery period.

MATERIAL AND METHODS

Design and study schedule

This was an observational, prospective cohort, level 2 evidence study in patients with end-stage OA, who underwent a TKA. Two weeks prior to the TKA, screening for eligibility and baseline measurements were performed to determine the preoperative clinical function. After the TKA procedure, patients visited the outpatient clinic for follow-up visits at 6 weeks and 6 months. Functional measurements were performed during the outpatient clinic visits and activity was monitored continuously up to 6 months.

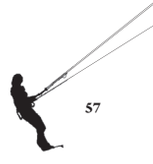
Participants

All patients were recruited from the TKA waiting list at the orthopedic department of the Alrijne Hospital (Leiden, The Netherlands), where the procedure and follow-up measurements took place. Inclusion criteria were a minimal age of 60 years; Indication for a unilateral, primary TKA, due to moderate to severe OA of the knee joint (grade 3-4 on the Kellgren and Lawrence scale); formerly able to walk independently; and ability to use a smartphone. Exclusion criteria were any planned surgery in the contralateral knee within 12 months after study enrollment; a history (within 3 months before screening) of alcohol consumption exceeding 2 standard drinks per day on average; and an underlying chronic disease, which would interfere with study participation or the validity of the measurements.

The study was approved by the independent ethics committee Stichting BEBO (Assen, the Netherlands) and conducted according to the principles of the Helsinki Declaration under registration number NL61972.056.17. Informed consent was obtained from all subjects prior to study enrollment.

Surgical technique

All TKAs were performed via medial parapatellar approach and with tourniquet application. Femoral and tibial components were cemented. Before closure, the knee capsule was locally infiltrated with lidocaine in order to facilitate immediate postoperative mobilization.[17]



Biopsy procedure

From each patient, two muscle tissue samples, 5×5×5mm each, were collected from the vastus lateralis muscle within 5 minutes after tourniquet application. Once collected, visible fat was removed and samples were put in two cryovials and immediately snap frozen in an ice bath of methanol and dry ice within 2 minutes and kept there until the end of the TKA procedure. Samples were transported on dry ice and stored at ≤ −80°C until analysis.

Mitochondrial enzyme assay

The mitochondrial enzyme assay was performed by Metabiolab (Lausanne, Switzerland). Muscle tissue was cut, weighed, crushed and sonicated in a dedicated buffer. Colorimetry to measure enzymatic activity was done on tissue homogenate using the Cobas Modular Analyzer Series (Roche Diagnostics, Basel, Switzerland). Citrate synthase (CS) abundancy was measured directly and used as reference value to calculate complex 1-5 activity. Complex 1 (NADH-ubiquinone reductase (CP1)) activity was measured following NADH's dizsappearance, using rotenone as a specific inhibitor to ensure specificity of the assay.[18] Complex 2 (succinate-ubiquinone reductase (CP2)) activity was measured through the reduction of 2,6-dichlorophenolindophenol, a final electron acceptor, after the addition of succinate.[18] The activity of complex 3 (ubiquinone-cytochrome C reductase (CP3)) was followed with the change of reduction of cytochrome c, whose absorbance is greater in its oxidized form than in its reduced form.[19] Complex 4 (cytochrome c oxidase (CP4)) activity assessment was based on the same assessment, with the use of potassium cyanide to inhibit the activity of the enzyme.[18] Complex 5 (ATP synthase (CP5)) activity was based on the regeneration of ATP through the action of pyruvate kinase and phosphoenolpyruvate carboxykinase.[20]

Knee injury and Osteoarthritis Outcome Score (KOOS)

The KOOS questionnaire evaluates the functional status and quality of life of patients with knee injury and is considered a valid and reliable outcome measure in TKA.[21] The Dutch translation of the questionnaire was used.[22] It was measured prior to surgery and 6 weeks and 6 months after surgery during outpatient visits. The ADL domain has shown best content validity for older patients and best represents functional disability.[23] Therefore, for this study, the total and ADL sub-scale score was used as outcome.

Timed up-and-go test

The TUG is a simple test to reliably assess mobility and balance, in TKA patients.[24] The test consists of measuring the time (in sec) it takes for an individual to get up from a standard chair, walk a distance of 3 m (marked on the floor), turn around, walk back to the chair, and sit down. Patients are not allowed to use their arms to get up from the chair.

Quadriceps strength

Maximal voluntary strength testing of the quadriceps muscle of the operated leg was assessed using a handheld dynamometer (CITEC, type CT 3001, C.I.T. Technics, Haren, The Netherlands). The subjects were positioned on an examining table in prone position with the right knee flexed to a 90° angle with the dynamometer placed against the instep. Maximal voluntary strength in Newton (N) in the quadriceps was exerted by extending the knee joint with the investigator keeping the position of the dynamometer fixed. Subjects were verbally motivated to apply maximal voluntary eccentric force, which was previously shown to produce reliable results.[25] Measurements were performed three times per occasion by the same investigator and the highest force was used for further analysis.

Grip strength

Grip strength has been used in geriatric studies as a measurement of overall physical condition and has shown to be associated with outcome after surgical procedures.[26] Handgrip strength was measured using the Jamar Plus dynamometer device (Patterson Medical, Nottinghamshire, United Kingdom). Each subject was positioned in a straight-backed chair with both feet placed flat on the floor. The force (in kg) was determined of the dominant hand. Subjects were instructed to keep an upright posture, with the elbow flexed at 90° and the forearm and wrist in neutral position. Subject were verbally motivated to provide maximum grip force. Out of three attempts, the highest force was used for further analysis.

Activity tracker

Consumer health devices have been evaluated in monitoring physical activity and show good reliability and validity in measuring parameters such as the daily number of steps.[27] The wrist mounted Nokia Go activity tracker was continuously

worn from 2 weeks before the TKA up to 6 months after. The Nokia Health platform is Android or IOS smartphone-based and the app, the Nokia Health Mate. The patient's own smartphone was used and set-up in such a way to contain no personal data other than height, weight and date of birth. An iPhone was supplied in case a patient did not own a smartphone. Via the app, data were automatically stored online on a secure Nokia server without any intervention required from the patient.

Definition of activity and recovery

Activity was defined as the mean daily number of steps, measured over the period of one week. Baseline activity was the mean number of daily steps over the 2 weeks before TKA. Recovery was defined as the weekly change of the mean daily number of steps over a period of 6 months, starting from the first week after TKA during which activity was down to a minimum and all patients were at home.

Power calculation

Based on the variability in electron transport chain complex IV activity in muscle tissue reported in a previous study with sedentary elderly (coefficient of variability = 60.8%), a sample size of 30 was found to be sufficient to detect an estimated correlation with clinical outcome variables of R = 0.4 with a power of 80% and a two-sided significance level of 0.05, taking into account possible drop-out of patients.[28]

Statistical method

Data were collected by printed data collection forms and entered into Promasys, an electronic data capturing system. Statistics were performed using SPSS v25 (IBM corp., Armonk, NY, USA). The significance of change of clinical measurements between 6 weeks and 6 months after TKA was determined by paired samples t-tests. Activity was correlated to clinical measurements with repeated measures (at 6 weeks and at 6 months after TKA), with a random intercept but no random slope. The relation of baseline predictors (activity, KOOS-A score, quadriceps strength, TUG completion time, grip strength, mitochondrial function, statin use, age, sex and BMI) on recovery was determined using a multivariate regression model with backwards elimination. Results are reported in the change of the number of steps increase per week (i.e. recovery) per unit of the predicting factor. Relation per predictor on recovery was depicted with scatter plots, showing the predicted recovery for minimum, mean and maximum predictor values. Significant predictors were then combined to formulate an algorithm to predict recovery. No correction for multiple testing was made, due to the exploratory nature of the study. Level of significance was p 0.05.

RESULTS

Demographics

A total of 30 patients (20 females and 10 males) were enrolled into the study with a median age of 71 years (range 60-83 years). All enrolled patients completed the required study procedures and were included in the analysis. Demographics are listed in Table 3.1.

Mitochondrial enzymes in muscle

CS and CP 1-5 abundancies at time of surgery are listed in Supplementary Table 3.4. Variability in mitochondrial function between patients was large with ranges of 166 to 558 (CS), 7.7 to 188.8 (CP1), 14.1 to 75.7 (CP2), 0.28 to 7.02 (CP3), 2.8 to 58.2 (CP4) and 229 to 1182 (CP5).

Clinical measurements

Clinical measurements before TKA, at 6 weeks after and 6 months after TKA are depicted in Figure 3.2A-D.

Knee injury and Osteoarthritis Outcome Score

After TKA, scores in all sub-scales were improved at 6 weeks, compared to preoperative, with a further improvement at the last follow-up at 6 months. On the ADL sub-scale, the group had a median score of 48.5 (range 20.6 to 89.7) prior to TKA, a median score of 80.9 (range 41.2 to 95.6,) at 6 weeks postoperatively and a median score of 86.0 (range 57.4 to 100,) at 6 months postoperatively (change from preoperative to 6 months after: CI95% -43 to -28; p<0.001).

Activity and recovery

Activity before surgery showed a group median of 4431 (range 1632 to 10340) daily steps. Directly after surgery, activity was decreased to a minimum of 1237 daily steps which increased during the 6 months thereafter to 4864 (Figure 3.1, change from pre-operative to 6 months after: CI95% -669 to 912; p=0.76). Graphs with individual activity data and a linear fit reflecting the recovery are depicted in Supplementary Figure 3.5. Recovery ranged from 32 to 392 steps per week with a median of 101.

Timed up-and-go test

Prior to surgery, the median TUG time to completion was 12.1 seconds (range 8.0 to 28.1 seconds). During the postoperative period, it decreased to 10.8 seconds (range 8.0 to 28.7) at 6 weeks and eventually to 8.3 seconds (range 6.1 to 26.3) at 6 months (change from pre-operative to 6 months after: CI95% 2.7 to 4.8; p<0.001).

Quadriceps strength

Quadriceps strength decreased after surgery and increased again over the follow-up period. Before surgery median strength was 149 N (range 48 to 265), deceased at 6 weeks to 119 N (range 51 to 245) and increased to 153 N (range 87 to 244) at 6 months (change from pre-operative to 6 months after: CI95% -23 to 9; p=0.39).

Grip strength

Grip strength before surgery (median 31.2, range 14.7 to 60.1) did not significantly change at 6 weeks (median 31.3, range 15.0 to 59.3) and 6 months after (median 32.3, range 14.8 to 57.5, change from pre-operative to 6 months after: CI95% -1.8 to 1.2; p=0.65).

Correlations between activity and clinical function measurements

Measured during the recovery period after TKA, Activity correlated significantly with KOOS ADL (r=0.55, p=0.009) and TUG (r=-0.61, p=0.001), but not to quadriceps strength and grip strength (Table 3.2).

Multivariate regression model to predict recovery

Through backward elimination, the following baseline predictors were significantly correlated with recovery: activity (r=0.51, p=0.004), grip strength (r=0.42, p=0.02) and CP5 abundancy (r=0.38, p=0.05). Separate association plots between predictors and recovery are displayed in Figure 3.3A-C. Combining the predictors, recovery can be estimated by the following algorithm:

$$Recovery = -111.92 + (0.02 \times [activity\ before\ surgery]) + (0.13 \times [CP5\ abundancy]) + (2.89 \times [grip\ strength])$$

For every 1 unit of CP5 abundancy, recovery increases with 0.13 steps/week (p=0.04). For every 1 step of activity, recovery increases with 0.02 steps/week (p=0.005). For every 1 kg of grip strength, recovery increases with 2.89 steps/week (p=0.02). The relation of predictors on the recovery (regression weights) is listed in Table 3.3 and in depicted in Figure 3.4A-C (per predictor, the other two predictors are fixed to the median value).

DISCUSSION

We showed that mitochondrial function (CP5 abundancy), grip strength and activity (mean daily number of steps) before surgery were found to be significantly related to the postoperative recovery in daily number of steps, measured with an activity tracker. Additionally, activity during the recovery period correlated well with traditional patient reported and functional measurements after surgery (KOOS-ADL score, TUG completion score and quadriceps strength).

Age-related declines in walking speed are strongly associated with impairments in mitochondrial function and indices of mitochondrial biogenesis[29,30] and it is known that a sedentary lifestyle itself can reduce mitochondrial function in skeletal muscle.[28] Mitochondrial function, including CP5, was found to be decreased in sedentary, pre-frail elderly, when compared to age-matched active elderly.[28] CP5, or ATP synthase, is the final step in the oxidative phosphorylation process. Congenital deficiency of the enzyme causes severe neuromuscular impairment shortly after birth.[31] The other complexes were not correlated to recovery, but the correlation coefficients between the different complexes were all significant

and ranged from 0.50 to 0.89. The exact mechanism is not fully understood, but it is thought that exercise increases mitophagy, which is the way for the cell to remove dysfunctional mitochondria to be replaced by healthy ones.[28] Although early mobilization after knee replacing surgery has proven to shorten hospital stay, increasing physical activity by exercise before surgery has not proven to significantly improve postoperative recovery.[32,33] Nonetheless, we showed that a higher activity before surgery was correlated to a faster recovery after surgery. The latter may indicate that keeping up a certain degree of mobility prevents sarcopenia. Additionally, adults with a sedentary lifestyle have a higher likelihood for smoking and living with a chronic disease,[34] which also relates to the recovery after surgery. Therefore, activity in terms of mobility might be more informative about outcome after surgery than the amount of exercise per se.

Grip strength reflects the general physical condition of an individual[35-37] and is an important tool in the assessment of frailty in elderly patients.[38] Additionally a low grip strength is associated to a higher mortality in the general population.[39] Grip strength in elderly patients before receiving surgery for a hip fracture correlates to walking recovery.[40] We showed that higher grip strength was correlated to a faster recovery. Grip strength is easy to measure in a clinical setting and could therefore be of value to predict recovery after a TKA.

Measuring mitochondrial function prior to surgery could be a way to select candidates for pre-surgery rehabilitation, but this is not possible in the routine clinical setting due to lack of reliable non-invasive measurements. Monitoring activity is easier and more realistic. Using wearable technology for this is not new and has been shown to be an objective method to measure postoperative recovery.[41] Nevertheless, it is rarely used in routine clinical practice. Due to the increasing availability of modern smartphones, monitoring a patient's activity as part of postoperative recovery is now readily available. The built-in accelerometer and GPS are able to measure not only activity, but also the traveled distance and quality of walking (e.g. slow, fast and sitting), accurately. The data can automatically be shared online with the treating surgeon, providing objective data on mobility and still minimizing patient burden. Knowing patient's activity might lead to a more personalized physiotherapy program early after surgery to have an optimal recovery after this extensive arthroplasty surgery.

Monitoring activity provides the physician with these additional patient data, other than PROMS or functional measurements. PROM outcomes are based on perceived patient satisfaction, which is a very important parameter after TKA, but lack objectivity.[42] It is known that patient satisfaction after a surgical intervention can be negatively influenced by unmet patient expectations[43] , but in our cohort the KOOS-ADL questionnaire correlated to activity. In practice, activity data could be used as a reference on progress to both patient as physician in addition to questionnaire data to gauge recovery after TKA.

Quadriceps strength and TUG completion time also provide objective data and both parameters correlated to activity. This confirms that a simple pedometer can be used to objectively measure recovery after TKA. The correlation to quadriceps strength was borderline moderate, which could be attributed to the fact that it is known to be negatively influenced by voluntary muscle activation [3]. The most important advantage of measuring activity instead of quadriceps strength and TUG completion time is that it can be done daily using just the patient's smartphone, instead of a visit to the outpatient clinic and that it is not affected by voluntary muscle activation. Also, the patient would be able to monitor his/her own recovery, which could work motivational.

Predicting recovery after TKA with an algorithm would provide surgeons in how to follow-up their patients after surgery. For instance, arrangements for intensified physiotherapy or additional visits to the outpatient clinic could be made beforehand. Note that the algorithm should be further validated in a large and independent (and ideally prospective) dataset. Improving these predictive factors prior to surgery might improve postoperative recovery. Although activity would be the easiest factor to measure clinically, multimorbidity or motivational issues may keep patients from improving their sedentary lifestyle.[44] Pharmacologically improving mitochondrial function prior to TKA and in the course of the recovery phase might therefore improve recovery. Several (pre)clinical trials with mitochondrial function enhancing compounds have already shown promising results in treating diseases, in which mitochondrial dysfunction plays a role (such as heart failure, chronic renal failure and myopathy) by protecting mitochondria from oxidation, inducing mitophagy or stabilizing the electron transport chain.[45-48] Clinical trials to improve muscle function by improving mitochondrial function with or without physical exercise in sedentary elderly are currently in the making.[49,50] A possible limitation of this study may be the relatively small number of patients. However, the outcome of this pilot study demonstrates that, contrary to traditional questionnaire based and intermittent, methods of evaluation wearable technology provides meaningful data on individual recovery. Additionally, correlations with measures of muscle strength and mitochondrial function were found

in this study. Although our data have to be confirmed in larger studies we have demonstrated that wearable continuous measurements may allow conclusions drawn from smaller studies and determine the individual value of interventions rather than for groups.

In conclusion, the level of recovery after TKA was significantly related to the level of CP5 abundancy, grip strength and activity before surgery. Activity also strongly correlated to traditional measures for recovery, which could be considered to validate the outcome measure of the activity tracker. Pharmacologically improving mitochondrial function before surgery might be a potential way to improve recovery after TKA.

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REFERENCES

1 Register DA. LRO1 annual report 2019. 2019.
2 Argenson JN, Boisgard S, Parratte S, et al. Survival analysis of total knee arthroplasty at a minimum 10 years' follow-up: a multicenter French nationwide study including 846 cases. Orthopaedics & traumatology, surgery & research: OTSR. 2013;99(4):385-390.
3 Mizner RL, Petterson SC, Snyder-Mackler L. Quadriceps strength and the time course of functional recovery after total knee arthroplasty. The Journal of orthopaedic and sports physical therapy. 2005;35(7):424-436.
4 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.
5 Dunbar MJ, Richardson G, Robertsson O. I can't get no satisfaction after my total knee replacement: rhymes and reasons. The bone & joint journal. 2013;95-b(11 Suppl A):148-152.
6 van de Water RB, Leichtenberg CS, Nelissen R, et al. Preoperative Radiographic Osteoarthritis Severity Modifies the Effect of Preoperative Pain on Pain/Function After Total Knee Arthroplasty: Results at 1 and 2 Years Postoperatively. The Journal of bone and joint surgery American volume. 2019;101(10):879-887.
7 Mizner RL, Petterson SC, Stevens JE, Axe MJ, Snyder-Mackler L. Preoperative quadriceps strength predicts functional ability one year after total knee arthroplasty. |The Journal of Rheumatology. 2005;32(8):1533.
8 Bokshan SL, DePasse JM, Daniels AH. Sarcopenia in Orthopedic Surgery. Orthopedics. 2016;39(2):e295-300.
9 Marzetti E, Calvani R, Cesari M, et al. Mitochondrial dysfunction and sarcopenia of aging: from signaling pathways to clinical trials. The international journal of biochemistry & cell biology. 2013;45(10):2288-2301.
10 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.
11 Waters DL, Mullins PG, Qualls CR, Raj DS, Gasparovic C, Baumgartner RN. Mitochondrial function in physically active elders with sarcopenia. Mechanisms of ageing and development. 2009;130(5):315-319.
12 Lanza IR, Nair KS. Muscle mitochondrial changes with aging and exercise. The American journal of clinical nutrition. 2009;89(1):467s-471s.
13 Fernandez-Vizarra E, Enriquez JA, Perez-Martos A, Montoya J, Fernandez-Silva P. Tissue-specific differences in mitochondrial activity and biogenesis. Mitochondrion. 2011;11(1):207-213.
14 Stevens-Lapsley JE, Schenkman ML, Dayton MR. Comparison of self-reported knee injury and osteoarthritis outcome score to performance measures in patients after total knee arthroplasty. PM & R: the journal of injury, function, and rehabilitation. 2011;3(6):541-549; quiz 549.
15 Omura JD, Carlson SA, Paul P, Watson KB, Fulton JE. National physical activity surveillance: Users of wearable activity monitors as a potential data source. Preventive medicine reports. 2017;5:124-126.
16 Hammer HB, Uhlig T, Kvien TK, Lampa J. Pain Catastrophizing, Subjective Outcomes, and Inflammatory Assessments Including Ultrasound: Results From a Longitudinal Study of Rheumatoid Arthritis Patients. Arthritis care & research. 2018;70(5):703-712.
17 Andersen LO, Husted H, Otte KS, Kristensen BB, Kehlet H. High-volume infiltration analgesia in total knee arthroplasty: a randomized, double-blind, placebo-controlled trial. Acta anaesthesiologica Scandinavica. 2008;52(10):1331-1335.
18 Kramer KA, Oglesbee D, Hartman SJ, et al. Automated spectrophotometric analysis of mitochondrial respiratory chain complex enzyme activities in cultured skin fibroblasts. Clinical chemistry. 2005;51(11):2110-2116.
19 Krahenbuhl S, Talos C, Wiesmann U, Hoppel CL. Development and evaluation of a spectrophotometric assay for complex III in isolated mitochondria, tissues and fibroblasts from rats and humans. Clinica chimica acta; international journal of clinical chemistry. 1994;230(2):177-187.
20 Guerrieri F, Capozza G, Kalous M, Papa S. Age-related changes of mitochondrial FoF1 ATP synthase. Annals of the New York Academy of Sciences. 1992;671:395-402.
21 Roos EM, Toksvig-Larsen S. Knee injury and Osteoarthritis Outcome Score (KOOS)-validation and comparison to the WOMAC in total knee replacement. Health and Quality of Life Outcomes. 2003;1:17.
22 de Groot IB, Favejee MM, Reijman M, Verhaar JAN, Terwee CB. The Dutch version of the knee injury and osteoarthritis outcome score: A validation study. Health and Quality of Life Outcomes. 2008;6:16.
23 Collins NJ, Prinsen CA, Christensen R, Bartels EM, Terwee CB, Roos EM. Knee Injury and Osteoarthritis Outcome Score (KOOS): systematic review and meta-analysis of measurement properties. Osteoarthritis and cartilage. 2016;24(8):1317-1329.
24 Yuksel E, Kalkan S, Cekmece S, Unver B, Karatosun V. Assessing Minimal Detectable Changes and Test-Retest Reliability of the Timed Up and Go Test and the 2-Minute Walk Test in Patients With Total Knee Arthroplasty. The Journal of arthroplasty. 2017;32(2):426-430.
25 Koblbauer IF, Lambrecht Y, van der Hulst ML, et al. Reliability of maximal isometric knee strength testing with modified hand-held dynamometry in patients awaiting total knee arthroplasty: useful in research and individual patient settings? A reliability study. BMC musculoskeletal disorders. 2011;12:249.

26 Sultan P, Hamilton MA, Ackland GL. Preoperative muscle weakness as defined by handgrip strength and postoperative outcomes: a systematic review. BMC Anesthesiology. 2012;12:1.

27 Kooiman TJM, Dontje ML, Sprenger SR, Krijnen WP, van der Schans CP, de Groot M. Reliability and validity of ten consumer activity trackers. BMC Sports Science, Medicine and Rehabilitation. 2015;7.

28 Andreux PA, van Diemen MPJ, Heezen MR, et al. Mitochondrial function is impaired in the skeletal muscle of pre-frail elderly. Scientific reports. 2018;8(1):8548.

29 Joseph AM, Adhihetty PJ, Buford TW, et al. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. Aging cell. 2012;11(5):801-809.

30 Coen PM, Jubrias SA, Distefano G, et al. Skeletal muscle mitochondrial energetics are associated with maximal aerobic capacity and walking speed in older adults. The journals of gerontology Series A, Biological sciences and medical sciences. 2013;68(4):447-455.

31 Kucharczyk R, Zick M, Bietenhader M, et al. Mitochondrial ATP synthase disorders: molecular mechanisms and the quest for curative therapeutic approaches. Biochimica et biophysica acta. 2009;1793(1):186-199.

32 Wang L, Lee M, Zhang Z, Moodie J, Cheng D, Martin J. Does preoperative rehabilitation for patients planning to undergo joint replacement surgery improve outcomes? A systematic review and meta-analysis of randomised controlled trials. BMJ open. 2016;6(2):e009857.

33 Yakkanti RR, Miller AJ, Smith LS, Feher AW, Mont MA, Malkani AL. Impact of early mobilization on length of stay after primary total knee arthroplasty. Annals of translational medicine. 2019;7(4):69.

34 Tudor-Locke C, Craig CL, Thyfault JP, Spence JC. A step-defined sedentary lifestyle index: <5000 steps/day. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2013;38(2):100-114.

35 Allard JP, Keller H, Teterina A, et al. Lower handgrip strength at discharge from acute care hospitals is associated with 30-day readmission: A prospective cohort study. Clinical nutrition (Edinburgh, Scotland). 2016.

36 Davies CW, Jones DM, Shearer JR. Hand grip–a simple test for morbidity after fracture of the neck of femur. Journal of the Royal Society of Medicine. 1984;77(10):833-836.

37 Shyam Kumar AJ, Beresford-Cleary N, Kumar P, et al. Preoperative grip strength measurement and duration of hospital stay in patients undergoing total hip and knee arthroplasty. European journal of orthopaedic surgery & traumatology: orthopedie traumatologie. 2013;23(5):553-556.

38 Cruz-Jentoft AJ, Baeyens JP, Bauer JM, et al. Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. Age and ageing. 2010;39(4):412-423.

39 Cooper R, Kuh D, Hardy R. Objectively measured physical capability levels and mortality: systematic review and meta-analysis. BMJ (Clinical research ed). 2010;341:c4467.

40 Savino E, Martini E, Lauretani F, et al. Handgrip strength predicts persistent walking recovery after hip fracture surgery. The American journal of medicine. 2013;126(12):1068-1075.e1061.

41 Lebleu J, Poilvache H, Mahaudens P, De Ridder R, Detrembleur C. Predicting physical activity recovery after hip and knee arthroplasty? A longitudinal cohort study. Brazilian journal of physical therapy. 2019.

42 Konan S, Hossain F, Patel S, Haddad FS. Measuring function after hip and knee surgery: the evidence to support performance-based functional outcome tasks. The bone & joint journal. 2014;96-b(11):1431-1435.

43 Harris IA, Harris AM, Naylor JM, Adie S, Mittal R, Dao AT. Discordance between patient and surgeon satisfaction after total joint arthroplasty. The Journal of arthroplasty. 2013;28(5):722-727.

44 Layne AS, Hsu FC, Blair SN, et al. Predictors of Change in Physical Function in Older Adults in Response to Long-Term, Structured Physical Activity: The LIFE Study. Archives of physical medicine and rehabilitation. 2017;98(1):11-24.e13.

45 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.

46 Andreux PA, Houtkooper RH, Auwerx J. Pharmacological approaches to restore mitochondrial function. Nature reviews Drug discovery. 2013;12(6):465-483.

47 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.

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

49 Layne AS, Krehbiel LM, Mankowski RT, et al. Resveratrol and exercise to treat functional limitations in late life: design of a randomized controlled trial. Contemporary clinical trials communications. 2017;6:58-63.

50 Andreux PA, Blanco-Bose W, Ryu D, et al. The mitophagy activator urolithin A is safe and induces a molecular signature of improved mitochondrial and cellular health in humans. *Nature Metabolism*. 2019;1(6):595-603.

TABLE 1 **Demographics.** Demographics of study patients (n=30)

Variable	Value
Age (years)	71 (60.0 – 83)
Female sex	20 of 30
Weight (kg)	87 (65 – 113)
BMI (kg/m^2)	29 (22 – 38)
Caucasian	30 of 30

TABLE 2 **Correlations.** Correlations between activity and clinical function measurements, using repeated measures correlation.

Variable	R	p-value
Activity vs KOOS-A	0.55	0.009
Activity vs TUG	-0.61	0.001
Activity vs quadriceps	0.40	0.07

TABLE 3 **Regression weights.** Regression weights per significant predictor, determined by the multivariate regression model.

Predictor	Regression weight	p-value
CP5 abundancy	0.13	0.04
Activity	0.02	0.005
Grip	2.89	0.02

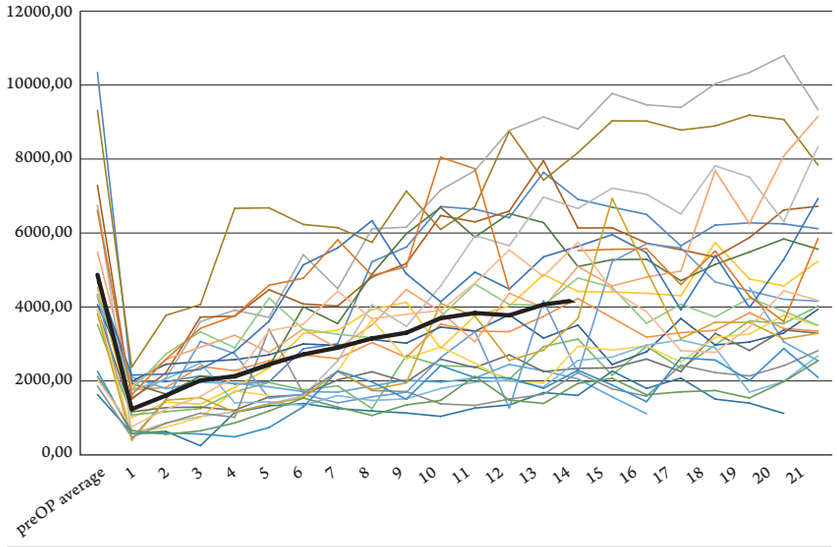
CP5 =complex 5.

TABLE 4 **Mitochondrial enzymes.** Listing of abundance of mitochondrial enzymes.

	Mean (SD)	Min, max
Citrate synthase abundance (U/g prot)	337 (± 111)	166, 558
Complex 1 abundance (U/g prot)	61.4 (± 49.2)	7.7, 188.8
Complex 2 abundance (U/g prot)	30.1 (± 16.0)	14.1, 75.7
Complex 3 abundance (U/g prot)	1.92 (± 1.64)	0.28, 7.02
Complex 4 abundance (U/g prot)	24.3 (± 13.3)	2.8, 58.2
Complex 5 abundance (U/g prot)	497 (± 196)	229, 1182

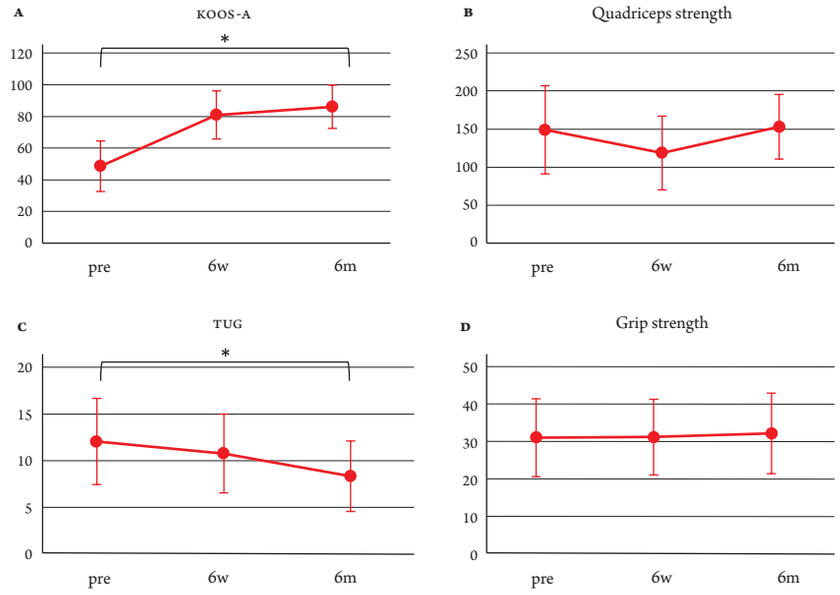
SD = standard deviation, U = unit, Prot = protein.

FIGURE 1 Activity tracker. Activity before surgery until 6 months after (separate line per patient). Activity before TKA was measured over a 2-week period. Activity dropped to a minimum after TKA for all patients.



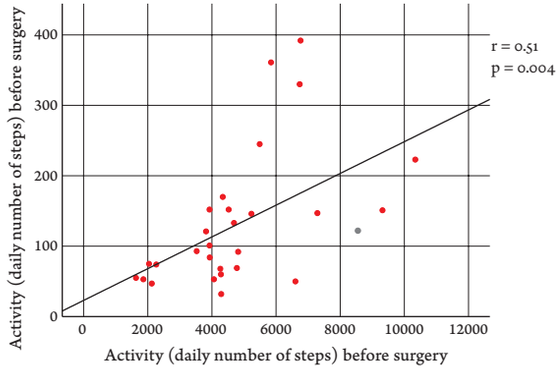
The thick black line represents the group average.

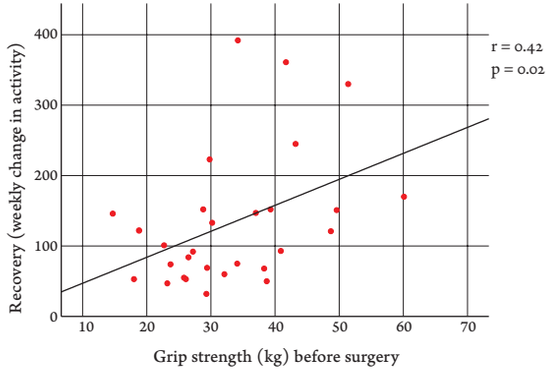
FIGURE 2 A-D Clinical measurements. Clinical measurements over time (median values with SD). A. KOOS ADL, B. quadriceps strength, C. Timed up-and-go completion time and D. grip strength.

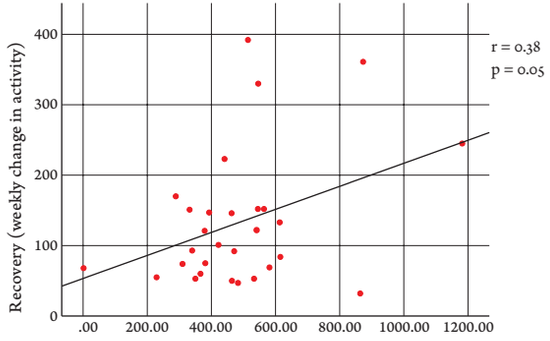


* = $p < 0.05$.

FIGURE 3 A-C **Correlations.** Pearson correlations between recovery (weekly change in activity) and predictors before surgery. Moderate associations with recovery were observed between A. activity; B. grip strength; and C. CP5 abundancy.

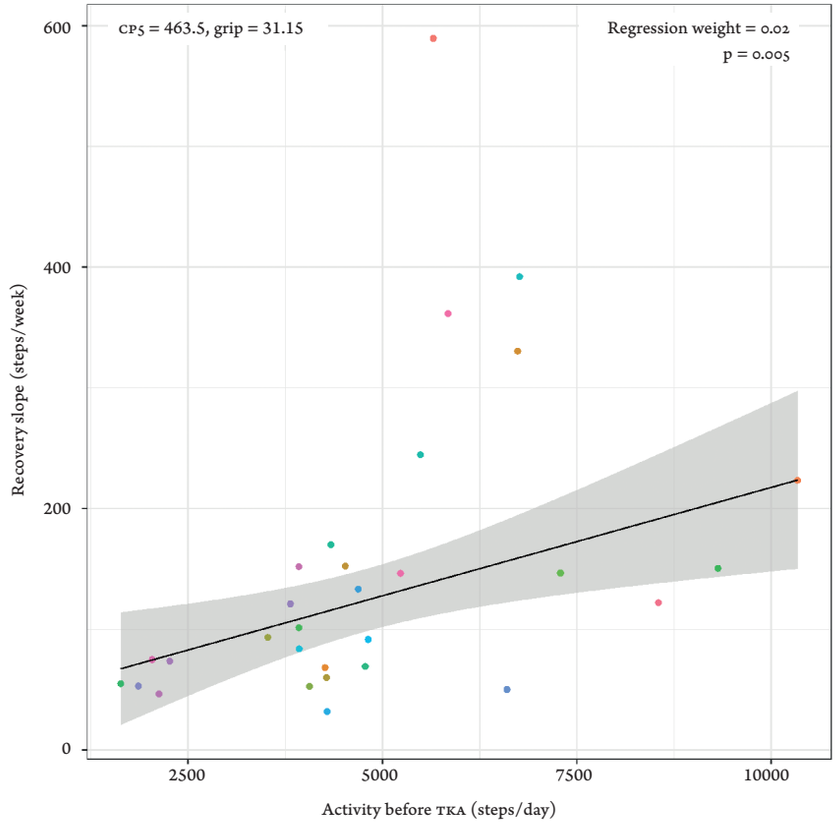






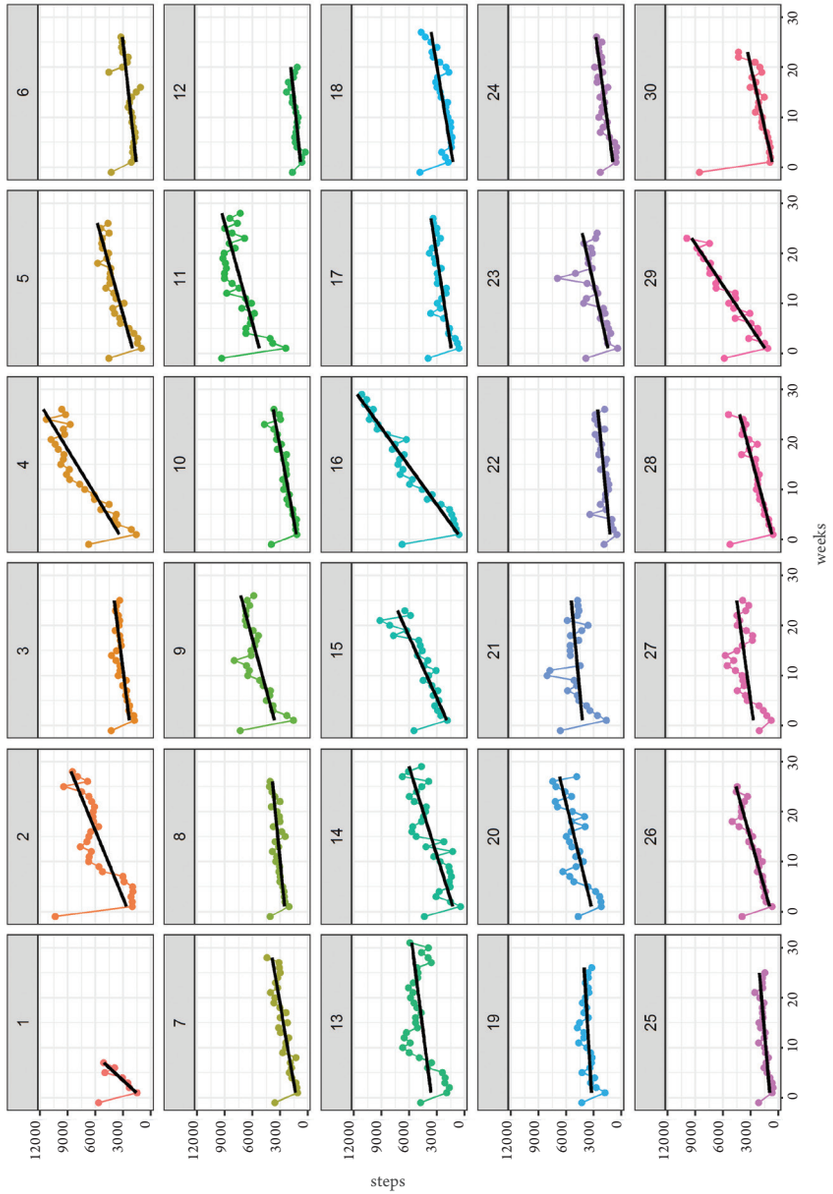
R=0.51, p=0.004. R= 0.42, p=0.02. R=0.38, p=0.05.

FIGURE 4 **Regression model.** Predicted recovery plots with regression weights for different values of correlating predictors. A. activity, B. grip strength and C. CP5 abundancy. 95% confidence intervals depicted as shaded area. Regression weights and p values in legend per graph.



CP5=463.5 , grip=31.15. Regression weight 0.02, p=0.005.

FIGURE 5 **Individual activity.** Individual step count data with linear fit reflecting recovery.



CHAPTER IV

VALIDATION OF A PHARMACOLOGICAL MODEL FOR MITOCHONDRIAL DYSFUNCTION IN HEALTHY SUBJECTS USING SIMVASTATIN: A RANDOMIZED PLACEBO-CONTROLLED PROOF-OF-PHARMACOLOGY STUDY

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Marcus P J van Diemen[1] ⋮ Cécile L Berends[1] ⋮ Naila Akram[1] ⋮ Joep Wezel[2] ⋮ Wouter M Teeuwisse[2] ⋮ Bert G Mik[3] ⋮ Hermien E Kan[2] ⋮ Andrew Webb[2] ⋮ Jan Willem M Beenakker[2] ⋮ Geert Jan Groeneveld[1] ⋮⋮⋮ *1. Centre for Human Drug Research, Leiden, NL ⋮ 2. C.J. Gorter Center for High-field MRI, Leiden, NL ⋮ 3. Erasmus Medical Center, Department of Anesthesiology, Rotterdam, NL*

INTRODUCTION Proof-of-pharmacology models to study compounds in healthy subjects offer multiple advantages. Simvastatin is known to induce mitochondrial dysfunction at least partly by depletion of co-enzyme Q10. The goal of this study was to evaluate a model of simvastatin-induced mitochondrial dysfunction in healthy subjects and to determine whether mitochondrial dysfunction could be pharmacologically reversed by treatment with co-enzyme Q10 (ubiquinol).

METHODS Subjects received simvastatin 40 mg/day for 8 weeks. After 4 weeks, subjects were randomized to receive ubiquinol 300 mg/day or placebo in a double-blinded fashion. Mitochondrial function was assessed by measuring the phosphocreatine recovery time (τPCR) using phosphorous Magnetic Resonance Spectroscopy (31P-MRS) after in-magnet exercise.

RESULTS After 4 weeks of simvastatin treatment, τPCR prolonged with 15.2% compared to baseline, (CI95%, 2.5 to 29.4%; P=0.018). After 8 weeks, τPCR further prolonged to 37.27 seconds in the placebo group (prolongation of 18.5% compared to baseline, still significantly prolonged, CI95%, 1.1 to 38.9%; P=0.037), but shortened to 33.81 seconds in the ubiquinol group (prolongation of 9.1% compared to baseline, no longer significantly prolonged, CI95%, -7.9 to 29.2%; P=0.31). At 8 weeks, there was no significant difference between groups (difference of 8.2%, CI95%, -14.5 to 37.0%; P=0.51).

CONCLUSION Simvastatin induces subclinical mitochondrial dysfunction in healthy subjects, which can be partly reversed by treatment with ubiquinol. This model of pharmacologically induced and reversed mitochondrial dysfunction can be used to study the effects of compounds that enhance mitochondrial function in healthy subjects.

INTRODUCTION

Evidence is growing that dysfunctional mitochondria play a central role in many age-related diseases, such as neurodegenerative diseases, sarcopenia and type 2 diabetes.[1-4] The burden of age-related diseases on elderly and society are significant: in 2000, estimated healthcare costs attributable to sarcopenia in the United States alone were $18.5 billion.[5] Finding new and innovative drug targets in this population is much needed. Mitochondrial dysfunction (MD) is therefore becoming an increasingly important drug target for development by the pharmaceutical industry.[6]

Proof-of-Pharmacology (PoP) studies are designed to identify the viability of candidate molecules for full clinical development in an early phase, by detecting pharmacology on a pathophysiologically relevant mechanism.[7] To keep inter-subject variability at a minimum and drug development costs lower, a POP study is ideally conducted in healthy subjects. A challenge model, pharmacological or non-pharmacological, is typically used, such as scopolamine to induce lower than normal cognitive function, or tryptophan depletion to induce a depressed mood.[8,9] No challenge model yet exists to study mitochondrial dysfunction. Here, we describe a model in healthy subjects, using simvastatin to induce MD and subsequently ubiquinol, the reduced form of co-enzyme Q10 (CoQ10), to reverse it. The induction of MD by statins is based on work from Wu *et al.*, who showed MD in statin users after restarting their therapy, reportedly by inhibition of the CoQ10 biosynthesis, which is the main electron carrier in the mitochondrial electron transport chain (ECT).[10-13] Reversibility of the induced effect, to make sure that the pharmacological effect of the candidate drug or food compound can be shown, is important for a POP model and a vital additional step.[10] Statins have been reported to cause MD by down-stream inhibition of the CoQ10 biosynthesis.[11-13] CoQ10 functions as electron carrier in the mitochondrial electron transport chain (ECT). Primary and secondary deficiencies of CoQ10 result in clinical disease, typically affecting muscular and neurological systems, which highly dependent on mitochondria for energy.[14]

We used 31-phosphorus Magnetic Resonance Spectroscopy (31P-MRS) as gold standard to determine the phosphocreatine (PCR) recovery time (τPCR), which has been validated by *in vitro* respirometry.[15,16] We also determined mitochondrial function using several less burdensome and cheaper alternatives. Oxygen consumption has been proposed to reflect mitochondrial function.[17,18] We determined the oxygen consumption rate (mVO_2) in muscle tissue, using Near Infrared Spectroscopy (NIRS), and mitochondrial oxygen tension ($MitoPO_2$) in the skin, using Protoporphyrin-9 Triplet State Lifetime Technique (PPIX-TSLT). We hypothesized to induce subclinical MD in healthy subjects and to show the pharmacological effect of ubiquinol in reversing the induced MD.

METHODS

The study was conducted as a single center, randomized, double-blind, parallel, placebo-controlled trial. The subject number was estimated based on published study by Wu *et al.* in 10 statin users, in which 4 weeks of treatment with a statin (simvastatin 20 or 40 mg/day, atorvastatin 5 or 10 mg/day or rosuvastatin 5 mg/day) led to MD measured by 31P-MRS.[10] In this study, the mean τPCR increased from 28.1 seconds to 55.4 seconds with an SD of 23.4. The assumption underlying our hypothesis was that ubiquinol suppletion for a period of 4 weeks would completely restore mitochondrial function and would therefore lead to a complete return to baseline τPCR. In order to demonstrate a difference in mean τPCR between ubiquinol and placebo of 27.5 seconds, at least 12 subjects per treatment arm were needed assuming that the common standard deviation is 23, using a two-group t-test with a .05 two-sided significance level. Because of potential drop-outs, a sample size per treatment arm of n=14 was chosen.

Thirty subjects were included (14 females and 14 males, Figure 1), with two subjects dropping out within two weeks after study start. Subjects were medically screened up to 28 days prior to study enrolment for eligibility. Inclusion criteria included; aged between 40 and 70 years and BMI 18-32 kg/m^2. Exclusion criteria included a clinically relevant disease; clinically significant abnormalities on routine chemistry and haematology laboratory; plasma creatine kinase (CK) levels >145 U/L (for females) or > 170 U/L (for males); history of myopathy; diabetes mellitus and/or lower extremity peripheral vascular disease; recent (within 14 days) use of medications with known mitochondrial toxicity (i.e. metformin, statins, paracetamol and Non-Steroidal Anti-Inflammatory Drugs) and vitamin supplements; any contraindication to have a MRI scan; pregnancy in females; a history (within 3 months of screening) of alcohol consumption exceeding 2 units per day on average; a sedentary lifestyle; smoking within 12 hours of the study visits; alcohol consumption within 24 hours of the study visits; and excessive physical activity within 48 hours of the study visits. The study was approved by the independent ethics committee Stichting Bebo (Assen, the Netherlands) according to the principles of the Helsinky Declaration under number NL48758.058.14, and informed consent was obtained from all subjects.

At study enrolment, the subjects were fully randomized by an independent and unblinded statistician, using a random seed in SAS for Windows V9.4 (SAS Institute, Inc., Cary, NC, USA), within two blocks of 14 subjects (ubiquinol or placebo), which were stratified for sex. Jars containing study medication and matching placebos were prepared and labeled by an unblinded pharmacy. The blinded study-physician enrolled the subjects by awarding subject numbers, which were linked to a randomization code. All subjects were treated with film-coated simvastatin 40 mg tablets (Teva Pharmaceutical Industries Ltd, Petah Tikva, Israel) daily for 8 weeks. The dose was chosen to keep adverse effects, most notably statin-associated myopathy, at a minimum based on a large clinical trial comparing simvastatin 20 mg to 80 mg.[19] After 4 weeks of simvastatin treatment, ubiquinol 300 mg capsules (Kaneka QH, Kaneka Corporation, Japan) or matching placebo were administrated daily in parallel for the remaining 4 weeks. Ubiquinol 300mg was chosen, due to the superior bioavailability of ubiquinol and proven safety for a dose up to 300mg.[20] All dosings were orally administered by the subjects at home around dinner time with sufficient still water. Times and dates of administration were noted by the subjects in a medication diary. Compliance with the drug regimen was checked by pill count during each study visit. Throughout the study, subjects with complaints of severe myopathy were excluded.

Subjects were admitted to the Clinical Research Unit of the Centre for Human Drug Research (CHDR, Leiden, the Netherlands) at day 0 (baseline visit before simvastatin treatment), day 14, day 28 (baseline visit before and ubiquinol/placebo treatment) and day 56 (end of treatment period). Measurements (31P-MRS, NIRS, PPIX-TSLT and Jamar dynamometry) were performed during all 4 visits. The 31P-MRS measurements were performed at the Gorter Center for high-field MRI (Leiden University Medical Center, Leiden, the Netherlands). Subjects were contacted by telephone no longer than 10 days after the last visit. Adverse events and concomitant medications were continuously registered throughout the entire study period. Plasma creatine kinase (CK) was measured at baseline, day 14, day 28 and day 56 to monitor sub-clinical signs of statin-induced myopathy.

Phosphorus Magnetic Resonance Spectroscopy (31P-MRS)

31P-MRS was performed on a 7-tesla MRI scanner (Phillips, Best, The Netherlands) on the right posterior calf, using a custom-built 8×6 cm 31P surface coil. An MRI-compatible pedal allowed the subjects to perform isometric plantar flexion exercise while supine. The right foot was strapped firmly to the pedal using non-elastic Velcro straps proximal to the base of the fifth digit with the right knee supported. Additional straps across the mid-thigh and mid-lower leg assured to isolate usage of the posterior calf muscles. Subjects were instructed to near-maximally contract the calf muscles, decreasing the PCR levels to around 50% of baseline, which could be monitored real-time by the investigator. Exercise took 3 minutes with rest intervals between plantar flexions (2.5 seconds contraction, 1.5 seconds rest), in order to keep changes to the blood flow to a minimum. The scanning protocol consisted of localizer sequences and the acquisition of a field map for shimming purposes using a custom-built outer partial volume coil, tuned to the proton frequency. Thereafter, 31P-MRS data were acquired before, during and after exercise with a time resolution of 1 second.

Peak integrals of the inorganic phosphate (PI), PCR and ATP signals were obtained using the JMRUI software package (version 5.0, JMRUI Consortium). The frequency difference between PCR and PI was used to calculate tissue pH. Recovery curves were fitted to a mono-exponential function to determine the τPCR using a custom made MatLab script (version 2012b). Outlying data, deviating more than 5% from the plotted curve over all data points, resulting from noise due to a high amount of overlying subcutaneous fat were manually removed using the MatLab script. Up to 10% could be removed, keeping a sufficient amount of data points to fit the mono-exponential curve.

Protoporphyrin-9 Triplet State Lifetime Technique (PPIX-TSLT)

This novel technique makes use of the oxygen-dependent delayed fluorescence of protoporphin-9, a precursor protein in the heme synthesis, which takes place in the mitochondria. The technique was shown to reflect mitochondrial function in animal models, but has not yet been applied in humans.[21-23] Mitochondrial oxygen tension ($MitoPO_2$) in the skin was measured using the PPIX-TSLT. Before start of the measurements, an Alacare patch (containing 8 mg of 5-aminolevulinic acid hydrochloride (ALA), (Spirig Pharma AG, Germany) was applied at the skin over the sternum, directly below the sternal angle and for 4 hours. Topical application of ALA induces the endogenous synthesis of PPIX in the mitochondria in order for the fluorescent signal, sufficient for detection.[24] The skin was scrubbed with medical fine-grained sandpaper (Prep Skin Red Dot Sand Paper Tape, 3M, Maplewood, Minnesota, United States) and shaved if necessary for improved ALA absorption.

After 4 hours, the patch was removed and a measurement probe was placed on the sternum. After excitation with a pulsed green light, oxygen-dependent red delayed fluorescence was emitted by PpIX. The lifetime of this delayed fluorescence is inversely related to MitoPO_2, which can be calculated using a method described in published work.[22] mitoVO_2 was determined by repeated measurements during local vasoconstriction by applying local pressure with the measurement probe. Pressure was applied after 20 baseline measurements for ±90 seconds. Analysis was performed by fitting an adapted Michaelis-Menten kinetics algorithm to determine the oxygen disappearance rate.[21]

Near Infrared Spectroscopy (NIRS)

MVO_2 was determined by NIRS (InSpectra™ StO_2 Monitor model 325, Hutchinson Technology, Hutchinson, United States), measuring tissue oxygen saturation (StO_2) over time, with a temporal resolution of 2 seconds. The NIRS optode was placed and secured on the left thenar muscle mass. Baseline StO_2 was recorded for 2 minutes before inflating a blood pressure cuff at the upper left arm to 250-300mmHg, inducing vaso-occlusion for 3 minutes. The mVO_2 was determined by the slope of the downward StO_2 curve (%/min) and reflects mitochondrial function.[18] To filter out possible blood-pooling and a plateau phase at the end of the vaso-occlusion, the first and last 30 seconds from the 2-minute vaso-occlusive period were not taken into account in calculating the slope.

Grip Strength

Grip strength was measured using the Jamar dynamometer (Patterson Medical, Nottinghamshire, United Kingdom). Each subject was positioned in a straight-backed chair with both feet placed flat on the floor. Grip strength (in kilograms) was determined in the dominant hand. Subjects were instructed to keep an upright posture, with the elbow flexed at 90° and the forearm and wrist in neutral position. The subject was verbally motivated to provide maximum grip force. The highest grip strength out of two attempts was used for analysis.

Plasma biochemical tests and ubiquinol

Several routine biochemical tests were performed by the chemical clinical laboratory of the Leiden University Medical Center (LUMC, Leiden, The Netherlands). Blood samples were collected at baseline, after 4 weeks and after 8 weeks. CK was performed for safety monitoring, being known as a marker for subclinical myopathy.[25] Triglycerides and (HDL and LDL) cholesterol were measured as a target of simvastatin. In particular, LDL cholesterol concentration was determined since this is a target of statin drugs as well as the main carrier for CoQ10 transport.[26]

Blood for plasma CoQ10 concentration was collected in a Vacutainer® K2EDTA tube (Vacutainer, BD, Franklin Lakes, US), samples were put on ice immediately after collection and centrifuged for 10 minutes at a speed of 2000g and temperature of 4°C. Plasma was divided over 2 aliquots of 1 ml and stored at -80°C. CoQ10 concentration was analyzed in bulk by the Analytical Biochemical Laboratory (Assen, the Nertherlands). using the 'Coenzyme Q10 in serum/plasma/whole blood kit' of Chromsystems according to the instructions in the kit (Chromsystems Instruments & Chemicals GMBH, Gräfelfing, Germany). This reagent kit allowed the chromatographic determination of CoQ10 in an isocratic HPLC run using UV detection. The total CoQ10 was determined in its oxidized form, ubiquinone. During sample preparation, any remaining traces of the reduced CoQ10 (i.e. ubiquinol) were oxidized and the total CoQ10 was analyzed after sample clean-up and sample concentration using solid phase extraction. The responses of the calibration sample included in the kit were used to set the integration parameters whereas two plasma control samples at the levels I (target concentration 520 µg/L) and II (target concentration 884 µg/L) were analyzed to monitor the accuracy and precision of the assay. The overall accuracy (expressed as absolute bias) was < 1.5% and the inter-assay variability (precision) was 8.6% maximally.

Statistical analysis

To establish whether significant effects could be detected on the repeatedly measured pharmacodynamic parameters, each parameter was analysed with a mixed model analysis of covariance (ANCOVA) with treatment, time, sex and the interactions as fixed factors and subject as random factor and the (average) baseline measurement as covariate. Comparisons were made between groups (at 8 weeks) and within groups (between baseline, 4 weeks and 8 weeks). The Kenward-Roger approximation was used to estimate denominator degrees of freedom and model parameters were estimated using the restricted maximum likelihood method. The general treatment effect and specific contrasts were reported with the estimated difference and the 95% confidence interval, the Least Squares Means (LSM) estimates and the p-value. LSM estimates were used to correct for missing data. A p-value of <0.05 was considered to reflect a statistical significant difference. All calculations were performed using SAS for windows V9.4.

RESULTS

Demographics

In total, 28 subjects included for analysis (Figure 1). Baseline characteristics did not differ between the groups (Table 1).

τPCr

An example of a PCR curve is depicted in Figure 2. In 4 occasions, the scan was repeated after 15 minutes of rest due to a pH of below 6.8. The mean τPCR at baseline was 31.22 seconds and prolonged to 35.96 seconds after 4 weeks of simvastatin treatment (prolongation of 15.2% compared to baseline, CI95%, 2.5 to 29.4%; P=0.018, Figure 3). After 8 weeks, the mean τPCR further prolonged to 37.27 seconds in the placebo group (prolongation of 18.5% compared to baseline, still significantly prolonged, CI95%, 1.1 to 38.9%; P=0.037), but shortened to 33.81 seconds in the ubiquinol group (prolongation of 9.1% compared to baseline, no longer significantly prolonged, CI95%, -7.9 to 29.2%; P=0.31). At 8 weeks, there was no significant difference between groups (difference of 8.2%, CI95%, -14.5 to 37.0%; P=0.51). There was no effect of age (P=0.22) or sex (P=0.84).

MitoVO2

The mean mitoVO2 increased from 7.53 mmHg/second to 8.88 mmHg/second over the first 4 weeks of simvastatin administration (increase of 13%, CI95%, -0.014 to 2.716%; P=0.052, Figure 4). Subjects treated with ubiquinol did not show any difference compared to those treated with placebo at 8 weeks (CI95%, -2.446 to 3.060; P=0.82).

mVO2

For all subjects, the mean mVO2 showed a trend of increase from 12.10 %StO_2/min at baseline to 13.50 %StO_2/min after 4 weeks of simvastatin administration (CI95%, -0.383 to 3.181%StO_2/min; P=0.12, Figure 5). In the placebo group, the mVO_2 further increased to 14.58 %StO_2/min compared to baseline (CI95%, -0.274 to 4.884%StO_2/min; P=0.079), whereas in the ubiquinol group it decreased to 12.45%StO_2/min (CI95%, -2.058 to 3.102 %StO_2/min; P=0.55). Difference within or between groups at 8 weeks was not significant.

Grip Strength

Peak grip strength did not change within the first 4 weeks of simvastatin treatment (36.56 to 37.07kg , CI95% -0.854 to 1.633kg; P=0.42) and afterwards in the ubiquinol group (36.60 to 35.67kg, CI95%, -2.687 to 0.830kg; P=0.30) . Interestingly, the peak grip strength increased in the placebo group, compared to baseline (36.52 to 38.56kg, CI95%, 0.277 to 3.795kg; P=0.023), although the clinical relevance of this increase cannot be viewed as significant. The difference between groups at 8 weeks was not significant.

Plasma CoQ10 concentration

Plasma CoQ10 concentration decreased after the first 4 weeks of simvastatin administration from 773.6 mmol/L (N=28) at baseline to 539.2 mmol/L at week 4 (CI95%, -363.2 to 105.6, P=0.0006, Figure 6). At 8 weeks, plasma concentrations increased in the ubiquinol group to 2305.8 mmol/L (CI95%, 1346 to 2173; P<0.0001) and continued being decreased in the placebo group at 436.5 mmol/L (CI95%, -194.8 to 2.4; P=0.06).

Plasma biochemical tests

In 8 subjects (5 males, 3 females) the blood CK level was elevated above the upper limit (171 U/L for males and 145 U/L for females, Table 2). None of these subjects reported muscle related adverse effects. Regarding lipid metabolism, no subjects with subclinical lipid dysmetabolism was present in this cohort (Table 3).

Safety

Adverse effects were recorded during all visits and a follow-up telephone call 7 to 10 days after the 8 weeks visit. In total, 7 subjects (2 males, 5 females) reported muscle related symptoms, such as muscle fatigue or myalgia. There was no relationship between the symptoms and mitochondrial function. Symptoms were mild and resolved with continued simvastatin administration. One male subject experienced intolerable myalgia after 4 days of simvastatin administration, likely related to simvastatin, and the subject was replaced. Two male subjects reported severe adverse events, unrelated to simvastatin. One suffered an acute myocardial infarction and was replaced. The other experienced a mild transient ischemic

attack (TIA). At the first follow-up visit, which occurred 13 days after the TIA, no abnormalities were found on neurological examination. This subject continued the study. No treatment-related adverse effects were noted for ubiquinol.

DISCUSSION

Our main goal was to evaluate a model for subclinical mitochondrial dysfunction (MD) in healthy subjects, using simvastatin to induce MD and ubiquinol to pharmacologically reverse this. Ubiquinol has a higher bioavailability than ubiquinone when administered in the same dose and was therefore chosen.[20] After 4 weeks of simvastatin administration, the τPCR prolonged, which is indicative of MD. This prolongation was lower in magnitude than what was observed in the study of Wu *et al.,* which might be explained by the study population of statin users and the inclusion of two outliers (τPCR of 116.9 and 147 seconds).[10] After 8 weeks, the MD in the ubiquinol group was no longer significantly different from baseline, but continued to be significantly different from baseline in the placebo group. No differences between the groups at 8 weeks were noted, however.

Simvastatin can induce MD in healthy middle-aged subjects, which can be used to demonstrate pharmacological effects of mitochondrial enhancing compounds. Statins inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-COA) reductase and thus to decrease the biosynthesis of cholesterol from mevalonate.[27] The COQ10 biosynthesis shares this common pathway and it has been shown that statins lower the plasma COQ10 concentration.[13,28] COQ10 plays a vital role in the mitochondrial electron transport chain by accepting electrons formed at complex I and II.[29] Although the literature is ambivalent on the matter, there is evidence that, mainly via this route, statins cause MD and the accompanying myopathy.[12,25,30-32] This is in line with secondary deficiencies of COQ10[33-35] and a decrease in the muscle COQ10 concentration after simvastatin administration in a clinical trial by Paiva *et al.*[36] The administration of simvastatin was not placebo controlled, due to these well-known effects.

Glycolysis is another source of ATP and could replace mitochondrial ATP synthesis. As Blei *et al.* explain in their article, glycolysis causes an intra-muscular acidification.[37] In a recent article, Fiedler *et al.* showed that glycolytical ATP synthesis *in vivo* was marginally activated in exercises reaching less than 65% of PCR depletion.[38] Scans with an end-of-exercise pH of <6.8 and/or PCR depletion of less than 25% (or more than 60%) of pre-exercise baseline were excluded from the analysis, as determination of the τPCR in this situation is unreliable[39] and subjects were rescanned once with a minimal time of 15 minutes in between scans.
In a study that was recently performed by Buettner *et al.* and which was published during the performance of our study the investigators also induced MD using simvastatin administration and measured the effects using 31P-MRS.[40] They also found there to be a partial attenuation of MD by additional administration of ubiquinol. However, the two studies were designed with different aims in mind. Our aim was to show feasibility of a POP to study novel drug candidates in healthy volunteers. Firstly, Buettner *et al*. did not study healthy subjects, but long-term statin users. Secondly, the administered statin type and dose varied between subjects. Thirdly, the additional ubiquinol was administered directly from baseline and not after inducing MD. Therefore, our study adds knowledge in developing a pharmacological challenge model to be used in POP studies with drugs that are being developed to treat diseases, where MD plays a pathophysiological role.

Apart from the COQ10 inhibiting effect, simvastatin seems to have a direct effect on the enzyme complexes of the ECT.[41-43] Recently, Schirris *et al.* showed the inhibitory effect of several statins on complex III.[41] *In vitro*, they measured 84% inhibition of complex III activity in C2C12 rat myoblasts, in which the cytotoxic effect of statins resulted in apoptosis. They found that binding of the lactone form of statins to the Q0 binding site inhibited the transfer of electrons from COQ10 to cytochrome c_1, also known as complex III subunit 4, thereby inhibiting the reduction of cytochrome c_1 and disrupting the electron flow between complex III and complex IV. The inhibitory effect was also shown in muscle tissue from human statin users with symptoms of myopathy, in which the inhibition was 18%.[41] Statins are administrated in the acid form and metabolized into the lactone form by uridine 5'-diphospho-glucuronosyl-transferase. Interestingly, due to the polymorphic nature of uridine 5'-diphospho-glucuronosyl-transferases, this might explain the large inter-individual variation in statin-induced myopathy.[44]

Importantly, the direct effect of statins on complex III might well explain the partial reversal of mitochondrial function after administration of ubiquinol. The sample size calculation was made with a full reversal in mind, thereby limiting statistical significance between the ubiquinol and placebo groups at week 8 with our samples size of N=28.

Summarized, the simvastatin-induced MD model affects several key components of the ETC, namely complexes I, II and III. The model therefore provides multiple targets within mitochondria for candidate compounds to improve and to show pharmacology. Proving pharmacology in an early phase is important in

developing a compound, because failing this is the leading cause to discontinue new compounds in phase 2 and 3 trials.[45,46] To prove pharmacology in patients is difficult, because MD is already too severe and variability within the population is too large.[1,2] A pharmacologically lowered mitochondrial function in healthy subjects does not have these limitations.

This study was not without limitations. First, the amount of force exerted by the calf muscles during the 31P-MRS measurement was not standardized. Instead, the decreases in PCR and increase in inorganic phosphate were monitored real-life during the exercising period and subjects were instructed to either increase or decrease their efforts based on the perceived rate of change in PCR. Although a subject of debate, Larson-Meyer *et al.* have demonstrated that the τPCR after exercising with a force of 70% of maximal voluntary contractions compared to 100% is not significantly different.[47] Our instructions to produce near-maximal force, combined with the facts that the PCR decrease was sufficient and the intramuscular pH was kept above the threshold of 6.8 in the analyzed measurements, however, underscore the reliability of the data. Second, The NIRS device that was used did not measure myoglobin, which plays an important role in muscle oxygenation and failing to measure this could underestimates the total oxygen consumption.[48] Finally, mitochondria from different tissues have been shown to vary in activity.[49] The PPIX-TSLT method has been shown to measure mitochondrial oxygen consumption in the skin before and our results suggest that it can be influenced by mitotoxical medications, such as simvastatin.[50] More research is needed to make a full correlation to mitochondrial function in muscle.

Conclusions

In conclusion, we developed a model for MD in healthy subjects using simvastatin to induce dysfunction and ubiquinol to partially reverse. The reason for only a partial reversal is probably due to the direct effect of simvastatin on complex III of the electron transport chain. This pharmacological challenge model can be used to demonstrate proof of pharmacology early in the development of a mitochondrial function-enhancing drug or food compounds.

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REFERENCES

1 Saft C, Zange J, Andrich J, et al. Mitochondrial impairment in patients and asymptomatic mutation carriers of Huntington's disease. Movement disorders: official journal of the Movement Disorder Society. 2005;20(6):674-679.

2 Yan MH, Wang X, Zhu X. Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. Free radical biology & medicine. 2013;62:90-101.

3 Victor VM, Rocha M, Herance R, Hernandez-Mijares A. Oxidative stress and mitochondrial dysfunction in type 2 diabetes. Current pharmaceutical design. 2011;17(36):3947-3958.

4 Konopka AR, Sreekumaran Nair K. Mitochondrial and skeletal muscle health with advancing age. Molecular and cellular endocrinology. 2013;379(1-2):19-29.

5 Janssen I, Shepard DS, Katzmarzyk PT, Roubenoff R. The healthcare costs of sarcopenia in the United States. Journal of the American Geriatrics Society. 2004;52(1):80-85.

6 Andreux PA, Houtkooper RH, Auwerx J. Pharmacological approaches to restore mitochondrial function. Nature reviews Drug discovery. 2013;12(6):465-483.

7 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.

8 Baakman AC, Rissmann R, Klaassen ES, van Gerven JMA, Groeneveld GJ. PK and PD of a nicotinic anticholineric challenge with mecamylamine in comparison to scopolamine. Clinical Therapeutics.37(8):e10-e11.

9 Gijsman HJ, van Gerven JM, de Kam ML, et al. Placebo-controlled comparison of three dose-regimens of 5-hydroxytryptophan challenge test in healthy volunteers. Journal of clinical psychopharmacology. 2002;22(2):183-189.

10 Wu JS, Buettner C, Smithline H, Ngo LH, Greenman RL. Evaluation of skeletal muscle during calf exercise by 31-phosphorus magnetic resonance spectroscopy in patients on statin medications. Muscle & nerve. 2011;43(1):76-81.

11 Bouitbir J, Charles AL, Rasseneur L, et al. Atorvastatin treatment reduces exercise capacities in rats: involvement of mitochondrial impairments and oxidative stress. Journal of applied physiology. 2011;111(5):1477-1483.

12 Diebold BA, Bhagavan NV, Guillory RJ. Influences of lovastatin administration on the respiratory burst of leukocytes and the phosphorylation potential of mitochondria in guinea pigs. Biochimica et biophysica acta. 1994;1200(2):100-108.

13 Littarru GP, Langsjoen P. Coenzyme Q10 and statins: biochemical and clinical implications. Mitochondrion. 2007;7 Suppl:S168-174.

14 Quinzii CM, Hirano M. Primary and secondary CoQ(10) deficiencies in humans. BioFactors (Oxford, England). 2011;37(5):361-365.

15 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.

16 Bendahan D, Mattei JP, Guis S, Kozak-Ribbens G, Cozzone PJ. [Non-invasive investigation of muscle function using 31P magnetic resonance spectroscopy and 1H MR imaging]. Revue neurologique. 2006;162(4):467-484.

17 Ryan TE, Southern WM, Reynolds MA, McCully KK. A cross-validation of near-infrared spectroscopy measurements of skeletal muscle oxidative capacity with phosphorus magnetic resonance spectroscopy. Journal of applied physiology. 2013;115(12):1757-1766.

18 Kemp GJ, Roberts N, Bimson WE, et al. Mitochondrial function and oxygen supply in normal and in chronically ischemic muscle: a combined 31P magnetic resonance spectroscopy and near infrared spectroscopy study in vivo. Journal of vascular surgery. 2001;34(6):1103-1110.

19 Armitage J, Bowman L, Wallendszus K, et al. Intensive lowering of LDL cholesterol with 80 mg versus 20 mg simvastatin daily in 12,064 survivors of myocardial infarction: a double-blind randomised trial. Lancet. 2010;376(9753):1658-1669.

20 Langsjoen PH, Langsjoen AM. Comparison study of plasma coenzyme Q10 levels in healthy subjects supplemented with ubiquinol versus ubiquinone. Clinical Pharmacology in Drug Development. 2014;3(1):13-17.

21 Harms FA, Voorbeijtel WJ, Bodmer SI, Raat NJ, Mik EG. Cutaneous respirometry by dynamic measurement of mitochondrial oxygen tension for monitoring mitochondrial function in vivo. Mitochondrion. 2013;13(5):507-514.

22 Harms FA, Bodmer SI, Raat NJ, Stolker RJ, Mik EG. Validation of the protoporphyrin IX-triplet state lifetime technique for mitochondrial oxygen measurements in the skin. Optics letters. 2012;37(13):2625-2627.

23 Harms FA, de Boon WM, Balestra GM, et al. Oxygen-dependent delayed fluorescence measured in skin after topical application of 5-aminolevulinic acid. Journal of biophotonics. 2011;4(10):731-739.

24 Grishko V, Xu M, Ho R, et al. Effects of hyaluronic acid on mitochondrial function and mitochondria-driven apoptosis following oxidative stress in human chondrocytes. The Journal of biological chemistry. 2009;284(14):9132-9139.

25 Thompson PD, Clarkson P, Karas RH. Statin-associated myopathy. JAMA: the journal of the American Medical Association. 2003;289(13):1681-1690.

26 Berthold HK, Naini A, Di Mauro S, et al. Effect of ezetimibe and/or simvastatin on coenzyme Q10 levels in plasma: a randomised trial. Drug safety. 2006;29(8):703-712.

27 Tobert JA. Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. Nature reviews Drug discovery. 2003;2(7):517-526.

28 Rundek T, Naini A, Sacco R, Coates K, DiMauro S. Atorvastatin decreases the coenzyme Q10 level in the blood of patients at risk for cardiovascular disease and stroke. Archives of neurology. 2004;61(6):889-892.

29 Ernster L, Dallner G. Biochemical, physiological and medical aspects of ubiquinone function. Biochimica et biophysica acta. 1995;1271(1):195-204.

30 Pierno S, De Luca A, Tricarico D, et al. Potential risk of myopathy by HMG-CoA reductase inhibitors: a comparison of pravastatin and simvastatin effects on membrane electrical properties of rat skeletal muscle fibers. The Journal of pharmacology and experimental therapeutics. 1995;275(3):1490-1496.

31 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.

32 Dai YL, Luk TH, Siu CW, et al. Mitochondrial dysfunction induced by statin contributes to endothelial dysfunction in patients with coronary artery disease. Cardiovascular toxicology. 2010;10(2):130-138.

33 Cotan D, Cordero MD, Garrido-Maraver J, et al. Secondary coenzyme Q10 deficiency triggers mitochondria degradation by mitophagy in MELAS fibroblasts. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2011;25(8):2669-2687.

34 Horvath R, Schneiderat P, Schoser BG, et al. Coenzyme Q10 deficiency and isolated myopathy. Neurology. 2006;66(2):253-255.

35 Lalani SR, Vladutiu GD, Plunkett K, Lotze TE, Adesina AM, Scaglia F. Isolated mitochondrial myopathy associated with muscle coenzyme Q10 deficiency. Archives of neurology. 2005;62(2):317-320.

36 Paiva H, Thelen KM, Van Coster R, et al. High-dose statins and skeletal muscle metabolism in humans: a randomized, controlled trial. Clinical pharmacology and therapeutics. 2005;78(1):60-68.

37 Blei ML, Conley KE, Kushmerick MJ. Separate measures of ATP utilization and recovery in human skeletal muscle. The Journal of physiology. 1993;465:203-222.

38 Fiedler GB, Schmid AI, Goluch S, et al. Skeletal muscle ATP synthesis and cellular H(+) handling measured by localized (31)P-MRS during exercise and recovery. Scientific reports. 2016;6:32037.

39 van den Broek NM, De Feyter HM, de Graaf L, Nicolay K, Prompers JJ. Intersubject differences in the effect of acidosis on phosphocreatine recovery kinetics in muscle after exercise are due to differences in proton efflux rates. American journal of physiology Cell physiology. 2007;293(1):C228-237.

40 Buettner C, Greenman RL, Ngo LH, Wu JS. Effects of Coenzyme Q10 on Skeletal Muscle Oxidative Metabolism in Statin Users Assessed Using 31P Magnetic Resonance Spectroscopy: a Randomized Controlled Study. Journal of nature and science. 2016;2(8).

41 Schirris TJ, Renkema GH, Ritschel T, et al. Statin-Induced Myopathy Is Associated with Mitochondrial Complex III Inhibition. Cell metabolism. 2015;22(3):399-407.

42 Kaufmann P, Török M, Zahno A, Waldhauser KM, Brecht K, Krähenbühl S. Toxicity of statins on rat skeletal muscle mitochondria. Cellular and Molecular Life Sciences CMLS. 2006;63(19):2415-2425.

43 Acosta MJ, Vazquez Fonseca L, Desbats MA, et al. Coenzyme Q biosynthesis in health and disease. Biochimica et biophysica acta. 2016;1857(8):1079-1085.

44 Stormo C, Bogsrud MP, Hermann M, et al. UGT1A1*28 is associated with decreased systemic exposure of atorvastatin lactone. Molecular diagnosis & therapy. 2013;17(4):233-237.

45 Arrowsmith J. Trial watch: phase III and submission failures: 2007-2010. Nature reviews Drug discovery. 2011;10(2):87.

46 Arrowsmith J. Trial watch: Phase II failures: 2008-2010. Nature reviews Drug discovery. 2011;10(5):328-329.

47 Larson-Meyer DE, Newcomer BR, Hunter GR, Hetherington HP, Weinsier RL. 31P MRS measurement of mitochondrial function in skeletal muscle: reliability, force-level sensitivity and relation to whole body maximal oxygen uptake. NMR in biomedicine. 2000;13(1):14-27.

48 Spires J, Lai N, Zhou H, Saidel GM. Hemoglobin and Myoglobin Contributions to Skeletal Muscle Oxygenation. Advances in experimental medicine and biology. 2011;701:347-352.

49 Fernandez-Vizarra E, Enriquez JA, Perez-Martos A, Montoya J, Fernandez-Silva P. Tissue-specific differences in mitochondrial activity and biogenesis. Mitochondrion. 2011;11(1):207-213.

50 Harms F, Stolker RJ, Mik E. Cutaneous Respirometry as Novel Technique to Monitor Mitochondrial Function: A Feasibility Study in Healthy Volunteers. PloS one. 2016;11(7):e0159544.

TABLE 1 **Demographics.** Baseline characteristics of subjects included for analysis.

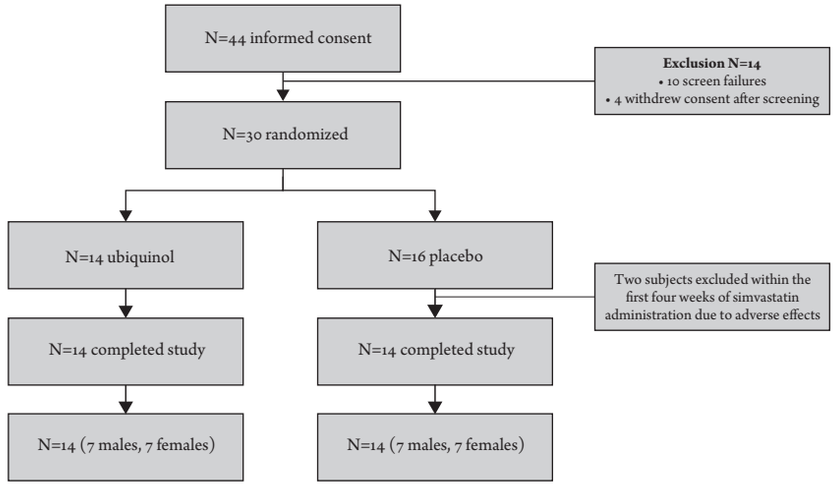
	Ubiquinol	Placebo
Age (years)	61.36 (range 40 – 66)	55.92 (range 44 – 70)
Male (n)	7 (50%)	7 (50%)
Female (n)	7	7
Body mass index (kg/m^2)	25.80 (range 18.29 – 30.37)	25.70 (range 21.13 – 30.98)

TABLE 2 **Plasma creatine kinase and triglycerides.** Creatine Kinase (CK) levels (U/L) and Triglyceride (TG) levels (mmol/L) per subject over the study period.

subject	group	sex	total cholesterol [mmol/l]				LDL cholesterol (Friedewald) [mmol/l]				triglycerides [mmol/l]				creatine phosphokinase [U/L]			
			0 wks	2 wks	4 wks	8 wks	0 wks	2 wks	4 wks	8 wks	0 wks	2 wks	4 wks	8 wks	0 wks	2 wks	4 wks	8 wks
1	ubi	M	4.93	3.08	3.51	3.22	2.94	1.15	1.42	1.24	0.79	0.57	0.74	0.83	68	63	80	81
2	plac	M	5.37	3.38	3.08	3.48	3.32	1.51	1.23	1.30	1.93	1.28	1.43	1.67	89	66	72	83
3	plac	M	5.35	4.02	3.83	2.23	3.63	2.28	2.08	0.95	1.45	1.25	1.57	0.90	108	101	99	101
4	ubi	M	5.17	2.58	3.93	3.50	2.57	0.97	1.70	1.19	3.31	1.11	2.36	2.38	87	113	94	74
6	plac	M	5.64	3.36	3.82	3.69	3.7	1.79	1.92	2.02	1.71	1.11	1.53	1.28	63	2874	56	47
7	ubi	M	6.37	4.22	4.47	4.10	3.72	1.84	2.15	1.67	2.89	2.45	2.21	2.65	43	41	50	53
8	plac	M	5.64	3.94	4.14	4.04	3.36	1.94	2.01	1.90	1.01	0.78	0.88	0.80	66	106	147	123
9	ubi	M	5.32	4.46	3.47	4.03	2.72	1.67	1.62	1.34	1.16	1.18	0.79	0.98	155	183	146	176
11	ubi	M	5.19	3.97	3.52	3.75	3.07	1.74	1.33	1.40	0.92	0.83	0.98	0.83	87	63	94	86
12	plac	M	4.41	2.99	2.77	2.64	2.70	1.39	1.40	1.08	0.63	0.73	0.68	0.76	157	150	142	193
13	plac	M	4.75	3.19	3.44	3.45	3.23	1.78	1.96	1.88	1.00	0.78	0.78	1.00	108	83	94	108
14	ubi	M	5.95	4.56	5.1	4.54	3.58	2.03	2.59	2.35	2.12	2.42	2.28	1.17	71	82	78	187
105	ubi	M	6.54	4.80	4.42	3.96	4.64	2.90	2.41	2.49	1.15	1.14	1.46	0.70	148	160	190	155
110	plac	M	5.73	4.13	3.78	4.12	3.26	2.05	1.93	2.15	1.70	0.78	0.93	1.03	162	126	109	105
51	ubi	F	4.49	2.78	3.21	3.39	2.26	0.79	0.95	1.14	0.46	0.36	0.45	0.47	73	69	57	83
52	plac	F	4.32	3.21	3.14	3.21	2.47	1.36	1.31	1.22	0.96	0.74	0.7	0.73	58	60	61	116
53	ubi	F	4.40	3.99	3.99	3.70	2.57	2.15	1.96	1.70	0.91	0.91	0.91	0.62	39	30	36	40
54	plac	F	4.84	3.52	3.71	3.35	2.91	1.50	1.73	1.12	0.98	0.93	1.03	0.76	70	92	66	138
55	plac	F	4.56	3.25	3.16	3.46	1.9	0.89	0.95	1.18	0.63	0.45	0.45	0.58	76	101	83	152
56	ubi	F	6.33	4.02	4.44	4.36	3.54	1.29	1.62	1.48	0.68	0.56	0.71	0.62	39	34	50	34
57	plac	F	6.38	4.35	4.89	4.53	4.53	2.34	2.96	2.72	1.86	1.99	1.67	1.47	72	MD	84	63
58	ubi	F	4.57	3.59	4.17	4.36	2.01	1.05	1.19	1.62	0.81	0.89	1.01	0.67	82	54	56	54
59	ubi	F	6.44	4.38	5.94	5.69	3.94	1.86	3.34	3.56	0.79	0.69	0.84	0.67	103	94	288	68
60	plac	F	6.78	4.25	4.03	4.19	4.44	2.49	2.1	2.49	2.64	1.02	1.54	0.90	73	68	70	127
61	plac	F	7.4	4.76	4.77	4.28	4.63	2.41	2.56	2.12	3.18	1.94	1.44	2.39	102	110	135	145
62	ubi	F	6.88	5.04	5.43	5.11	4.55	2.69	2.95	2.73	1.48	1.16	1.2	1.37	100	115	114	109
63	ubi	F	6.54	3.92	3.91	3.88	3.87	1.47	1.36	1.22	1.28	0.96	1.17	1.37	112	139	148	152
64	plac	F	5.01	3.61	3.44	3.47	2.59	1.20	1.17	1.16	1.3	0.89	0.76	0.65	56	53	55	127

MD=missing data.

FIGURE 1 **Subject allocation.** Study flow diagram.



Exclusion N=14. 10 screen failures. Withdrew consent after screening. Two subjects excluded within the first four weeks of simvastatin administration due to adverse effects.

FIGURE 2 **Example of phosphocreatine curve .** Example of phosphocreatine curve. Plantar flexion exercising was performed for 3 minutes (between the red and green vertical lines), followed by a period of relaxation. The red line within the curve depicts the fit, from which the PCr recovery time was calculated.

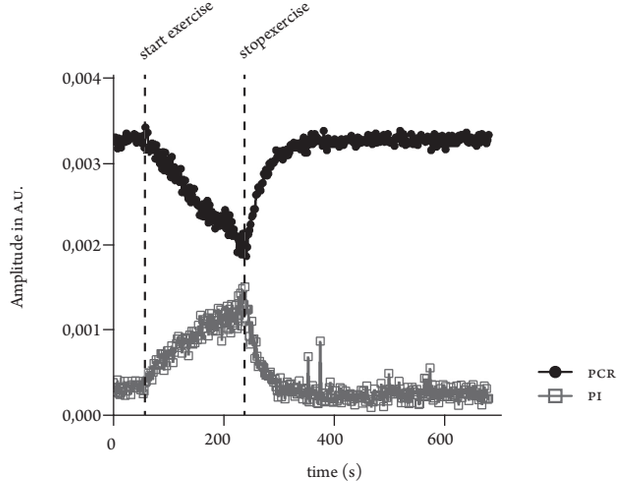
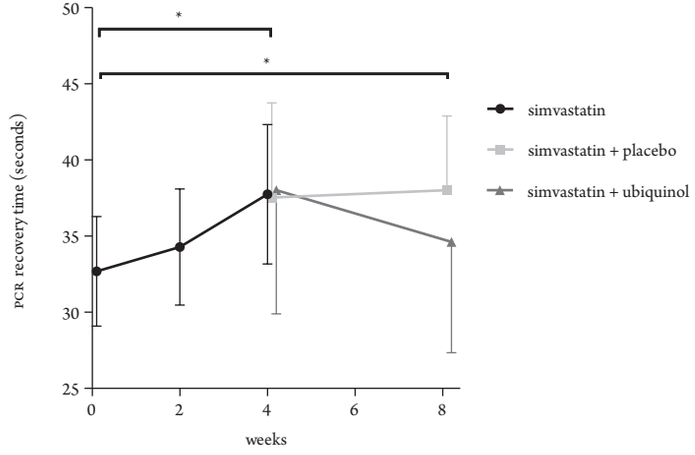


FIGURE 3 **Phosphocreatine recovery time.** τ-PCr after 4 weeks of simvastatin treatment was significantly prolonged compared to baseline by 15.2%. After 8 weeks, the τ-PCr had further prolonged compared to baseline to 18.5% in the placebo group. In the ubiquinol group, however, the τ-PCr prolongation had decreased to 9.1% compared to baseline and was no longer significantly different from baseline. No differences were noted between groups at 8 weeks. Error bars depict 95% confidence interval.



* *p<0.05.*

FIGURE 4 **Mitochondrial oxygen consumption.** Mitochondrial oxygen consumption, measured by the Protoporphyrin IX Triplet State Lifetime Technique (PpIX-TSLT). The different baseline values before addition of placebo or ubiquinol to the simvastatin treatment are depicted. Error bars depict 95% confidence interval. No differences were noted between groups or weeks.

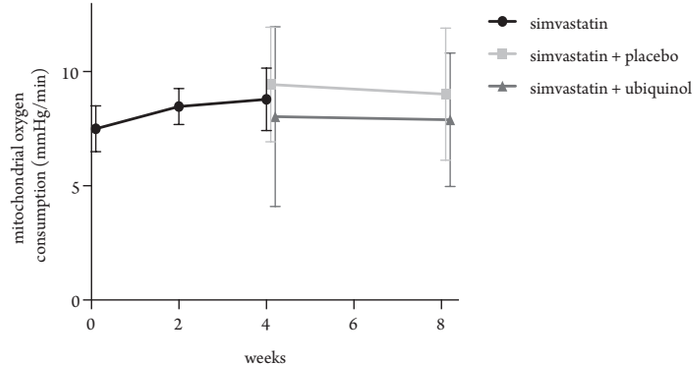


FIGURE 5 **Muscular oxygen consumption.** Decrease in oxygen saturation (mVO_2) in the thenar muscle. Error bars depict 95% confidence interval. No differences were noted between groups or days.

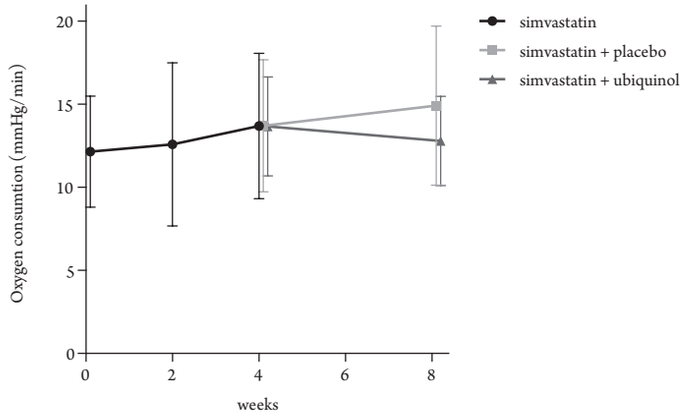
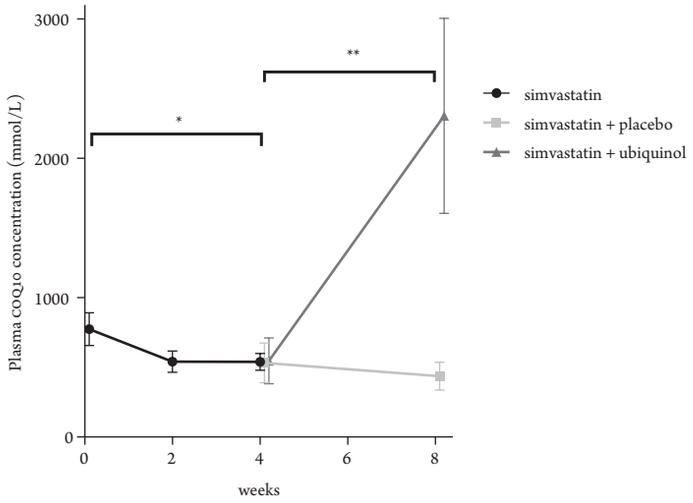


FIGURE 6 **Plasma CoQ10 concentration.** Plasma CoQ10 concentration over time. Triangles: first 4 weeks of simvastatin treatment; dots: simvastatin + placebo treatment; squares: simvastatin + ubiquinol treatment. Concentration significantly increased in the ubiquinol group and decreased further in the placebo group.



*Error bars depict 95% confidence interval. * p<0.05, ** p<0.0001.*

CHAPTER V

SAFETY, PHARMACOKINETICS AND PHARMACODYNAMICS OF SBT-020 IN PATIENTS WITH EARLY STAGE HUNTINGTON'S DISEASE, A TWO-PART STUDY

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Marcus PJ van Diemen[1*] ⋮ Ellen P Hart[1] ⋮ Anthony Abbruscato[4] ⋮ Liz Mead[4] ⋮ Ilse van Beelen[1] ⋮ Sandrin C Bergheanu[4] Pieter W Hameeteman[1] ⋮ Emma Coppen[5] ⋮ Jessica Y Winder[5] ⋮ Matthijs Moerland[1] ⋮ Hermien Kan[2] ⋮ Jeroen van der Grond[3] ⋮ Andrew Webb[2] ⋮ Raymund AC Roos[5] ⋮ Geert Jan Groeneveld[1*] ⋮⋮ *1. Centre for Human Drug Research, Leiden, NL* ⋮ *2. Gorter Centre for high-field MRI, Leiden University Medical Center, Leiden, NL* ⋮ *3. Radiology Research Center, Department of Radiology, Leiden University Medical Center, Leiden, NL* ⋮ *4. Stealth BioTherapeutics Inc., Newton, Massachusetts* ⋮ *5. Department of Neurology, Leiden University Medical Center, Leiden, NL*

INTRODUCTION Huntington's disease (HD) is a neurodegenerative disease with cognitive, motor and psychiatric symptoms. Toxic accumulation of misfolded mutant huntingtin protein induces mitochondrial dysfunction, leading to a bioenergetic insufficiency in neuronal and muscle cells. We evaluated the safety, pharmacokinetics and pharmacodynamics of SBT-020, a novel compound to improve mitochondrial function, in a two-part study in early stage HD patients.

METHODS Part 1 consisted of 7-day multiple ascending dose study to select the highest tolerable dose for Part 2, a 28-day multiple dose study. Mitochondrial function was measured in the visual cortex and calf muscle, using phosphorous magnetic resonance spectroscopy, and in circulating peripheral blood mononuclear cells (PBMCS).

RESULTS Treatment-emergent adverse events were mild and more present in the SBT-020 group. Injection site reactions occurred in 91% in Part 1 and 97% in Part 2. Mitochondrial function in calf muscle, PBMCS or visual cortex was not changed overall due to treatment with SBT-020. In a post hoc analysis, patients with a higher degree of mitochondrial dysfunction (below the median ($\Delta\Psi m < 3412$ and $\tau PCr > 42.5$ s)) showed more improvement than patients with a relatively lower level of mitochondrial dysfunction.

DISCUSSION SBT-020 was safe at all doses, but no significant differences in any of the pharmacodynamic measurements between the treatment groups and placebo group could be demonstrated. The data suggest that the better than expected mitochondrial function in our patient population at baseline might explain the lack of effect of SBT-020.

INTRODUCTION

Huntington's disease (HD) is a hereditary, progressive neurodegenerative disorder, characterized by motor, cognitive and psychiatric deficits. It is caused by an elongated CAG (glutamine) expansion in the gene coding for the huntingtin protein[1] and there is currently no disease-modifying treatment. Its prevalence within Caucasians is approximately 10 per 100,000.[2] Mitochondrial dysfunction plays a central role in the pathogenesis of HD through toxic accumulation of misfolded/mutant huntingtin protein (HTT).[3] *In vivo* assessment of phosphorous metabolism, using phosphorous magnetic resonance spectroscopy (31P-MRS), has previously shown a decreased bioenergetic profile in muscle and brain of (pre) manifest HD gene carriers when compared to healthy volunteers.[4,5] Cardiolipin plays a central role in oxidative phosphorylation by organizing the complexes of the mitochondrial Electron transport chain (ETC), thereby improving the electron flow between complexes. SBT-020 (aka SS-20, H-Phe-D-Arg-Phe-Lys-NH_2) is one of the Szeto-Schiller (SS) proteins, a novel class of small tetra-peptides of which SS-31 (also known as elamipretide) is furthest in clinical development.[6,7] SS-31 and SBT-020 both improve mitochondrial respiration by binding to cardiolipin, a phospholipid which is uniquely expressed on the inner mitochondrial membrane.[8] Cardiolipin peroxidation through cytochrome c in the early stage of apoptosis is essential for the transduction of apoptotic signals and formation of the mitochondrial permeability transition pore (MPTP), a key element to cell apoptosis.[9-11] SBT-020-bound cardiolipin is protected from peroxidation, which optimizes mitochondrial bioenergetics and prevents triggering apoptosis.[11,12] In a 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) induced mouse model of Parkinson's disease, SBT-020 was effective in attenuating injury and improving neurotransmitter release when given systemically,[13] protecting against loss of dopaminergic neurons and causing normalization of dopamine and its metabolites. Additionally, SBT-020 improved cell viability and reduced apoptosis in cultured SN4741 cells (dopaminergic neurons derived from the substantia nigra of transgenic mouse embryos) when exposed to MPTP.[13] The efficacy of SS-31, the predecessor of SBT-020, was shown in a preclinical HD model of cultured mutant HTT expressing nigrastriatal neurons (STHDHQ111/Q111) by normalizing mitochondrial structure and function.[14]

The primary objectives in the current study were assessment of safety, tolerability and pharmacokinetics of SBT-020 in early-stage HD. The secondary objectives were to assess the effect of SBT-020 on central and peripheral mitochondrial function through *in vivo* 31P-MRS measurements, and on mitochondrial membrane potential ($\Delta\Psi m$) measurements in peripheral blood mononuclear cells (PBMCS). Finally, effects on motor and neurocognitive functioning were assessed through the UHDRS and a battery of neurocognitive tests.

MATERIAL AND METHODS

Trial design

This phase II study was conducted at the Centre for Human Drug Research (CHDR, Leiden, The Netherlands) as a single-center, randomized, double-blinded, placebo-controlled trial in patients with early stage HD. It consisted of a 7-day multiple ascending dose-determination part (Part 1) followed by a 28-day multiple dose part (Part 2). In Part 1, 24 patients were randomized into one of three dose cohorts (5mg, 15mg or 25mg) of 8 patients each (6 active, 2 placebo). For Part 2, the same patients were re-randomized into 12 placebo and 12 active, to receive the dose selected from Part 1.

Dosing rationale

The dose of SBT-020 was chosen based on pre-clinical and clinical studies. In a pharmacodynamic, pre-clinical study on the neuroprotective effects of SBT-020 in 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) treated mice, a single dose of 4 mg/kg of SBT-020 attenuated 40% of the MPTP-induced dopamine depletion.[13] In a pre-clinical study on ischemia/reperfusion damage in rats, a single dose of 4 mg/kg SBT-020 significantly reduced infarct size and myocardial lipid peroxidation.[15] This corresponds to a Human Equivalent Dose (HED) of 0.76 mg/kg. For a 60kg human this corresponds to a starting dose of 4.5mg.

5, 10, 20 and 30mg doses were assessed in a subcutaneously administered single and multiple ascending dose study in healthy volunteers, which proved safe and tolerable, but with dose related occurrence of injection site reactions. In this patient study, 25mg was hypothesized to be the effective and safe dose for a 28-day multiple dose study. For safety assessment, a 7-day multiple ascending dose study with 5, 15 and 25mg single and multiple dose part was performed prior to the 28-day multiple dose part.

Study schedule

Part 1 of the study consisted of a screening period for eligibility, a 7-day treatment period and a follow-up visit. Patients were screened for medical status (interview, physical examination, vitals, laboratory and ECG), motor and functional status (UHDRS assessment) and peripheral mitochondrial function (31P-MRS scan of the calf muscles). The 31P-MRS scans were performed at the Leiden University Medical Center (LUMC, Leiden, NL). After randomization, SBT-020 or placebo was subcutaneously administered once daily for 7 days. For the administrations on days 1, 2, and 7, patients were admitted at the Clinical Research Unit (CRU) of CHDR in order to perform pharmacokinetic (PK) and pharmacodynamic (PD) measurements. The administrations on days 3, 4, 5, and 6 were performed at the patient's home by trained staff. Safety (including blood samples for plasma histamine concentrations) and PK measurements were performed continuously on days 1, 2, 7, and 8. PD measurements, 31P-MRS of the calf muscle and blood sampling for measurement of $\Delta\Psi m$, were performed during screening (31P-MRS), 1 hour before dosing on day 1 ($\Delta\Psi m$) and 1.5 hours after the final dose administration on day 7 (31P-MRS and $\Delta\Psi m$). There was a wash-out period of at least 1 month between the end of Part 1 and the start of Part 2 for each patient. Dose escalation in Part 1 was evaluated after completing each dose cohort based on PD, PK and safety.

Part 2 of the study consisted of a re-assessment of eligibility, a 28-day treatment period and a follow-up visit. The set of PD measurements in Part 2 was expanded with central mitochondrial function assessment (31P-MRS scan of the brain) and neurocognitive testing, in addition to the PD assessments included in Part 1. PD measurements, 31P-MRS of skeletal muscle and visual cortex were performed on day -1 (before the first dose administration of Part 2), 1.5 hours after dose administration on day 27 (31P-MRS of the calf muscles and brain and $\Delta\Psi m$ measurements in PBMCS) and 1.5 hours after final dose administration on day 28 (neurocognitive and motor testing). Patients were admitted to the CRU at day 1, 2, 27, and 28 and visited at home on days 7, 14, and 21 for safety assessments (vitals, laboratory and ECG) and trough PK sampling. On the days that the patients were not scheduled to visit the CRU, the daily drug administration was performed at home.

Participants

Patients with mild to moderate HD were included. The main inclusion criteria were: a genetically confirmed CAG repeat expansion of 36 or more repeats in the HTT gene; Total Motor Score (TMS) of 5 or more and Total Functional Capacity Score (TFC) of 7 or more as assessed by the UHDRS; and a time constant of phosphocreatine recovery (τPCR) after a bout of exercise of at least 40 seconds, measured by dynamic 31P-MRS of the calf muscles. This threshold was based on earlier work with 31P-MRS in HD patients, to ensure sufficient mitochondrial dysfunction.[5] The threshold was later lowered to 32.4 seconds to better reflect the early stage HD patient population (see the section on sample size calculation).

The main exclusion criteria were: positive test for drugs of abuse; history (within 3 months of screening) of alcohol consumption exceeding 2 standard drinks per day on average; smoking more than half a pack of cigarettes daily; history of active malignancy within the last 5 years, with the exception of localized or in situ carcinoma (e.g., skin basal or squamous cell carcinoma); positive Hepatitis B surface antigen, Hepatitis C antibody, or human immunodeficiency virus antibody; aspartate transaminase, alanine transaminase, gamma glutamyl transferase or total bilirubin levels >1.5 times the upper limit of normal; renal insufficiency (defined as eGFR < 60 mL/min); history of photosensitive epilepsy; any contraindication to have MRI scans performed; significant cardiac abnormalities on the resting ECG (QTCF >450 or <300 msec, evidence of atrial fibrillation, atrial flutter, complete branch block, Wolf-Parkinson-White Syndrome or cardiac pacemaker); any confirmed significant allergic reactions (urticaria or anaphylaxis) against any drug, or multiple drug allergies (non-active hay fever was acceptable).

Concomitant medications

Paracetamol (up to 4 g/day) and ibuprofen (1 g/day) were allowed before and during the study period. Medications with an effect on cognitive functioning (e.g. antidepressants) were allowed on a stable dose, but medications with known mitochondrial toxicity (e.g. statins and metformin) were not allowed until the end of the study period and needed to be discontinued 21 days before study enrolment if applicable. Use of other medications were allowed under scrutiny of the investigator. The use of hormonal contraceptives was allowed during the study.

PK sample collection

During Part 1 and Part 2, blood and urine samples were collected at various time points to measure plasma concentrations of SBT-020 (Supplementary Table 5.9) in Part 1 a 24-hour profile at day 1 and at steady state was performed, in Part 2 only

trough samples at steady state were measured to assess potential accumulation. Urine was collected for 24 hours during day 1 and 7 of Part 1 to assess renal clearance of SBT-020. Aliquots for plasma and containers for urine were spiked with a 5% formic acid aqueous solution to prevent the compound from binding to the collection materials.

PK concentration measurement

Concentrations of SBT-020 were measured by a validated LC/MS method for both plasma and urine. Sample analysis was performed for patients receiving SBT-020 and not for patients receiving placebo. The lower levels of quantification were 2.5 ng/mL in plasma and 50 ng/mL in urine.

Pharmacodynamics

During Part 1 and 2, measurements for PD were performed at various time points to assess effects on mitochondrial and clinical functioning (Supplementary Table 5.10). Mitochondrial function measurements were performed prior to initiation of drug treatment and at the end of drug treatment in each part of the study. Measurements for central mitochondrial function and motor and neurocognitive function were performed at the start and end of Part 2 only.

Skeletal muscle

Dynamic 31P-MRS in skeletal muscle before, during and after exercise was performed in a 7 Tesla MRI scanner (Phillips, Best, The Netherlands) with surface coil and custom-built MRI-compatible pedal ergometer. The ergometer was designed to allow the patients to perform isometric plantar flexion exercise by pressing against a foot pedal while supine. The foot was strapped firmly to the ergometer and the subject's lower extremity was secured to the MRI table with straps across the mid-thigh and mid-lower leg in order to isolate usage of the posterior calf muscles. The scanning protocol consisted of localizer sequences and the acquisition of a field map for shimming purposes. Thereafter, 31P-MRS data was acquired before, during and after exercise using a pulse-acquire sequence with a time resolution of 2 seconds (flip angle 45 degrees, surface coil localization, 1 signal average). Peak integrals of inorganic phosphate (PI), PCR and ATP signals were obtained using JMRUI software (version 5.0, JMRUI Consortium) and the τPCR was determined by mono-exponential fit using a custom made MatLab script (version 2012b). The frequency difference between PCR and PI was used to calculate tissue pH. The τPCR is considered unreliable when tissue pH is below 6.8[16] and rescanning after a 10-minute break was allowed to reach an end-exercise pH of >6.8. Outlying data (up to 10% of total), deviating more than 5% from the plotted curve overall data points, resulting from noise due to a high amount of overlying subcutaneous fat were removed using the MatLab script.

Visual cortex

31P-MRS of the brain was performed on a 3 Tesla MRI scanner (Philips, Best, The Netherlands). A custom-made 6cm 31P transmit/receive surface coil was used to detect signals from the visual cortex while limiting muscle contamination. A small sphere (Æ 10mm) filled with water was placed below the coil along the coil axis to verify and adjust the positioning of the 31P RF coil on 1H images. An adiabitic pulse-acquire sequence (TR 2 s, flip angle 90°) was used to collect free induction decays (FIDS) for 4 minutes at rest (128 signals averaged), 8 minutes during visual activation (256 signals averaged), and 8 minutes after visual stimulation (256 signals averaged). Analysis of the 31P spectra using JMRUI allowed quantification of the following resonances: βATP, αATP, γATP, PCR, and PI, from which the ratios of PCR/ATP, PI/PCR, and PI/ATP were calculated as well as the pH. The spectra were analyzed in the time domain using AMARES in the JMRUI software. AMARES allowed the inclusion of prior knowledge about relations between peaks (derived from the method of Mochel *et al.*)[4]

Peripheral blood mononuclear cells

The ΔΨm can be used as a general outcome for mitochondrial health, because most mitochondrial inhibition or damage results in a decrease of ΔΨm.[17] The ΔΨm of live PBMCS was assessed using the JC-1 dye and flowcytometry (method described elsewhere).[18] Healthy mitochondria emit different fluorescent (FL-2) than dysfunctional mitochondria (FL-1). Treating a small fraction of cells with the uncoupling agent CCCP carbonyl cyanide m-chlorophenyl hydrazine (CCCP) to act as positive control, the ΔΨm was calculated:

$$[(\Delta\Psi)]\_M = ((FL2/FL1) / ([FL2]\_CCCP / [FL1]\_CCCP)) \times 100$$

In Part 2 of the study, the 'stressability' of the ΔΨm was additionally assessed by *ex vivo* titration of mitotoxic medications verapamil and carvedilol.[19] Verapamil decreases the calcium fluctuation under stress by increasing sensitivity to H_2O_2, and enhances oxidative stress by increasing ROS levels.[20] Carvedilol has an adverse effect on mitochondrial complex I, resulting in a decreasing activity of this complex and, therefore, an increase in ROS production.[21] When challenged with cyanide (an inhibitor of complex IV), the ΔΨm of HD patients collapsed to a much greater extent than in heathy controls.[22] The same was found in a study using Ca^2+ as a stressor.[23] Freshly isolated PBMCS were incubated with a concentration range (0mM, 0.125mM, 0.25mM, 0.5mM, 1mM and 2mM) of verapamil and carvedilol, at pre-dose baseline and after 27 days of SBT-020 administration. With the titration curve, we calculated the half maximal inhibitory concentration (IC50) values per timepoint, per mitotoxic compound.

Motor and neurocognitive assessments

To assess the neurocognitive and motor functioning, we used a comprehensive set of tests (Supplementary Table 5.10). The assessments were selected as they have been proven sensitive to detect cognitive and motor deterioration in HD.[24-31] The Single Digit Modalities Task (SDMT), Stroop, and Trail Making Test (TMT) were paper-and-pencil tasks, the Sustained Attention to Response Task (SART), Adaptive Tracking, and Visual Verbal Learning Test (VVLT) were computerized and were administered using the CHDR's NeuroCart®.

Unified Huntington's Disease Rating Scale (UHDRS)

The UHDRS is a clinical rating scale, which is used to assess the total motor score (TMS, range 0-124) and the total functional capacity (TFC, range 0-13) (described in detail elsewhere).[32,33] The UHDRS was performed by certified physicians. A higher TMS indicates increased motor symptoms and a lower TFC indicates increased functional disability.

Sample size calculation

Sample size was calculated based on τPCR data in the literature. The effect size (9.8s) was set on the difference between the means of asymptomatic HD patients (43.0s)[5] and heathy controls (33.2s), because the goal of the treatment was to normalize mitochondrial function in HD patients. The variability was set on the standard deviation (8.2s) of pre-frail sedentary elderly.[34] This meant that a sample size of 12 in each group would have a power of 0.80 using a two-sample t-test with a 0.05 two-sided significance level.

The threshold for inclusion in the study was set on a τPCR of 40s, enabling at least half of the screened patients to be eligible. However, data from the literature did not reflect our patient population. After the first 11 patients were screened, the median τPCR was 32.4s instead of the earlier reported mean value of 43s, which led to exclusions of most of the screened patients. However, the standard deviation was considerably lower in our measurements (4.0s instead of the reported 8.2s).[5] Therefore, we amended the study protocol to set the inclusion threshold on at least 32.4s, in order to include the 50% of patients with a τPCR above average (median) and re-performed the sample size calculation. Based on these new data, the sample size of 12 patients per treatment arm would have a power of 0.833 to detect a difference in means of 5.0s, using a two-sample t-test with a 0.05 two-sided significance level. It was subsequently decided to not change the sample size.

Statistical methods

Statistics were performed using SAS, version 9.4, by a study-independent, CHDR statistician. To establish whether significant treatment effects could be detected on the repeatedly measured biomarker parameters (mitochondrial function), each parameter was analyzed with a mixed model analysis of covariance (ANCOVA) with treatment, time and treatment by time as fixed factors and subject as random factor and the baseline measurement as covariate. To establish whether significant treatment effects could be detected on the single measured efficacy and PD endpoints (neurocognitive and motor function) each parameter was analyzed with a mixed model ANCOVA with treatment as fixed factor and the baseline measurement as covariate. There was no adjustment for multiplicity due to the exploratory nature of the study.

Post hoc analysis

A post hoc analysis was performed on the PD data from Part 2 in order to assess the effect of SBT-020 on the patients with relatively low versus relatively high mitochondrial function. To divide the active cohort (n = 11), the median values of the τPCR (42.4s) and ΔΨm (3412) prior to drug administration in Part 2 were

used as cutoff values. Patients with a low mitochondrial function were defined as a τPCR > 42.4s and ΔΨm < 3412 and patients with a high mitochondrial function as a τPCR < 42.4s and ΔΨm > 3412.

Randomization procedure

The randomization code was generated by a study-independent CHDR statistician using SAS v9.4. The randomization code could be broken and made available for data analysis only after study closure, i.e., when the study was completed, the protocol deviations determined, and the clinical database declared complete, accurate and locked. The randomization code was kept strictly confidential. Individual randomization codes, per subject and per treatment, were placed in a sealed envelope containing the labelled 'emergency decoding envelopes' and kept in a locked cabinet.

Pharmacokinetic analysis

PK analysis was performed, using SAS v9.4, by a study-independent CHDR statistician. Plasma PK parameters were derived by non-compartmental analysis of the plasma concentration data. Data below the limit of quantification (BLOQ) before T_{max} were replaced with zero, data after T_{max} were excluded from the analysis. No outlying data were removed.

RESULTS

Demographics

Supplementary Figure 5.1 summarizes the disposition of patients. A total of 24 patients enrolled in the study (mean age 47.5 years, range 20-64 ; mean CAG repeat number 44.3, range 39-60). At baseline, patients had a mean TMS of 18.9 (range 6-47), mean TFC of 9.9 (range 7-13) and mean τPCR of 40.2s (range 33.3-57.5). Demographics and baseline values are listed in Table 5.1. All 24 patients successfully passed rescreening for Part 2, but 1 patient dropped out due to SAEs before drug administration and 1 patient withdrew consent after inclusion due to the perceived study burden. Hence 22 patients completed Part 2.

SAFETY

Adverse events

The frequency of treatment-emergent adverse events (TEAEs) per treatment is listed in Table 2 (with a detailed overview in Supplementary Table 5.5). The grand majority of TEAEs (91% in Part 1 and 97% in Part 2) were injection site reactions (ISR; erythema, swelling, pain and pruritus). All ISRs were mild, generally developed within a few minutes of dosing and resolved within the hour. Injection site pruritus and pain were most often seen in the 25mg dose cohort.

There were two SAEs in one patient, after the follow-up visit of Part 1 (54 days), but prior to the drug administration in Part 2: a pneumonia followed by a pulmonary embolism. Both SAEs were deemed unrelated to the study treatment, due to the extended time between receiving the last dose of SBT-020 and the start of symptoms. There were no clinically significant findings in any laboratory assessments, (including plasma histamine), vital signs, ECGs or physical examinations.

Pharmacokinetics

A non-compartmental PK analysis (Supplementary Table 6 for plasma and Supplementary Table 7 for urine) was performed for SBT-020 concentration (Plasma PK is depicted in Figure 2). In Part 1, concentrations were measured for 24 hours after the first and last administration. SBT-020 was rapidly absorbed and an early T_{max} (around 1h post-dose) was observed in all subjects, independent of dose. Plasma concentration vs time profiles were consistent with extravascular dosing and the variability was less than 26% for both C_{max} and $AUC_{0\text{-}last}$. At day 7, the median percentage of extrapolated AUC was less than 8% (max 12.5%) in the 5mg cohort and less than 2% in the 15mg and 25mg cohorts. Exposures between Day 1 and Day 7 were approximately 10% higher in the 25mg dose cohort, but within reasonable variability. The apparent elimination half-life at day 7 appeared to be fairly independent of dose (3.13h in the 5mg cohort, 3.94 in the 15mg cohort and 4.14h in the 25mg cohort). The apparent volume of distribution at day 7 was consistent across. Based on the trough samples taken weekly in Part 2, SBT-020 did not accumulate over a period of 28 days of daily drug administration.

Pharmacodynamics

A summary of the statistical analysis (results of the ANCOVA analysis and the least square means (LSM) change from baseline) of the mitochondrial function tests has been listed in Table 5.3 for Part 1 and in Table 5.4 for Part 2. The results of the neurocognitive and motor function tests can be found in Supplementary Table 5.8.

Part 1

No overall or dose-related effects were noted on τPCR, ΔΨm and the percentage of dysfunctional PBMCS after 7 days of treatment. The mean τPCR changed from 38.8s to 33.6s (placebo) 41.9s to 42.5s (5mg cohort), 40.0s to 43.1s (15mg cohort) and 39.2s to 38.8s (25mg cohort). For the mean ΔΨm the change was 3454 to 3372 (placebo), 2956 to 2948 (5mg cohort), 3316 to 3282 (15mg cohort) and 3715 to 5279 (25mg cohort). For the mean percentage of dysfunctional PBMCS the change was from 2.7% to 5.2% (placebo), 3.2% to 3.4% (5mg cohort), 4.3% to 3.8% (15 mg cohort) and 3.2% to 3.4% (25mg cohort).

Part 2

Mitochondrial function

No overall effects were noted on τPCR, ΔΨm and the percentage of dysfunctional PBMCS after 28 days of treatment. Mean τPCR in the active group did not change from 42.8 sec (Figure 5.3A). Mean τPCR in the placebo group also did not significantly change (36.5s to 36.0s). For the mean ΔΨm the change was from 3770 to 4124 in the active group and 3125 to 2991 in the placebo group. For the mean percentage of dysfunctional PBMCS the change was from 4.6% to 4.2% in the active group and 2.9% to 4.6% in the placebo group. No overall statistically significant effect of SBT-020 on brain mitochondrial function could be observed compared to placebo (Figure 5.3B). Furthermore, no effect on ΔΨm values and IC50 values for carvedilol and verapamil could be observed.

Post hoc analysis on mitochondrial function

In the low mitochondrial function group (τPCR > 42.4s) the PCR decreased with 3.6s, indicating an improvement in mitochondrial function, while in the high mitochondrial function group (τPCR of < 42.4s) the τPCR did not decrease.

Patients on active treatment with a low mitochondrial function (ΔΨm < 3412) had an average increase in mitochondrial membrane potential of 1931 while patients with a high mitochondrial function (ΔΨm > 3412) had a decrease in mitochondrial membrane potential of 959.

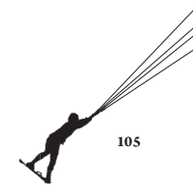
Motor and neurocognitive function

No effect of SBT-020 on cognition could be observed compared to placebo except for the total errors on the Visual Scanning Part of the TMT (CI95% -0.9–-0.015, p = 0.04). Also, no effect on motor function could be observed.

DISCUSSION

SBT-020 was safe in all dose levels during both parts of the study. Mild injection site reactions were observed frequently throughout the study, but other AEs were few and equally divided over the active and placebo groups. The exact mechanism of the ISRs is unknown, but plasma histamine levels measured 15 and 30 minutes after administration were not elevated. SBT-020 was rapidly absorbed following SC dosing, T_{max} was observed between 0.5 h and 1.0 h. SBT-020 did not accumulate following repeat dosing, as assessed by comparison of C_{max} and $AUC_{0\text{-}tau}$. Mean terminal T½ values were estimated between 3.13 h and 4.14 h following multiple doses (Day 7). The longer value for the highest dose group on Day 7 may simply reflect more quantifiable data at later time points. All dose levels were safe after single and multiple dosing with injection site reactions (ISR) being the most common adverse event. SBT-020 did not accumulate following repeat dosing, as judged by comparison of C_{max} and $AUC_{0\text{-}tau}$. Geometric mean terminal T½ values were estimated between 3.47 h and 3.74 h for a single dose (Part 1), and between 3.51 h and 5.26 h following multiple doses (Part 2, Day 7). Geometric mean CLr was estimated between 25.3 mL/min and 47.5 mL/min and did not indicate active secretion. There was no evidence that clearance, volume of distribution or bioavailability varied with dose or time. By 24 h post dose, between 27.5% and 44.9% of the dose was excreted unchanged in urine; the majority was excreted in the first 6 h post dose.

In our cohort of HD patients, we did not observe an effect of SBT-020 on mitochondrial function in calf muscle or brain. McGhee *et al.* advocate treatment during clinical trials for neurodegenerative disease such as Alzheimer's and Parkinson's disease, of at least 6 months, arguing that it is unlikely to observe



disease modification before that.[35] This is significantly longer than the 28 days in our study. However, mitochondrial dysfunction plays an important role in the pathophysiology of HD and SBT-020 was previously shown to improve mitochondrial function in pre-clinical studies in HD. Multiple factors could explain the lack of effect of SBT-020 on mitochondrial function. Most importantly, the mitochondrial function in our patients might not have been impaired sufficiently to be able to improve. Mitochondrial complex defects are present in the striatal cells of late stage HD patients and in late stage disease mouse models, but not in neostriatum and cerebral cortex cells of pre-symptomatic and mild stage HD patients and transgenic mice in which neuronal loss could not be documented.[36] Mean τPCR (40.2s) in calf muscle in our patients was longer than previously reported in untrained, healthy volunteers (31.2s),[37] but shorter than in another cohort of symptomatic HD patients (49.4s).[5] Mitochondrial function in our cohort was better than expected while both cohorts consisted of similar affected patients: TMS ranged 5-53 (mean 22.7) in our study versus TMS ranged 5-55 (mean 25.4) in the study by Saft *et al.* (range 5-55, mean 25.4, SD 14.4), which means that there is a poor correlation between UHDRS scores and τPCR.[5] It is important to mention that the aim of the study was primarily to prove the pharmacological principle of SBT-020 in its ability to improve mitochondrial function, whereas improving clinical symptoms was a secondary objective. The observed effects were not clinically meaningful.

SBT-020 does not improve normal functioning mitochondria, so the overall, relatively good, mitochondrial function might have been a relevant factor in the absence of an effect. Nonetheless, PD data in this study indicates that SBT-020 works best when targeting a higher level of mitochondrial dysfunction. When looking at mitochondrial function in PBMCS in Part 2, SBT-020 was most beneficial in the patients with the lowest $\Delta\Psi m$. Although these effects cannot be viewed as clinically meaningful, patients on active treatment with a $\Delta\Psi m < 3412$ had an average improvement of 1931 while patients with a $\Delta\Psi m > 3412$ had a decrease of 959, which indicates a potential pharmacological effect. Also, the patients with the longest τPCR at baseline (> 44 s) in Part 2 showed the highest improvement after 28 days of drug administration (improvement of 3.6 s versus a prolonging of 3 s for the patients on active treatment with a baseline τPCR of < 44 s). SBT-020 does not improve normal functioning mitochondria, so the overall, relatively good, mitochondrial function might have been a relevant factor in the absence of an effect.

Measuring mitochondrial function *in vivo* inside the brain is challenging. To date only 31P-MRS has approximated this by measuring the bio-energetics before, during and after visual stimulation.[4] Contrary to the τPCR resulting from exerting skeletal muscle, the bio-energetics in the visual cortex are harder to interpret. In healthy controls, the PI/PCR ratio increases during visual stimulation, whereas in HD patients the ratio stays the same, which underlines the difference in bioenergetics between the two groups.[4] Although no hard conclusion can be drawn from the results, the method is an important tool in assessing mitochondrial function as demonstrated in an earlier clinical trial in HD patients.[38] Another possible cause for a lack of clear effects in this study may be, that the drug did not reach a sufficient concentration at the target site of action in the CNS. In an acute model of CNS neurodegeneration induced by the mitochondrial toxin MPTP, peripheral administration of SBT-20 at 5mg/kg was sufficient to achieve neuroprotection in the striatum. However, this model may not fully recapitulate the progressive neurodegenerative decline observed in HD, where higher levels of drug exposure over more sustained intervals may be required. Additionally, MPTP itself can be damaging to the blood-brain-barrier (BBB),[39] causing leakage through which SBT-020 could have penetrated the BBB in this MTPT induced model. However, BBB integrity has been observed to be decreased and in patients with neurodegenerative disorders , including HD,[40] which increases the brain delivery of neuropharmaceuticals. Since lumbar punctures to measure SBT-020 concentration in cerebrospinal fluid (CSF), a well-known proxy for CNS tissue concentration, was deemed not feasible for this study, this is a possibility that cannot be excluded. A clinical trial with triheptanoin, C7 fatty acid oil, has previously been conducted in HD patients to improve bioenergetics the visual cortex.[38] The trial reported a normalization of the PI/PCR ratios between HD patients and healthy controls, although there was no correlation between the normalization and UHDRS score improvement. This proves that it should be possible to pharmacologically influence mitochondrial function in HD patients.

In conclusion, SBT-020 was safe during daily administration for 28 days up to a daily dose of 25mg in HD patients. PK analysis showed that once daily SC administration resulted in dose-proportional exposure and no accumulation over a 28-day administration period. No effects were observed on mitochondrial or clinical function and we suspect the mild degree of mitochondrial dysfunction in our patients and short treatment period to be responsible. It is worth mentioning that the results have provided a platform for further studies with SBT-020.

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REFERENCES

1 A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell.* 1993;72(6):971-983.

2 Rawlins MD, Wexler NS, Wexler AR, et al. The Prevalence of Huntington's Disease. *Neuroepidemiology.* 2016;46(2):144-153.

3 Quintanilla RA, Johnson GVW. Role of Mitochondrial Dysfunction in the Pathogenesis of Huntington's Disease. *Brain research bulletin.* 2009;80(4-5):242-247.

4 Mochel F, N'Guyen TM, Deelchand D, et al. Abnormal response to cortical activation in early stages of Huntington disease. *Movement disorders: official journal of the Movement Disorder Society.* 2012;27(7):907-910.

5 Saft C, Zange J, Andrich J, et al. Mitochondrial impairment in patients and asymptomatic mutation carriers of Huntington's disease. *Movement disorders: official journal of the Movement Disorder Society.* 2005;20(6):674-679.

6 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.

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

8 Szeto HH, Schiller PW. Novel therapies targeting inner mitochondrial membrane–from discovery to clinical development. *Pharmaceutical research.* 2011;28(11):2669-2679.

9 Kagan VE, Bayir HA, Belikova NA, et al. Cytochrome c/cardiolipin relations in mitochondria: a kiss of death. *Free radical biology & medicine.* 2009;46(11):1439-1453.

10 Kagan VE, Tyurin VA, Jiang J, et al. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nature chemical biology.* 2005;1(4):223-232.

11 Li K, Li Y, Shelton JM, et al. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell.* 2000;101(4):389-399.

12 Paradies G, Paradies V, De Benedictis V, Ruggiero FM, Petrosillo G. Functional role of cardiolipin in mitochondrial bioenergetics. *Biochimica et biophysica acta.* 2014;1837(4):408-417.

13 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.

14 Yin X, Manczak M, Reddy PH. Mitochondria-targeted molecules MitoQ and SS31 reduce mutant huntingtin-induced mitochondrial toxicity and synaptic damage in Huntington's disease. *Human molecular genetics.* 2016;25(9):1739-1753.

15 Cho J, Won K, Wu D, et al. Potent mitochondria-targeted peptides reduce myocardial infarction in rats. *Coronary artery disease.* 2007;18(3):215-220.

16 van den Bogaard SJ, Dumas EM, Teeuwisse WM, et al. Exploratory 7-Tesla magnetic resonance spectroscopy in Huntington's disease provides in vivo evidence for impaired energy metabolism. *Journal of neurology.* 2011;258(12):2230-2239.

17 Zorova LD. Mitochondrial membrane potential. 2018;552:50-59.

18 Cossarizza A, Salvioli S. Flow cytometric analysis of mitochondrial membrane potential using JC-1. *Current protocols in cytometry.* 2001;Chapter 9:Unit 9.14.

19 Finsterer J, Zarrouk Mahjoub S. Mitochondrial toxicity of antiepileptic drugs and their tolerability in mitochondrial disorders. *Expert opinion on drug metabolism & toxicology.* 2012;8:71-79.

20 Yu Q, Xiao C, Zhang K, et al. The Calcium Channel Blocker Verapamil Inhibits Oxidative Stress Response in Candida albicans. *Mycopathologia.* 2014;177:167-177.

21 Sgobbo P, Pacelli C, Grattagliano I, Villani G, Cocco T. Carvedilol inhibits mitochondrial complex I and induces resistance to H_2O_2-mediated oxidative insult in H9C2 myocardial cells. *Biochimica et Biophysica Acta – Bioenergetics.* 2007;1767:222-232.

22 Sawa A, Wiegand GW, Cooper J, et al. Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. *Nature medicine.* 1999;5(10):1194-1198.

23 Panov AV, Gutekunst C-A, Leavitt BR, et al. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nature Neuroscience.* 2002:731-736.

24 Stout JC, Jones R, Labuschagne I, et al. Evaluation of longitudinal 12 and 24 month cognitive outcomes in premanifest and early Huntington's disease. *Journal of neurology, neurosurgery, and psychiatry.* 2012;83(7):687-694.

25 Tian JR, Herdman SJ, Zee DS, Folstein SE. Postural control in Huntington's disease (HD). *Acta oto-laryngologica Supplementum.* 1991;481:333-336.

26 Hart EP, Dumas EM, Schoonderbeek A, Wolthuis SC, van Zwet EW, Roos RA. Motor dysfunction influence on executive functioning in manifest and premanifest Huntington's disease. *Movement disorders: official journal of the Movement Disorder Society.* 2014;29(3):320-326.

27 O'Rourke JJ, Beglinger LJ, Smith MM, et al. The Trail Making Test in prodromal Huntington disease: contributions of disease progression to test performance. *Journal of clinical and experimental neuropsychology.* 2011;33(5):567-579.

28 Lasker AG, Zee DS, Hain TC, Folstein SE, Singer HS. Saccades in Huntington's disease: initiation defects and distractibility. *Neurology.* 1987;37(3):364-370.

29 Hicks SL, Robert MP, Golding CV, Tabrizi SJ, Kennard C. Oculomotor deficits indicate the progression of Huntington's disease. *Progress in brain research.* 2008;171:555-558.

30 Henderson T, Georgiou-Karistianis N, White O, et al. Inhibitory control during smooth pursuit in Parkinson's disease and Huntington's disease. *Movement disorders: official journal of the Movement Disorder Society.* 2011;26(10):1893-1899.

31 Butters N, Wolfe J, Martone M, Granholm E, Cermak LS. Memory disorders associated with huntington's disease: Verbal recall, verbal recognition and procedural memory. *Neuropsychologia.* 1985;23(6):729-743.

32 Shoulson I, Fahn S. Huntington disease: clinical care and evaluation. *Neurology.* 1979;29(1):1-3.

33 Unified Huntington's Disease Rating Scale: reliability and consistency. Huntington Study Group. *Movement disorders: official journal of the Movement Disorder Society.* 1996;11(2):136-142.

34 Andreux PA, van Diemen MPJ, Heezen MR, et al. Mitochondrial function is impaired in the skeletal muscle of pre-frail elderly. *Scientific reports.* 2018;8(1):8548.

35 McGhee DJM, Ritchie CW, Zajicek JP, Counsell CE. A review of clinical trial designs used to detect a disease-modifying effect of drug therapy in Alzheimer's disease and Parkinson's disease. *BMC Neurology.* 2016;16.

36 Guidetti P, Charles V, Chen EY, et al. Early degenerative changes in transgenic mice expressing mutant huntingtin involve dendritic abnormalities but no impairment of mitochondrial energy production. *Experimental neurology.* 2001;169(2):340-350.

37 van Diemen MPJ, Berends CL, Akram N, et al. Validation of a pharmacological model for mitochondrial dysfunction in healthy subjects using simvastatin: A randomized placebo-controlled proof-of-pharmacology study. *European journal of pharmacology.* 2017;815:290-297.

38 Adanyeguh IM, Rinaldi D, Henry PG, et al. Triheptanoin improves brain energy metabolism in patients with Huntington disease. *Neurology.* 2015;84(5):490-495.

39 Zhao C, Ling Z, Newman MB, Bhatia A, Carvey PM. TNF-alpha knockout and minocycline treatment attenuates blood-brain barrier leakage in MPTP-treated mice. *Neurobiology of disease.* 2007;26(1):36-46.

40 Sweeney MD, Sagare AP, Zlokovic BV. Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nature reviews Neurology.* 2018;14(3):133-150.

41 Stroop JR. Studies of interference in serial verbal reactions. *Journal of Experimental Psychology.* 1935;18(6):643.

42 Reitan RM. Validity of the Trail Making Test as an Indicator of Organic Brain Damage. *Perceptual and Motor Skills.* 1958;8(3):271-276.

43 Ballard JC. Computerized assessment of sustained attention: A review of factors affecting vigilance performance. *Journal of clinical and experimental neuropsychology.* 1996;18(6):843-863.

44 Borland RG, Nicholson AN. Visual motor co-ordination and dynamic visual acuity. *British journal of clinical pharmacology.* 1984;18 Suppl 1:69s-72s.

45 Andrew JM. Delinquents and the Tapping Test. *Journal of clinical psychology.* 1977;33(3):786-791.

TABLE 1 Demographics. Demographics and baseline values for the UHDRS sub-scores and the PCr recovery time of 31P-MRS of the calf muscle.

	Mean	SD	Min	Max
Number of patients (n)	24			
Age (years)	47.5	9.3	20	64
Sex (% female)	50%			
BMI (kg/m²)	25.9 (4.8)	24.7	18.6	39.7
CAG repeat (number)	44.3	4.4	39	60
Age of disease onset (years)	40.6	9.7	19	59
Time since HD-related complaints (years)	28.5	21	1	60
UHDRS (score)				
• TMS	18.9	10.4	6	47
• TFC	9.9	1.8	7	13
τPCR (calf muscle, in s)	40.2	6.4	33.3	57.5

UHDRS = Unified Huntington's Disease Rating Scale, TMS = Total Motor Score, TFC = Total Functional Capacity, τPCr = PCr recovery time, SD = standard deviation.

TABLE 2 Adverse events. Occurrence of treatment emergent adverse events (TEAEs).

Treatment	Number of TEAEs	Number of patients that reported TEAEs (%)
PART 1		
5 mg (n=6)	41	6 (100)
15 mg (n=6)	64	6 (100)
25 mg (n=6)	99	6 (100)
Placebo (n=6)	15	5 (83)
PART 2		
25 mg (n=11)	423	11 (100)
Placebo (n=12)	67	11 (92)

TABLE 3 Pharmacodynamics Part 1. Summary of PD results in Part 1.

	LS Means				Contrasts (CI95%)			LS Means change from baseline		
Parameter	Placebo	5 mg SBT-020	15 mg SBT-020	25 mg SBT-020	Treatment P-value	5 mg SBT-020 Placebo	15 mg SBT-020 Placebo	25 mg SBT-020 Placebo	Placebo	5 mg SBT-020	15 mg SBT-020	25 mg SBT-020
τPCR with 31P-MRS (s)	34.3969	40.9016	43.2040	39.8743	0.3104	6.50472 (-3.4601, 16.4696) p=0.1844	8.80711 (-1.0294, 18.6436) p=0.0757	5.47741 (-5.5072, 16.4620) p=0.3047	-5.40847	1.09625	3.39864	0.06894
PCr/ATP resting phase 31P-MRS scan	3.8055	3.7838	3.8400	3.8510	0.9045	-.02172 (-.22404, 0.18060) p=0.8235	0.03449 (-.17248, 0.24146) p=0.7295	0.04551 (-.17953, 0.27054) p=0.6750	0.04631	0.02459	0.08080	0.09181
PCr/Pi resting phase 31P-MRS scan	10.4685	9.9289	11.5607	11.2289	0.2035	-.53955 (-2.2149, 1.13580) p=0.5060	1.09226 (-.68069, 2.86521) p=0.2110	0.76042 (-1.0773, 2.59818) p=0.3948	-0.30014	-0.83969	0.79212w	0.46028
Percentage of dysfunctional PMBCs (%)	5.80	3.82	2.80	3.75	0.0337	-1.980 (-3.819, -0.141) p=0.0363	-3.002 (-4.980, -1.025) p=0.0051	-2.052 (-3.894, -0.210) p=0.0310	2.410	0.430	-0.593	0.358
Mitochondrial membrane potential (Delta Psi)	2898.74	3162.27	2882.36	4411.95	0.0919	263.528 (-1151.3, 1678.31) p=0.7001	-16.375 (-1411.2, 1378.48) p=0.9806	1513.21 (114.067, 2912.36) p=0.0356	-457.692	-194.164	-474.067	1055.521

PCr = phosphocreatine, Pi = inorganic phosphate, 31P-MRS = phosphorous magnetic resonance spectroscopy, LS = least square, PBMCS = peripheral blood mononuclear cells, CI = confidence interval, ATP =adenosine triphosphate, τPCr = PCr recovery time.

TABLE 4 Pharmacodynamics Part 2. Summary of mitochondrial function PD results of Part 2.

	LS Means			Contrasts (CI95%)	LS Means change from baseline	
Parameter	**Placebo**	**25 mg SBT-020**	**Treatment P-value**	**25 mg SBT-020 Placebo**	**Placebo**	**25 mg SBT-020**
τPCR with 31P-MRS (sec)	38.0	40.8	0.63	2.8 (-9.3, 15.0) p=0.63	-1.7	1.2
PCR/ATP resting phase 31P-MRS scan	3.8	3.7	0.54	-.07 (-0.3, 0.2) p=0.54	0.03	-0.04
PCR/PI resting phase 31P-MRS scan	10.1	10.2	0.9	0.1 (-2.0, 2.1) p=0.94	0.2	0.3
Percentage of dysfunctional PMBCs (%)	4.83	4.08	0.62	-0.75 (-3.93, 2.42) p=0.62	1.062	0.308
Mitochondrial membrane potential (Delta Psi)	3025.07	4090.47	0.17	1065.4 (-495.2, 2626.0) p=0.17	-422.405	642.997
PCR/ATP after visual stimulation (central 31P-MRS)	0.9	0.9	0.57	-0.02 (-0.1, 0.05) p=0.57	-0.003	-0.02
PCR/ATP before visual stimulation (central 31P-MRS)	0.8	0.8	0.71	-0.01 (-0.09, 0.06) p=0.71	-0.04	-0.05
PCR/ATP during visual stimulation (central 31P-MRS)	0.8	0.8	0.41	-0.03 (-0.1, 0.04) p=0.41	-0.03	-0.06
PI/ATP after visual stimulation (central 31P-MRS)	0.2	0.2	0.66	0.0073 (-0.0270, 0.0415) p=0.6613	-0.01	0.0001
PI/ATP before visual stimulation (central 31P-MRS)	0.2	0.2	0.80	-0.002 (-0.02, 0.02) p=0.80	0.008	0.005
PI/ATP during visual stimulation (central 31P-MRS)	0.2	0.2	0.57	-0.004 (-0.02, 0.01) p=0.57	-0.007	-0.01
PI/PCR after visual stimulation (central 31P-MRS)	0.2	0.2	0.32	0.02 (-0.02, 0.06) p=0.32	-0.01	0.01
PI/PCR before visual stimulation (central 31P-MRS)	0.2	0.2	0.94	0.0007 (-0.02, 0.02) p=0.93	0.02	0.02
PI/PCR during visual stimulation (central 31P-MRS)	0.2	0.2	0.81	0.004 (-0.03, 0.03) p=0.81	0.003	0.006
PCR/ATP during-before visual stimulation (central 31P-MRS)	0.02	-0.03	0.22	-0.04 (-0.11, 0.03) p=0.23	0.02	-0.02
PCR/ATP during-after visual stimulation (central 31P-MRS)	-0.05	-0.05	0.92	-0.004 (-0.07, 0.06) p=0.92	-0.03	-0.03
PI/ATP during-before visual stimulation (central 31P-MRS)	-0.002	-0.01	0.42	-0.009 (-0.03, 0.01) p=0.42	-0.01	-0.02
PI/ATP during-after visual stimulation (central 31P-MRS)	-0.003	-0.02	0.49	-0.01 (-0.05, 0.02) p=0.49	0.0004	-0.01
PI/PCR during-before visual stimulation (central 31P-MRS)	-0.005	-0.006	0.97	-0.0004 (-0.03, 0.03) p=0.97	-0.02	-0.02
PI/PCR during-after visual stimulation (central 31P-MRS)	0.01	-0.005	0.52	-0.02 (-0.06, 0.03) p=0.52	0.01	-0.003

PCr = phosphocreatine, Pi = inorganic phosphate, 31P-MRS = phosphorous magnetic resonance spectroscopy, LS = least square, PBMCs = peripheral blood mononuclear cells, CI = confidence interval, ATP =adenosine triphosphate, tPCr = PCr recovery time.

TABLE 5 Adverse events detailed. Overview of treatment-emergent adverse events in Part 1&2.

Treatment	Organ system	Patients affected	TEAE	Frequency
		PART 1		
5 mg	General disorders	6/6	· Injection site erythema	23
			· Injection site swelling	9
			· Fatigue	1
			· Feeling cold	1
	Nervous system disorders	3/6	· Headache	3
			· Somnolence	1
	Eye disorder	1/6	· Ocular hyperaemia	1
	Musculoskeletal and connective tissue disorders	1/6	· Muscle strain	1
	Skin and subcutaneous tissue disorders	1/6	· Contact dermatitis	1
15 mg	General disorders	6/6	· Injection site erythema	38
			· Injection site swelling	11
			· Injection site pruritus	6
			· Injection site pain	5
			· Injection site haematoma	1
	Nervous system disorders	2/6	· Headache	1
			· Dizziness	1
	Gastrointestinal disorders	1/6	· Toothache	1
25 mg	General disorders	6/6	· Injection site erythema	37
			· Injection site swelling	22
			· Injection site pruritus	18
			· Injection site pain	12
			· Injection site warmth	1
			· Increased energy	1
	Nervous system disorders	2/6	· Paraesthesia	1
			· Somnolence	1
	Gastrointestinal disorders	1/6	· Diarrhea	1
	Psychiatric disorders	1/6	· Flat affect	1
		PART 2		
25 mg	General disorders	11/11	· Injection site erythema	77
			· Injection site swelling	72
			· Injection site pain	3
			· Injection site paraesthesia	2
			· Injection site haematoma	
			· Injection site irritation	2
			· Malaise	
			· Chest pain	1
				1
				1
	Infections and infestations	5/11	· Nasopharyngitis	3
			· Root canal infection	1
			· Lice infestation	1
	Nervous system disorders	3/11	· Headache	3

TABLE 6 Plasma PK analysis SBT-02. Results from non-compartmental PK analysis of SBT-020 in plasma.

Parameter	n	Median	SD	Min	Max
			DAY 1		
			5 MG COHORT		
C_{max} (ng/ml)	6	199.00	56.23	174.00	311.00
T_{max} (h)	6	0.62	0.25	0.50	1.00
T_{lag} (h)	6	0.00	0.00	0.00	0.00
$AUC_{0\text{-}last}$ (ng*h/ml)	6	861.47	221.21	601.29	1203.44
			15 MG COHORT		
C_{max} (ng/ml)	6	886.50	169.89	603.00	1020.00
T_{max} (h)	6	0.75	0.21	0.50	1.00
T_{lag} (h)	6	0.00	0.00	0.00	0.00
$AUC_{0\text{-}last}$ (ng*h/ml)	6	3374.82	942.05	2770.29	5260.16
			25 MG COHORT		
C_{max} (ng/ml)	6	973.00	165.33	858.00	1310.00
T_{max} (h)	6	0.75	0.23	0.50	1.03
tlag (h)	6	0.00	0.00	0.00	0.00
$AUC_{0\text{-}last}$ (ng*h/ml)	6	4618.57	489.18	4241.35	5475.74
			DAY 7		
			5 MG COHORT		
C_{max} (ng/ml)	6	211.50	55.14	132.00	276.00
$AUC_{0\text{-}last}$ (ng*h/ml)	6	817.12	279.30	597.96	1325.97
$AUC_{0\text{-}imf}$ (ng*h/ml)	6	882.24	281.19	653.23	1342.77
Terminal T½ (h)	6	2.99	0.55	2.70	4.17
V_F (L)	6	23.98	5.02	19.65	32.05
			15 MG COHORT		
C_{max} (ng/ml)	6	726.00	165.63	494.00	956.00
$AUC_{0\text{-}last}$ (ng*h/ml)	6	3301.97	733.75	2660.93	4510.37
$AUC_{0\text{-}imf}$ (ng*h/ml)	6	3332.43	743.28	2704.67	4583.36
Terminal T½ (h)	6	3.90	0.28	3.64	4.31
V–F (L)	6	24.40	5.82	17.78	34.45
			25 MG COHORT		
C_{max} (ng/ml)	6	1135.00	240.00	897.00	1610.00
$AUC_{0\text{-}last}$ (ng*h/ml)	6	5220.46	396.11	4805.33	5754.24
$AUC_{0\text{-}imf}$ (ng*h/ml)	6	5267.38	396.52	4871.52	5801.97
Terminal T½ (h)	6	4.00	0.39	3.80	4.68
V–F (L)	6	27.77	3.69	23.64	34.08

TABLE 7 Urine PK analysis SBT-02. SBT-020 urine PK analysis of day 1 and day 7 of all active cohorts in Part 1.

Time (h)	n	Median_Ae (ng)	SD_Ae (ng)	Mean_Ae (ng)	Median_Fe (%)	SD_Fe (%)	Mean_Fe (%)
5 MG COHORT							
5.833	6	1018333	400502	1084116	20.370	8.011	21.683
11.833	6	273721	105752	301853	5.475	2.115	6.035
23.833	5	91615	64678	110647	1.830	1.291	2.212
TotalSum	6	1351659	356971	1478175	27.030	7.146	29.562
15 MG COHORT							
5.833	6	5249174	1094235	5050779	34.995	7.293	33.673
11.833	6	1200864	370868	1221642	8.005	2.473	8.142
23.833	5	352929	124618	333208	2.350	0.833	2.220
TotalSum	6	6581210	1111212	6550094	43.870	7.409	43.665
25 MG COHORT							
5.833	6	8369072	2423278	7747313	33.475	9.693	30.988
11.833	6	1427505	606069	1458579	5.710	2.423	5.833
23.833	6	401561	274109	445017	1.605	1.097	1.780
TotalSum	6	9898159	2875633	9650909	39.590	11.502	38.602

Ae = amount excreted, Fe = fraction excreted.

TABLE 8 Neurocognitive and motor function. Summary of neurocognitive and motor function PD results of Part 2.

Parameter	Placebo	25 mg SBT-020	Treatment P-value	25 mg SBT-020 Placebo	placebo	25 mg SBT-020
Total score of SDMT paper task	40	38	0.1915	-1.9 (-5.0, 1.1) p=0.1915	2.2	0.2
Stroop: Number incorrect answers card 3	3	3	0.9195	00.1 (-1.4, 1.5) p=0.9195	-0.3	-0.2
Stroop: Time completing card 1 (sec)	68	65	0.4605	-2.8 (-10.6, 5.0) p=0.4605	1.9	-0.9
Stroop: Time completing card 2 (sec)	87	83	0.1960	-4.6 (-11.9, 2.6) p=0.1960	0.6	-4.1
Stroop: Time completing card 3 (sec)	135	133	0.8797	-1.6 (-24.0, 20.7) p=0.8797	-5.8	-7.4
Stroop: Difference in time card (3-2) (sec)	49	48	0.9149	-1.3 (-26.3, 23.7) p=0.9149	-4.2	-5.5
Total errors TMT visual Scanning	1	0	0.0435	-0.5 (-0.9,-0.0) p=0.0435	0.5	0.0
Time TMT visual Scanning (sec)	27	24	0.2316	-2.7 (-7.2, 1.8) p=0.2316	-0.8	-3.4
Total errors TMT Digit Sequencing	0	0	0.3789	-0.2 (-0.6, 0.2) p=0.3789	0.0	-0.2
Time TMT Digit Sequencing (sec)	42	42	0.9119	0.6 (-9.8, 10.9) p=0.9119	-8.0	-7.4
Total errors TMT Letter Sequencing	0	0	0.1707	-0.4 (-0.9, 0.2) p=0.1707	0.1	-0.3
Time TMT Letter Sequencing (sec)	46	46	0.9478	-0.4 (-11.5, 10.8) p=0.9478	-6.1	-6.5
Total errors TMT Letter-Digit Sequencing	1	1	0.6886	0.2 (-0.7, 1.1) p=0.6886	0.0	0.2
Time TMT Letter-Digit Sequencing (sec)	119	125	0.6695	5.2 (-19.7, 30.1) p=0.6695	-1.6	3.5
Time TMT Motor Speed (sec)	34	34	0.9329	-0.4 (-9.0, 8.3) p=0.9329	-4.0	-4.4
VVLT: Word recall correct 1	5.2	5.8	0.5005	0.61 (-1.24, 2.45) p=0.5005	-1.03	-0.42
VVLT: Word recall correct 2	6.9	8.3	0.1438	1.32 (-0.49, 3.14) p=0.1438	-0.48	0.84
VVLT: Word recall correct 3	8.8	10.1	0.2732	1.29 (-1.10, 3.69) p=0.2732	0.04	1.33
VVLT: Delayed word recall correct	4.0	6.2	0.1969	2.12 (-1.20, 5.44) p=0.1969	-0.33	1.79
VVLT: Delayed word recognition correct	20.1	21.7	0.4398	1.52 (-2.52, 5.56) p=0.4398	-0.40	1.13

TABLE 8 (*Continuation of previous page*)

Parameter	Placebo	25 mg SBT-020	Treatment P-value	25 mg SBT-020 Placebo	placebo	25 mg SBT-020
VVLT: Delayed word recognition RT correct (msec)	993.9	1066.2	0.3601	72.27 (-89.02, 233.55) p=0.3601	-2.36	69.91
SART total commission errors	10.2	9.0	0.4220	-1.13 (-4.02, 1.76) p=0.4220	1.34	0.21
SART mean RT correct	453.6	440.2	0.5321	-13.36 (-57.29, 30.57) p=0.5321	12.77	-0.59
SART total omission errors	13.0	12.3	0.8535	-0.71 (-8.67, 7.24) p=0.8535	-1.96	-2.67
SART post error slowing	0.3005	0.2844	0.8436	-.01609 (-.18442, 0.15225) p=0.8436	0.03848	0.02240
SART RT variability	0.3488	0.3205	0.4351	-.02830 (-.10257, 0.04598) p=0.4351	0.02201	-0.00629
SART total error score	23.1	21.4	0.6517	-1.72 (-9.59, 6.14) p=0.6517	-0.68	-2.41
Adaptive tracking (%)	16.20	16.51	0.8286	0.307 (-2.626, 3.239) p=0.8286	-1.394	-1.088
UHDRS: Total Motor Score	23	22	0.7335	-1.0 (-6.7, 4.8) p=0.7335	0.4	-0.6
UHDRS: Total Functional Capacity	9	9	0.9832	0.0 (-1.4, 1.5) p=0.9832	0.6	0.6
Tapping: Mean of 5 trials (taps/10 sec)	48.35	47.56	0.7411	-0.782 (-5.664, 4.100) p=0.7411	-2.509	-3.291
Saccadic eye movements: Inaccuracy (%)	8.4	7.5	0.4248	-0.93 (-3.33, 1.48) p=0.4248	0.36	-0.57
Saccadic eye movements: Peak Velocity (deg/s)	417.9	409.4	0.6510	-8.48 (-47.47, 30.51) p=0.6510	-27.62	-36.10
Saccadic eye movements: Reaction Time (sec)	0.255	0.274	0.0674	0.0192 (-0.0015, 0.0400) p=0.0674	0.0022	0.0214
Smooth Pursuit (%)	36.5	37.6	0.6208	1.08 (-3.41, 5.57) p=0.6208	-1.19	-0.11
Body sway (mm)	723.4	700.5	0.7987	-3.2% (-25.4%, 25.7%) p=0.7987	0.6%	-2.6%
IC50 of PMBCS in CAR concentration (mM)	0.445	0.485	0.4681	0.0401 (-0.0740, 0.1542) p=0.4681	0.0533	0.0935
IC50 of PMBCS in VER concentration (mM)	0.632	0.667	0.6327	0.0353 (-0.1173, 0.1879) p=0.6327	0.0534	0.0887
IC50 of ΔΨm in CAR concentration (mM)	0.348	0.329	0.6748	-0.0183 (-0.1082, 0.0716) p=0.6748	0.0765	0.0582
IC50 of ΔΨm in VER concentration (mM)	0.573	0.589	0.8434	0.0161 (-0.1522, 0.1845) p=0.8434	0.0298	0.0459

TABLE 9 **PK timepoints.** Timepoints of plasma samples for PK.

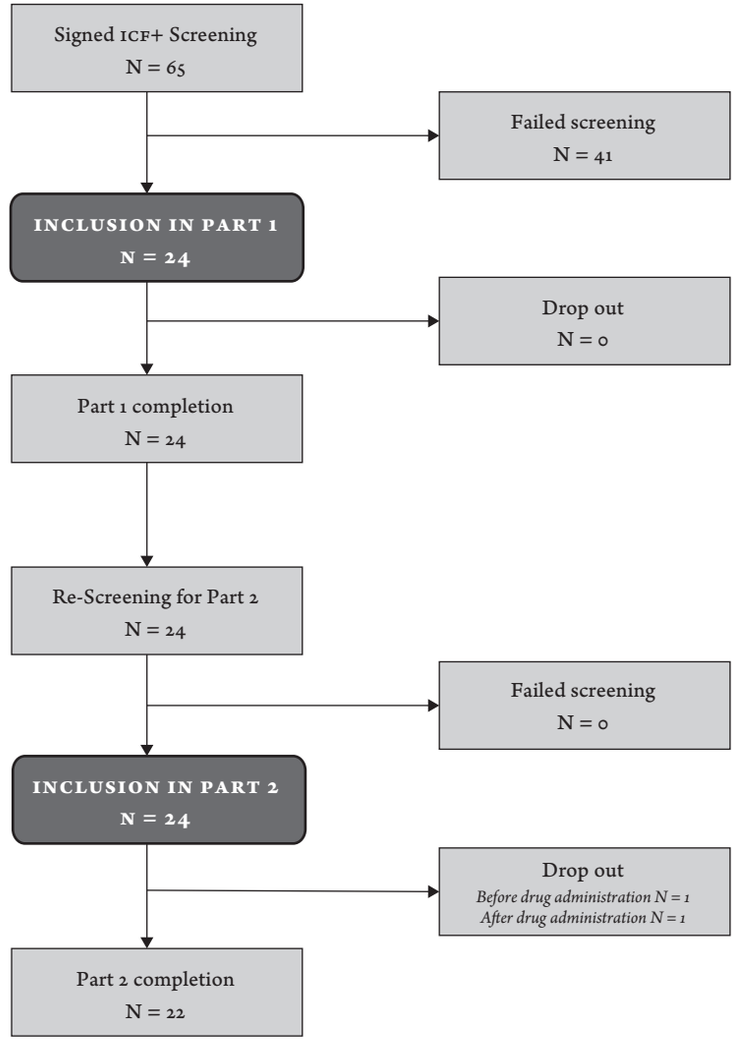
PART 1	
Day	**Time relative to drug administration**
Day 1	-15 min +30 min +45 min +1 h +2 h +4 h +6 h +8 h +10 h +24 h
Day 7	- 15 min +30 min +45 min +1.25 h +2.5 h +4 h +6 h +8 h +10 h +24 h

PART 2	
Day	**Time relative to drug administration**
Day 1	-1 h
Day 7	-15 min
Day 14	-15 min
Day 21	-15 min
Day 27	-5 min +1 h
Day 28	+1 h

TABLE 10 **Neurocognitive and motor test battery.** List of neurocognitive and motor tests and outcome parameters.

Test	Function evaluated	Method	Outcome parameter	Ref
NEUROCOGNITIVE ASSESSMENTS				
Symbol Digit Modalities Test (SDMT)	Speed of processing	Pairing symbols to numbers according to a preset key. A higher score indicates a better performance.	Total number of correct responses in 90 seconds.	26
Stroop test	Information processing and executive functioning, especially cognitive control and inhibitory processes	Color, word and interference tasks were used to determine. Higher scores indicate a better performance.	Total number of correct responses in 45 seconds per trial	41
Trail Making Test (TMT)	Attention and cognitive flexibility: perceptual processing, visual scanning, attention, executive functioning (response inhibition, set-SHIFTing), processing speed, and working memory	Connecting numbers and/or letters in ascending order.	Completion time in seconds and number of errors for each trial	42
Visual Verbal Learning Test (VVLT)	Various components of learning (including acquisition, consolidation, storage, and retrieval of memories).	Recall of words	Total number correct	31
Sustained Attention to Response Task (SART)	Attentional control.	Patients have to press a button when the number 3 appears on the screen, but withhold a response if 0-2 or 4-9 is shown 43 .	The total number of (commission and omission) errors and the mean reaction time of all correct response trials	16 17
Adaptive tracking	Pursuit tracking, in which the neocortex, basal nuclei, brain stem and cerebellum are involved.	Tracking a moving dot on a screen, using a joystick.	average performance (%)	44
MOTOR ASSESSMENTS				
Finger tapping task	Motor activation and fluency	Computerized finger tapping task (adapted from the Halstead Reitan Test Battery)	Mean tapping rate and standard deviation	45
Saccadic eye movements	Motor activation and fluency	Capturing eye movement following a horizontally moving light (jumping side-to-side) on a computer screen.	Saccadic reaction time (seconds), saccadic peak velocity (degrees/second), saccadic inaccuracy (%)	28 29
Smooth pursuit	Motor activation and fluency	Capturing eye movement following a continuously moving light on a computer screen.	Percentage of time the eyes are in smooth pursuit of the target (%)	28 29
Body sway	Postural stability	Standing still with eyes closed, measuring sway with the Celesco® string potentiometer	Antero-posterior sway (in mm)	25

FIGURE 1 **Subject allocation.** Flow chart patient disposition Parts 1 and 2.



ICF = informed consent form.

FIGURE 2 A-B **Plasma SBT concentrations.** Plasma SBT-020 concentrations on A. day 1 and B. day 7 of Part 1 for the 3 different dose cohorts. Concentrations of individual patients are depicted.

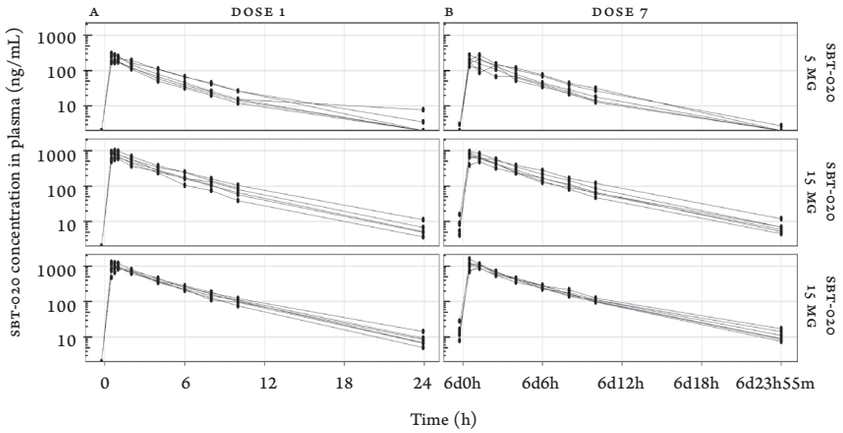
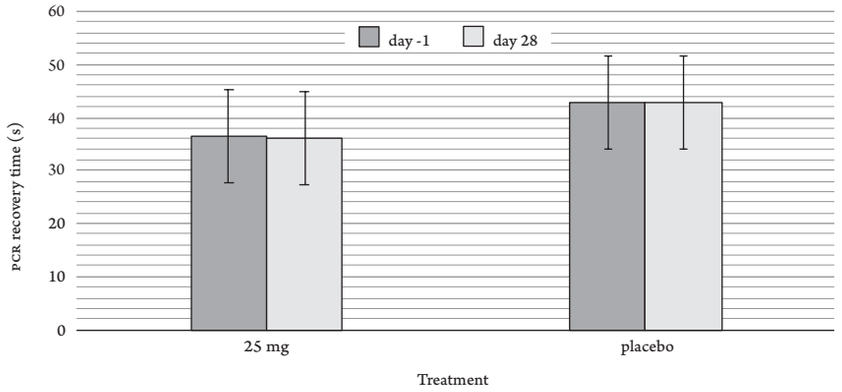


FIGURE 3 **Peripheral mitochondrial function.** Effect of daily administration of 25mg SBT-020 in Part 2 on peripheral mitochondrial function, measured with 31P-MRS. No differences between placebo and SBT-020 were observed.



PCr = phosphocreatine, Pi = inorganic phosphate, 31P-MRS.

CHAPTER VI

BRAIN BIO-ENERGETIC STATE DOES NOT CORRELATE TO MUSCLE MITOCHONDRIAL FUNCTION IN HUNTINGTON'S DISEASE

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Marcus PJ van Diemen, MD[1] ⋮ Ilse van Beelen, BSc[1] ⋮ Ellen P Hart, PhD[1] ⋮ Pieter W Hameeteman, MSc[1]
Emma M Coppen, MD[4] ⋮ Jessica Y Winder, MD[4] ⋮ Jonas den Heijer, MD[1] ⋮ Matthijs Moerland, PhD[1]
Hermien Kan, PhD[2] ⋮ Jeroen van der Grond, PhD[3] ⋮ Andrew Webb, PhD[2] ⋮ Raymund AC Roos, MD, PhD[4]
Geert Jan Groeneveld, MD, PhD[1,5*] ⋮⋮⋮ *1. Centre for Human Drug Research, Leiden, NL ⋮ 2. Gorter Centre for high-field MRI, Leiden University Medical Center, Leiden, NL ⋮ 3. Radiology Research Center, Department of Radiology, Leiden University Medical Center, Leiden, NL 4. Department of Neurology, Leiden University Medical Center, Leiden, The Netherlands ⋮ 5. Department of Anesthesiology, Leiden University Medical Center, Leiden, nl ⋮* corresponding author: ggroeneveld@chdr.nl*

BACKGROUND Huntington's disease (HD) is a neurodegenerative disease with cognitive, motor and psychiatric symptoms. A toxic accumulation of misfolded mutant huntingtin protein (HTT) induces mitochondrial dysfunction, leading to a bioenergetic insufficiency in neuronal and muscle cells. Improving mitochondrial function has been proposed as an opportunity to treat HD, but it is not known how mitochondrial function in different tissues relates. We explored associations between central and peripheral mitochondrial function in a group of mild to moderate staged HD patients.

METHODS We used phosphorous Magnetic Resonance Spectroscopy (31P-MRS) to measure mitochondrial function *in vivo* in the calf muscle (peripheral) and the bio-energetic state in the visual cortex (central). Mitochondrial function was also assessed *ex vivo* in circulating peripheral blood mononuclear cells (PBMCS). Clinical function was determined by the Unified Huntington's Disease Rating Scale (UHDRS) total motor score. Pearson correlation coefficients were computed to assess the correlation between the different variables.

RESULTS We included 23 manifest HD patients for analysis. There was no significant correlation between central bio-energetics and peripheral mitochondrial function. Central mitochondrial function at rest correlated significantly to the UHDRS total motor score (R=–0.45 and –0.48), which increased in a subgroup with the largest number of CAG repeats.

DISCUSSION We did not observe a correlation between peripheral and central mitochondrial function. Central, but not peripheral, mitochondrial function correlated to clinical function. Muscle mitochondrial function is a promising biomarker to evaluate disease-modifying compounds that improve mitochondrial function, but Huntington researchers should use central mitochondrial function to demonstrate proof-of-pharmacology of disease-modifying compounds

BACKGROUND

Huntington's disease (HD) is a progressive neurodegenerative disease, characterized by motor, cognitive and psychiatric signs and symptoms, caused by an inherited CAG-repeat (glutamine) expansion in the gene coding for the huntingtin (HTT) protein, which leads to misfolding.[1] Accumulation of misfolded HTT is toxic for cells and results in the characteristic neuronal loss of the striatal region in the brain.[1] The exact mechanism for this degeneration is not fully understood, but evidence from HD mouse models points towards changes in metabolism and/or bioenergetics (reviewed by Polyzos *et al.* in).[2] Mitochondria supply over 90% of cells' energy needs, are especially sensitive to accumulation of mutant HTT and disturbances of mitochondrial function are suspected to play an important role in the pathogenesis of the disease.[3] Observed in HD mouse models, mitochondrial dysfunction occurs during the later stages of the disease.[4-6] Striatal cells have a very high energy demand and heavily rely on well-functioning mitochondria.[7] Therefore, it is likely that a disturbance in mitochondrial bio-energetics within striatal cells can lead to neuronal loss. A similar mechanism of mitochondrial dysfunction is thought to be involved in Parkinson's disease (PD), another neurodegenerative disease, which results from neuronal loss of the also high energy demanding cells that form the substantia nigra. In animals, HD and PD phenotypes, including pathology in the brain and disease specific symptoms, can be induced by administrating mitochondrial toxins.[8-11] Mutant HTT has indeed been shown to localize to mitochondria[12-14] and to interact with the mitochondrial protein import machinery in striatal cells in a mutant HTT knock-in mouse model and in postmortem brain sections of patients with grade 2 HD.[15] Furthermore, mutant HTT directly inhibits mitochondrial protein import *in vitro*, triggering mutant HTT induced cell death of primary neurons.[15] In patients with HD, several studies have observed a decreased mitochondrial function when compared to healthy volunteers: mitochondrial function in the calf muscle and circulating lymphoblasts and the bio-energetic state of the visual cortex.[16-18] Accumulation of mutant HTT is not specific for the striatum, but also occurs in cells outside the brain, such as muscle cells. Using immunohistology, myofibers of HD patients were found to stain intensely for granules of HTT, whereas myofibers of healthy controls only contained a few granules, which correlated to mitochondrial function.[16] This suggests mitochondria as a pharmacological target to treat HD. Indeed, several clinical trials have targeted mitochondrial dysfunction in an attempt to treat HD.[19-21]

In order to prove pharmacology of new compounds in development for the treatment of mitochondrial dysfunction, *in vivo* biomarkers, that reflect mitochondrial function, are necessary. Ideally, mitochondrial function of the affected part of the brain is measured, which is currently technically not yet possible. The best *in vivo* alternative available, measuring the bio-energetic state of the visual cortex, indirectly measures mitochondrial function and has not yet been correlated to robust *in vitro* or *ex vivo* methods, unlike muscle mitochondrial function, for which these correlations were previously described.[22] However, it is not known how central and peripheral mitochondrial function relate to each other. In this study we explore the relationship between peripheral mitochondrial function in the calf muscle (*in vivo*) and circulating lymphocytes (*ex vivo*), central bio-energetics in the visual cortex (*in vivo*) and clinical function in a cohort of manifest HD patients.

MATERIAL AND METHODS

Participants

All patients were participants in a randomized, double blind, placebo controlled study with the small peptide SBT-020 from Stealth Biotherapeutics (https://www.clinicaltrialsregister.eu/ctr-search/trial/2016-003730-25/nl). The results of this study have not yet been accepted for publication. The interventional study consisted two parts: Part 1, which was a 7-days multiple, ascending dose study and Part 2, which was a 28-days multiple dose study with the highest tolerable dose from Part 1. There was a washout period of at least 1 month between both parts. Patients were re-randomized before entering Part 2. The data used in this manuscript were from the baseline measurement before dose administration in Part 2. Data from all patients were used in this comparative study. The main inclusion criteria were: DNA confirmed diagnosis of HD with a CAG-repeat expansion of 36 or more; Total Motor Score (TMS) of 5 or more and Total Functional Capacity Score (TFC) of 6 or more on the Unified Huntington Disease Rating Scale (UHDRS); absence of any significant comorbidity.

Medication with an effect on cognitive functioning (e.g. antidepressants) needed to be stable for at least 30 days before study enrolment. Medication with known mitochondrial toxicity (e.g. statins and metformin) were not allowed within 21 days before study enrolment until the end of the study period.

The study was approved by the independent ethics committee Stichting BEBO (Assen, the Netherlands) according to the principles of the Helsinki Declaration

under registration number NL59198.056.16, and informed consent was obtained from all subjects prior to study enrollment.

Mitochondrial function

Peripheral mitochondrial function (calf muscle)

Dynamic 31P-MRS in skeletal muscle (i.e. the gastrocnemius muscle of the right leg) was performed at a field strength of 7 Tesla using a surface coil on a custom-built MRI-compatible pedal ergometer (made by the technical instrumentation division of the LUMC, Leiden, The Netherlands). The exercise ergometer was designed to allow the patients to perform isometric plantar flexion exercise by pressing against a foot pedal while lying in the supine position. The foot was strapped firmly to the exercise device using non-elastic Velcro straps proximal to the base of the fifth digit, and the knee was supported. The subject's lower extremity was secured to the MRI table with straps across the mid-thigh and mid-lower leg in order to isolate usage of the posterior calf muscles. The scanning protocol consisted of localizer sequences and the acquisition of a field map for shimming purposes. Thereafter, 31P-MRS data was acquired before, during and after exercise with a time resolution of 2 s Peak integrals of the inorganic phosphate (PI), phosphocreatine (PCR) and adenine triphosphate (ATP) signals were obtained using the JMRUI software package. The frequency difference between PCR and PI was used to calculate tissue pH. A pH of at least 6.8 was required for a reliable τPCR.[23]

Peripheral mitochondrial function (circulating lymphocytes)

The mitochondrial membrane potential (ΔΨm) is the major force driving protons across the inner membrane,[24,25] which is in turn the central intermediate of aerobic energy production, and driving force of other physiological processes in mitochondria, such as Ca2+ uptake, antioxidant defence (NADPH generation at the transhydrogenase) or heat production of brown fat. The ΔΨm of a lymphocyte subset of live peripheral blood mononuclear cells (PBMCS) was assessed with flow cytometry as the ratio of fluorescent signals after treatment of the cells with the fluorescent dye JC-1.[26] Attracted by the positive charge of the inner mitochondrial space, accumulation of the dye leads to a change in fluorescence ('green' to 'red') and a higher ratio of red to green corresponds to a higher ΔΨm. Additionally, we determined the stressability of the mitochondria by treating the PBMCS *ex vivo* with two medications with mitotoxic properties (carvedilol and verapamil). The technical aspects of the methods can be found in the supplement.

Central bio-energetic state (visual cortex)

31P-MRS of the brain was performed on a 3 Tesla whole-body MRI scanner (Philips Achieva, Best, The Netherlands). A custom-made 6-cm 31P transmit/receive surface coil was used to detect signals from the visual cortex while limiting muscle contamination. Technical details of the method are described in the supplementary section. Using a checkerboard animation (a projection of alternating black and red lights), neurons in the visual cortex are being activated and this activity is measured by PI/PCR and PI/ATP ratios.[17] The ratios are a direct marker for the bio-energetic state within the mitochondria.[27-33] In healthy controls, the ratios increase during visual stimulation, whereas in HD patients the ratios stay the same, which reflects the difference in bio-energetics between the two groups.[17]

Unified Huntington's Disease Rating Scale (UHDRS)

The UHDRS is a clinical assessment scale, which is used as the gold standard for motor, cognitive, behavioural capacity and function of HD patients. The method is described in detail elsewhere.[34] The UHDRS was performed by one of three certified physicians to assess the sub-scores total motor functioning (TMS) and the total functional capacity (TFC).[34,35] A higher TMS indicates increased motor symptoms and a lower TFC indicates increased functional disability. Apart from assessing drug efficacy, the scores were used as inclusion criteria to ensure that patients were symptomatic, but could still function well enough to participate.

Statistical methods

The statistical analysis was performed using SPSS v25. All missing or incomplete data were treated as such. Pearson correlation coefficients were used to assess correlation between parameters. The threshold for statistical significance was set at p-value < 0.05.

RESULTS

Demographics

A total of 23 patients (10 females and 13 males, Table 6.1) with manifest HD (TMS ranged 5-53 ; TFC ranged 6-11) were enrolled in the study, with a mean age of 47.6 years (range 20 to 64 years) and on average HD-related complaints for 7.7 years (ranged 1 to 27 years).

Mitochondrial function

In vivo mitochondrial function was assessed in the calf muscles (peripheral) and bio-energetics in the visual cortex (central) using 31P-MRS. A detailed overview of the results is displayed in Supplementary Table 6.3.

Mitochondrial function in skeletal muscle

The mean τPCR in calf muscle was 39.5 seconds (SD 8.8, min 26.3, max 55.4 seconds). A longer duration for the PCR signal to return to baseline after exercise reflects a lower mitochondrial function.[36]

Ex vivo mitochondrial health of lymphocytes

Lymphocytes were assessed for mitochondrial health by measuring the percentage of dysfunctional cells, the absolute membrane potential ΔΨm and the membrane potential collapse induced by carvedilol and verapamil. The average percentage of dysfunctional lymphocytes was 3.6% (range 0.3-23.5%) and the average ΔΨm was 3518 (range 802-6082). The average IC50 for induced membrane potential collapse was 0.27 mmol for carvedilol (range 0.13-0.48 mmol), and 0.54 mmol for verapamil (range 0.34-0.78 mmol).

Bio-energetic state in the visual cortex

The bio-energetics in the visual cortex was assessed by determining different ratios in energy metabolism in rest, during and after visual stimulation (see Figure 6.1). The ratios are listed in Supplementary Table 6.4. During rest, the mean PI/PCR ratio was 0.21 (SD 0.03, min 0.16, max 0.28) and the mean PI/ATP ratio was 0.19 (SD 0.03, min 0.12, max 0.23). The ratios did not significantly differ when measured before, during or after the visual stimulation, which is in line with literature. Importantly, in healthy volunteers, the ratios would increase during visualization and decrease again during recovery).[17]

Correlation peripheral versus central mitochondrial bio-energetics

None of the variables for peripheral mitochondrial capacity or health (31P-MRS of skeletal muscle and collapse of ΔΨm in lymphocytes after titration with carvedilol or verapamil) showed correlation with central bio-energetics or clinical function. PI/PCR and PI/ATP ratios before visual stimulation showed a significant correlation to clinical function (TMS only), with an r of -0.45 (p = 0.02) to -0.48 (p = 0.03), respectively. The ratios during and after visual stimulation did not correlate to clinical function (TMS or TFC). Pearson correlation coefficients and p-values are shown in Table 6.2 and Figure 6.2.

Post hoc subgroup analysis

A post hoc analysis was performed in patients (n=11) with the highest CAG repeat number to investigate if mitochondrial dysfunction would be worse with higher disease load (Table 6.3). The subgroup consisted of the 11 patients with the highest CAG repeat number (range 43-60). TMS strongly correlated to PI/ATP ratio at rest (r = –0.76, p = 0.006) and PI/PCR ratio at rest (r = –0.66, p = 0.03). TFC correlated to PI/ATP ratio at rest (r = 0.63, p = 0.04) and PI/PCR ratio at rest (r = 0.68, p = 0.02). TFC also correlated to the difference in PI/PCR before and during activation (r = 0.67, p = 0.03). Mitochondrial function in muscle did not correlate to bio-energetic state in the visual cortex.

DISCUSSION

Central mitochondrial function based on the bio-energetic state in the visual cortex was not correlated to peripheral mitochondrial function in calf muscle. There was a moderate correlation between central mitochondrial bio-energetics and Total Motor Score within the full patient cohort. The data imply that a larger HD-related disability (higher TMS) correlated to a worse bioenergetic profile (lower

PI/PCR and PI/ATP ratios) in the visual cortex. When selecting patients with the highest CAG repeat number, the correlation between central mitochondrial capacity and Total Motor Score was strong, with a moderate correlation between central mitochondrial bio-energetics and Total Functional Capacity.

To our knowledge this is the first study, comparing *in vivo* central to peripheral mitochondrial function in HD patients. Apart from the striatal area, mitochondrial dysfunction in HD patients has been reported in both calf muscle (Saft *et al.*) as well as in the visual cortex (Mochel *et al.*).[16,17] However, we did not observe a correlation between the mitochondrial function in skeletal muscle and circulating lymphocytes and bioenergetics in the visual cortex. When comparing mitochondrial function to clinical function, we also observed a difference between peripheral and central function. We observed a significant negative correlation between TMS vs PI/PCR ratio (r = –0.45) and vs PI/ATP ratio (r = –0.48). In the patients with a higher CAG repeat number than the median, the correlation between TMS vs PI/PCR ratio (r = –0.66) and vs PI/ATP ratio (r = –0.76) was stronger, in addition to a significant positive correlation between TFC vs PI/PCR ratio (r = 0.68) and vs PI/ATP ratio (r = 0.63). These data show that patients with a higher degree of HD-related pathology have a lower mitochondrial function in the visual cortex. No correlations between clinical function and peripheral mitochondrial function were observed.

We expected a correlation between central and peripheral mitochondrial function, because MHTT is ubiquitously present in mitochondria of HD patients. However, it is known that mitochondrial function is affected differently in different tissues (reviewed by Polyzos *et al.*).[2] In postmortem brain samples, complex II, III and IV deficiencies have been observed, whereas in skeletal muscle and platelets complex I is more affected. Even within the brain, there are differences between more and less affected regions: using spectrophotometric assays, ETC enzymes were impaired in the basal ganglia of HD brains, while enzyme activities were unaltered in three regions relatively spared by HD pathology (frontal cortex, parietal cortex, and cerebellum).[37] In the most affected region of the HD brain, the striatum, complex II–III activity were markedly reduced in both caudate (–29%), while complex II–III (–67%), and IV (–62%) are altered in the putamen.[37] When combined with the fact that mitochondrial disturbances are only universally observed late in the disease progression of mouse models,[38] it is plausible that mitochondrial dysfunction is a consequence of a metabolic disturbance, rather than the cause. Additionally, the regional specificity implies that mitochondrial dysfunction develops in response to its changing environment. It is unfortunately not known what this might be. However, suppleting essential substrates to the Krebs cycle through administration of triheptanoin – a long fatty acid, which is metabolized into acetyl-CoA and propionyl-CoA – in a pilot study led to the normalization of bioenergetics in the brain of HD patients.[19] A phase 2 study with triheptanoin for efficacy on clinical function measured by the UHDRS is currently running. Mitochondrial dysfunction might not be the direct cause of HD, but could potentially be used to alleviate symptoms.

In other neurodegenerative disorders, mitochondrial dysfunction also plays a role in the pathophysiology via a toxic accumulation of misfolded proteins (hyperphosphorylated tau and Ab plaques in AD, alpha-synuclein in PD, and MSOD1 in ALS). The same pattern between central and peripheral mitochondrial function has been observed. In a study comparing mitochondrial function in brain, skeletal muscle and platelet homogenates from idiopathic PD patients[39] , mitochondrial dysfunction was found to be part of the pathophysiology of PD (complex 1 deficiency), but limited to the substantia nigra, with normal mitochondrial function in skeletal muscle and platelets. In a study on mitochondrial dysfunction in post-mortem brains of AD, complex II, III and IV were reduced in activity in the temporal, parietal, entorhinal cortex and hippocampus, but succinate cytochrome c reductase was significantly reduced only in the parietal and temporal cortex.[40] Although mitochondrial dysfunction is a common phenomenon in neurodegenerative disorders, it is expressed differently. Inside the brain, the most affected areas differ between diseases, as well as the impairment of the mitochondrial respiratory chain complexes activity (complex I, III, and IV in AD ; complex I and IV deficiency in PD ; complex II, III, and IV deficiency in HD ; and complex I, II, III, and IV deficiency in ALS). Although mitochondrial dysfunction plays an important role, the pathology of neurodegenerative disorders is likely multifactorial.

A reason for the discrepancy between peripheral mitochondrial function and clinical function may be that mitochondrial function in muscle tissue is more influenced by physical activity. It is known that a sedentary lifestyle is associated to a lower mitochondrial function in skeletal muscle in otherwise healthy elderly, which can be improved by increased physical activity.[41,42] Exercise has also been shown to increase mitochondrial function in the muscle of HD patients.[43,44] Given that patients with a higher disease severity are more prone to have a sedentary lifestyle due to physical disability, it is difficult to know how much muscle mitochondrial dysfunction is directly disease related and how much is physical activity dependent. Being physically active could boost mitochondrial function in skeletal muscle, but not influence mitochondrial bio-energetics in the striatum, resulting in a lack of correlation.

Improving clinical function in patients with HD and other neurodegenerative diseases, such as Parkinson's disease, has proven difficult due to the progressive nature of neurological pathology, but improving mitochondrial function can be used to show proof-of-pharmacology in an early stage of compound development. Both peripheral and central mitochondrial function measurements could be used, but for different reasons. Peripheral mitochondrial function (31P-MRS of skeletal muscle) has the advantage of being better accessible than the brain and can be combined with other types of measurements (e.g. respirometry on muscle biopsy) and the mitochondrial membrane potential in lymphocytes has been explored as a systemic biomarker for diseases with mitochondrial dysfunction such as Alzheimer's disease, rheumatoid arthritis and diabetes type 2[45-47] , and as a tool to monitor mitotoxic drug effects, for example of anti-cancer drugs and volatile anesthetics.[48,49] However, any pharmacodynamic effect in skeletal muscle or circulating lymphocytes might not correlate to the central nervous system and thus not informative when disease modifying is the primary aim of the study. On the other hand, central mitochondrial function (31P-MRS of visual cortex) correlates to clinical function in HD patients and should therefore be used to evaluate pharmacology of compounds being developed for the disease modifying treatment of HD.

This study contains three limitations. First, the number of patients was relatively small, which needs to be considered when interpreting the results. Studies with larger cohorts are therefore needed to confirm the results. Second, caution is advised when comparing different aspects of mitochondrial function in different tissues. Earlier studies have separately investigated different aspects of mitochondrial function and have shown deficits. In this study we have combined these measurements within the same patients for the first time. Although we did not show any correlations between central and peripheral mitochondrial function, the complexity of mitochondrial dysfunction within the pathophysiology of HD warrants further research. Third, our 31P-MRS setup included a phosphorous surface coil, which limited the measurements to the visual cortex. Currently, *in vivo* 31P-MRS of the brain is an important way to gain insight into bioenergetics in HD patients. While measuring bioenergetics within the striatal area would in theory yield more relevant results to HD pathology, disturbances within the visual cortex have been described before.[17] However, the results from this study should be interpreted with this caution in mind. Additionally, future studies should try to include the striatal area, if technologically possible.

In conclusion, we did not observe a correlation between peripheral and central mitochondrial function in a group of mild to moderate HD patients. Central, but not peripheral, mitochondrial function correlated to clinical function. Mitochondrial function is a promising biomarker to evaluate disease-modifying compounds that improve mitochondrial function, but researchers should use central mitochondrial function instead of peripheral mitochondrial function to demonstrate proof-of-pharmacology of compounds intended as disease modifying.

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SUPPLEMENTARY TEXT

Technical details of the mitochondrial membrane potential measurements

The mitochondrial membrane potential ($\Delta\Psi m$) of live, circulating lymphocytes was assessed using a fluorescent dye (JC-1) and flow cytometry.[26] The dye is attracted to the positive charge of the proton gradient within the intermembrane space. When accumulation occurs, the dye aggregates and emits red fluorescence. In case of mitochondrial dysfunction, the $\Delta\Psi m$ is decreased and the dye stays in the cytosol as monomers, emitting green fluorescence. Cytometry was used to quantify the fluorescence. Using the ratio of the fluorescent signals, the $\Delta\Psi m$ was determined. A fraction of the lymphocytes was treated with the mitochondrial uncoupling agent carbonyl cyanide m-chlorophenyl hydrazine (CCCP), eliminating the proton gradient, to act as a positive control.

Gating strategy

The lymphocyte subset of the PBMCS is targeted based on the FSC and SSC (Figure 6.3, left panels). The positive control is used to target the dysfunctional lymphocytes, this gate is then copied to the sample (right panels). All the cells of the sample falling in this gate are dysfunctional and reported as a percentage of the parent population, the dysfunctional lymphocytes. The delta Psi was calculated as follows. The median fluorescence intensity (MFI) value for red (FL-2, PE-A B2-A) obtained without CCCP was divided by the MFI for green

(FL-1, FITC-A B1-A) without CCCP. In a second step, a correction for the positive control was performed. In particular, the ratio FL-2:FL-1 was calculated with the values obtained with the CCCP (positive control) and set as 100%. Finally, the value of the first ratio (without CCCP) was expressed as percentage of the second ratio (with CCCP).

$$\Delta\Psi_m = \left(\frac{FL2}{FL1} \Big/ \frac{FL2_{CCCP}}{FL1_{CCCP}} \right) \times 100$$

Additionally, the mitochondria were additionally assessed by measuring the 'stressability' of lymphocytes after *ex vivo* titration of verapamil and carvedilol (two medications with mitotoxic effects).[50] We incubated freshly isolated lymphocytes from the patients with a concentration range (0mM, 0.125mM, 0.25, 0.5mM, 1mM and 2mM) of verapamil and carvedilol. With the titration curve, we calculated the half maximal inhibitory concentration (IC50) values per timepoint, per mitotoxic compound.

Technical details of 31P-MRS of the visual cortex

A small sphere (10 mm in diameter) filled with water was placed below the coil along the coil axis to verify and adjust the positioning of the 31P RF coil on 1H images. An adiabitic pulse-acquire sequence (TR 2 s, flip angle 90°) was used to collect free induction decays (FIDS) for 4 minutes at rest (128 signals averaged), 8 minutes during visual activation (256 signals averaged), and 8 minutes after visual stimulation (256 signals averaged). Analysis of the 31P spectra using JMRUI allowed quantification of the following resonances: βATP, αATP, γATP, PCR, and PI, from which the ratios of PCR/ATP, PI/PCR, and PI/ATP were calculated as well as the pH. The spectra were analyzed in the time domain using AMARES in the JMRUI software package. AMARES allowed the inclusion of prior knowledge about relations between peaks (derived from the method of Mochel *et al.*).[17]

REFERENCES

1 A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell.* 1993;72(6):971-983.

2 Polyzos AA, McMurray CT. The chicken or the egg: mitochondrial dysfunction as a cause or consequence of toxicity in Huntington's disease. *Mechanisms of ageing and development.* 2017;161(Pt A):181-197.

3 Quintanilla RA, Johnson GVW. Role of Mitochondrial Dysfunction in the Pathogenesis of Huntington's Disease. *Brain research bulletin.* 2009;80(4-5):242-247.

4 Hamilton J, Brustovetsky T, Brustovetsky N. Oxidative metabolism and Ca(2+) handling in striatal mitochondria from YAC128 mice, a model of Huntington's disease. *Neurochemistry international.* 2017;109:24-33.

5 Pellman JJ, Hamilton J, Brustovetsky T, Brustovetsky N. Ca(2+) handling in isolated brain mitochondria and cultured neurons derived from the YAC128 mouse model of Huntington's disease. *Journal of neurochemistry.* 2015;134(4):652-667.

6 Brustovetsky N. Mutant Huntingtin and Elusive Defects in Oxidative Metabolism and Mitochondrial Calcium Handling. *Molecular neurobiology.* 2016;53(5):2944-2953.

7 PIckrell AM, Fukui H, Wang X, PInto M, Moraes CT. The striatum is highly susceptible to mitochondrial oxidative phosphorylation dysfunctions. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* 2011;31(27):9895-9904.

8 Beal MF, Brouillet E, Jenkins BG, et al. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* 1993;13(10):4181-4192.

9 Brouillet E, Hantraye P, Ferrante RJ, et al. Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. *Proceedings of the National Academy of Sciences of the United States of America.* 1995;92(15):7105-7109.

10 Khalil B, El Fissi N, Aouane A, Cabirol-Pol MJ, Rival T, Lievens JC. PINK1-induced mitophagy promotes neuroprotection in Huntington's disease. *Cell death & disease.* 2015;6:e1617.

11 Cannon JR, Tapias VM, Na HM, Honick AS, Drolet RE, Greenamyre JT. A highly reproducible rotenone model of Parkinson's disease. *Neurobiology of disease.* 2009;34(2):279-290.

12 Orr AL, Li S, Wang CE, et al. N-terminal mutant huntingtin associates with mitochondria and impairs mitochondrial trafficking. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* 2008;28(11):2783-2792.

13 Yu ZX, Li SH, Evans J, PIllarisetti A, Li H, Li XJ. Mutant huntingtin causes context-dependent neurodegeneration in mice with Huntington's disease. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* 2003;23(6):2193-2202.

14 Song W, Chen J, Petrilli A, et al. Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity. *Nature medicine.* 2011;17(3):377-382.

15 Yano H, Baranov SV, Baranova OV, et al. Inhibition of mitochondrial protein import by mutant huntingtin. *Nat Neurosci.* 2014;17(6):822-831.

16 Saft C, Zange J, Andrich J, et al. Mitochondrial impairment in patients and asymptomatic mutation carriers of Huntington's disease. *Movement disorders: official journal of the Movement Disorder Society.* 2005;20(6):674-679.

17 Mochel F, N'Guyen TM, Deelchand D, et al. Abnormal response to cortical activation in early stages of Huntington disease. *Movement disorders: official journal of the Movement Disorder Society.* 2012;27(7):907-910.

18 Sawa A, Wiegand GW, Cooper J, et al. Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. *Nature medicine.* 1999;5(10):1194-1198.

19 Adanyeguh IM, Rinaldi D, Henry PG, et al. Triheptanoin improves brain energy metabolism in patients with Huntington disease. *Neurology.* 2015;84(5):490-495.

20 Smith RA, Murphy MP. Animal and human studies with the mitochondria-targeted antioxidant MitoQ. *Annals of the New York Academy of Sciences.* 2010;1201:96-103.

21 Shults CW, Flint Beal M, Song D, Fontaine D. PIlot trial of high dosages of coenzyme Q10 in patients with Parkinson's disease. *Experimental neurology.* 2004;188(2):491-494.

22 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.

23 van den Bogaard SJ, Dumas EM, Teeuwisse WM, et al. Exploratory 7-Tesla magnetic resonance spectroscopy in Huntington's disease provides in vivo evidence for impaired energy metabolism. *Journal of neurology.* 2011;258(12):2230-2239.

24 Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. *Biochemical Journal.* 2011;435(2):297-312.

25 Nicholls DG. Mitochondrial membrane potential and aging. *Aging cell.* 2004;3(1):35-40.

26 Cossarizza A, Salvioli S. Flow cytometric analysis of mitochondrial membrane potential using JC-1. *Current protocols in cytometry.* 2001;Chapter 9:Unit 9.14.

27 Chance B, Leigh JS, Jr., Clark BJ, et al. Control of oxidative metabolism and oxygen delivery in human skeletal muscle: a steady-state analysis of the work/energy cost transfer function. *Proceedings of the National Academy of Sciences of the United States of America.* 1985;82(24):8384-8388.

28 Hands LJ, Bore PJ, Galloway G, Morris PJ, Radda GK. Muscle metabolism in patients with peripheral vascular disease investigated by 31P nuclear magnetic resonance spectroscopy. *Clinical science (London, England: 1979).* 1986;71(3):283-290.

29 Suzuki E, Kashiwagi A, Hidaka H, et al. 1H- and 31P-magnetic resonance spectroscopy and imaging as a new diagnostic tool to evaluate neuropathic foot ulcers in Type II diabetic patients. *Diabetologia.* 2000;43(2):165-172.

30 Taylor DJ, Bore PJ, Styles P, Gadian DG, Radda GK. Bioenergetics of intact human muscle. A 31P nuclear magnetic resonance study. *Molecular biology & medicine.* 1983;1(1):77-94.

31 Greenman RL, Panasyuk S, Wang X, et al. Early changes in the skin microcirculation and muscle metabolism of the diabetic foot. *Lancet.* 2005;366(9498):1711-1717.

32 Wiener DH, Maris J, Chance B, Wilson JR. Detection of skeletal muscle hypoperfusion during exercise using phosphorus-31 nuclear magnetic resonance spectroscopy. *Journal of the American College of Cardiology.* 1986;7(4):793-799.

33 Zochodne DW, Thompson RT, Driedger AA, Strong MJ, Gravelle D, Bolton CF. Metabolic changes in human muscle denervation: topical 31P NMR spectroscopy studies. *Magnetic resonance in medicine: official journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine.* 1988;7(4):373-383.

34 Unified Huntington's Disease Rating Scale: reliability and consistency. Huntington Study Group. *Movement disorders: official journal of the Movement Disorder Society.* 1996;11(2):136-142.

35 Shoulson I, Fahn S. Huntington disease: clinical care and evaluation. *Neurology.* 1979;29(1):1-3.

36 Walter G, Vandenborne K, McCully KK, Leigh JS. Noninvasive measurement of phosphocreatine recovery kinetics in single human muscles. *The American journal of physiology.* 1997;272(2 Pt 1):C525-534.

37 Browne SE, Bowling AC, MacGarvey U, et al. Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. *Annals of neurology.* 1997;41(5):646-653.

38 Polyzos A, Holt A, Brown C, et al. Mitochondrial targeting of XJB-5-131 attenuates or improves pathophysiology in HdhQ150 animals with well-developed disease phenotypes. *Human molecular genetics.* 2016;25(9):1792-1802.

39 Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AH, Marsden CD. Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. *Brain: a journal of neurology.* 1992;115 (Pt 2):333-342.

40 Reichmann H, Flörke S, Hebenstreit G, Schrubar H, Riederer P. Analyses of energy metabolism and mitochondrial genome in post-mortem brain from patients with Alzheimer's disease. *Journal of neurology.* 1993;240(6):377-380.

41 Andreux PA, van Diemen MPJ, Heezen MR, et al. Mitochondrial function is impaired in the skeletal muscle of pre-frail elderly. *Scientific reports.* 2018;8(1):8548.

42 Lanza IR, Nair KS. Muscle mitochondrial changes with aging and exercise. *The American journal of clinical nutrition.* 2009;89(1):467s-471s.

43 Mueller SM, Gehrig SM, Petersen JA, et al. Effects of endurance training on skeletal muscle mitochondrial function in Huntington disease patients. *Orphanet journal of rare diseases.* 2017;12(1):184.

44 Mueller SM, Petersen JA, Jung HH. Exercise in Huntington's Disease: Current State and Clinical Significance. *Tremor and other hyperkinetic movements (New York, NY).* 2019;9:601.

45 Moodley D, Mody G, Patel N, Chuturgoon AA. Mitochondrial depolarisation and oxidative stress in rheumatoid arthritis patients. *Clinical biochemistry.* 2008;41(16-17):1396-1401.

46 Leuner K, Schulz K, Schutt T, et al. Peripheral mitochondrial dysfunction in Alzheimer's disease: focus on lymphocytes. *Molecular neurobiology.* 2012;46(1):194-204.

47 Khan S, Raghuram GV, Bhargava A, et al. Role and clinical significance of lymphocyte mitochondrial dysfunction in type 2 diabetes mellitus. *Translational research: the journal of laboratory and clinical medicine.* 2011;158(6):344-359.

48 Salimi A, Roudkenar MH, Seydi E, et al. Chrysin as an Anti-Cancer Agent Exerts Selective Toxicity by Directly Inhibiting Mitochondrial Complex II and V in CLL B-lymphocytes. *Cancer investigation.* 2017;35(3):174-186.

49 Loop T, Dovi-Akue D, Frick M, et al. Volatile anesthetics induce caspase-dependent, mitochondria-mediated apoptosis in human T lymphocytes in vitro. *Anesthesiology.* 2005;102(6):1147-1157.

50 Finsterer J, Zarrouk Mahjoub S. Mitochondrial toxicity of antiepileptic drugs and their tolerability in mitochondrial disorders. *Expert opinion on drug metabolism & toxicology.* 2012;8:71-79.

TABLE 1 Demographics. Demographics and baseline values for the UHDRS sub-scores and the PCr recovery time of 31P-MRS of the calf muscle.

	Mean	SD	Min	Max
Number of patients (n)	23			
Age (years)	47.6	9.2	20	64
Sex (% female)	43.5%			
BMI (kg/m2)	26.2	4.7	18.8	40.3
CAG repeat (number)	44.3	4.4	39	60
Age of disease onset (years)	40.6	9.7	19	59
Time since HD-related complaints (years)	7.7	6.7	1	27
UHDRS (score)				
• TMS	22.7	11.1	5	53
• TFC	8.4	1.6	6	11
τPCR (calf muscle, in s)	40.2	6.4	33.3	57.5

UHDRS = Unified Huntington's Disease Rating Scale, TMS = Total Motor Score, TFC = Total Functional Capacity, τPCr = PCr recovery time.

TABLE 2 Pearson correlations. Pearson correlations between mitochondrial and clinical function in all patients.

		CAG repeats	PCR rec time	PCR/ATP ratio	PI/ATP ratio	PI/PCR ratio	TMS	TFC
CAG repeats	R	1	.334	-.325	-.158	.003	.283	-.178
	Sig. (2-tailed)		.120	.130	.472	.990	.190	.416
PCR Rec Time	R	.334	1	-.048	-.310	-.336	.386	-.252
	Sig. (2-tailed)	.120		.828	.151	.117	.069	.246
PCR/ATP ratio	R	-.325	-.048	1	.529**	.028	-.106	.030
	Sig. (2-tailed)	.130	.828		.009	.901	.629	.891
PI/ATP ratio	R	-.158	-.310	.529**	1	.860**	-.449*	.224
	Sig. (2-tailed)	.472	.151	.009		.000	.032	.304
PI/PCR ratio	R	.003	-.336	.028	.860**	1	-.478*	.276
	Sig. (2-tailed)	.990	.117	.901	.000		.021	.202
TMS	R	.283	.386	-.106	-.449*	-.478*	1	-.532**
	Sig. (2-tailed)	.190	.069	.629	.032	.021		.009
TFC	R	-.178	-.252	.030	.224	.276	-.532**	1
	Sig. (2-tailed)	.416	.246	.891	.304	.202	.009	

** Correlation is significant at the 0.05 level (2-tailed) / ** Correlation is significant at the 0.01 level (2-tailed).*

TABLE 3 **Post hoc analysis.** Pearson correlations between mitochondrial and clinical function in the 11 patients with highest CAG repeat number.

		CAG repeats	TMS	TFC	PCR rec time	PCR/ATP ratio	PI/ATP ratio	PI/PCR ratio
CAG repeats	R	1	.070	-.266	-.619*	-.540	-.309	.014
	Sig. (2-tailed)		.838	.429	.042	.086	.355	.968
TMS	R	.070	1	-.550	.186	.131	-.174	-.317
	Sig. (2-tailed)	.838		.080	.583	.700	.608	.342
TFC	R	-.266	-.550	1	-.116	-.165	-.143	-.029
	Sig. (2-tailed)	.429	.080		.735	.627	.675	.932
PCR rec time	R	-.619*	.186	-.116	1	.643*	.186	-.233
	Sig. (2-tailed)	.042	.583	.735		.033	.584	.491
PCR/ATP ratio	R	-.540	.131	-.165	.643*	1	.593	.023
	Sig. (2-tailed)	.086	.700	.627	.033		.054	.947
PI/ATP ratio	R	-.309	-.174	-.143	.186	.593	1	.816**
	Sig. (2-tailed)	.355	.608	.675	.584	.054		.002
PI/PCR ratio	R	.014	-.317	-.029	-.233	.023	.816**	1
	Sig. (2-tailed)	.968	.342	.932	.491	.947	.002	

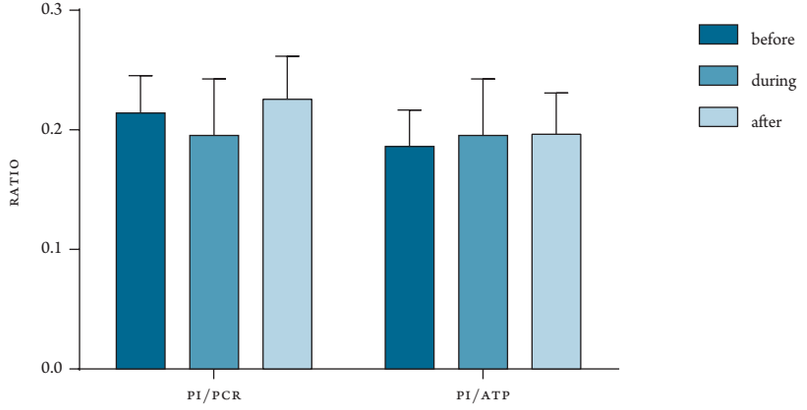
** Correlation is significant at the 0.05 level (2-tailed) / ** Correlation is significant at the 0.01 level (2-tailed).*

TABLE 4 **Central and peripheral mitochondrial function.** Results mitochondrial assessments (peripheral and central).

Mitochondrial function tests	Mean (SD)	SD	Min	Max
SKELETAL MUSCLE				
PCR recovery time after muscle exercise (in seconds)	39.5	8.8	26.3	55.4
LYMPHOCYTES				
Dysfunctional cells (%)	3.6	4.9	0.3	23.5
MMP (delta Psi)	3518.8	1542.2	801.9	6082.4
IC50 verapamil (mmol)	0.54	0.13	0.34	0.78
IC50 carvedilol (mmol)	0.27	0.09	0.13	0.46
VISUAL CORTEX				
PI/PCR ratio before visual stimulation	0.21	0.03	0.16	0.28
PI/ATP ratio before visual stimulation	0.19	0.03	0.12	0.23
PI/PCR ratio during visual stimulation	0.23	0.04	0.15	0.32
PI/ATP ratio during visual stimulation	0.20	0.05	0.11	0.31
PI/PCR ratio after visual stimulation	0.23	0.04	0.15	0.31
PI/ATP ratio after visual stimulation	0.20	0.03	0.15	0.27

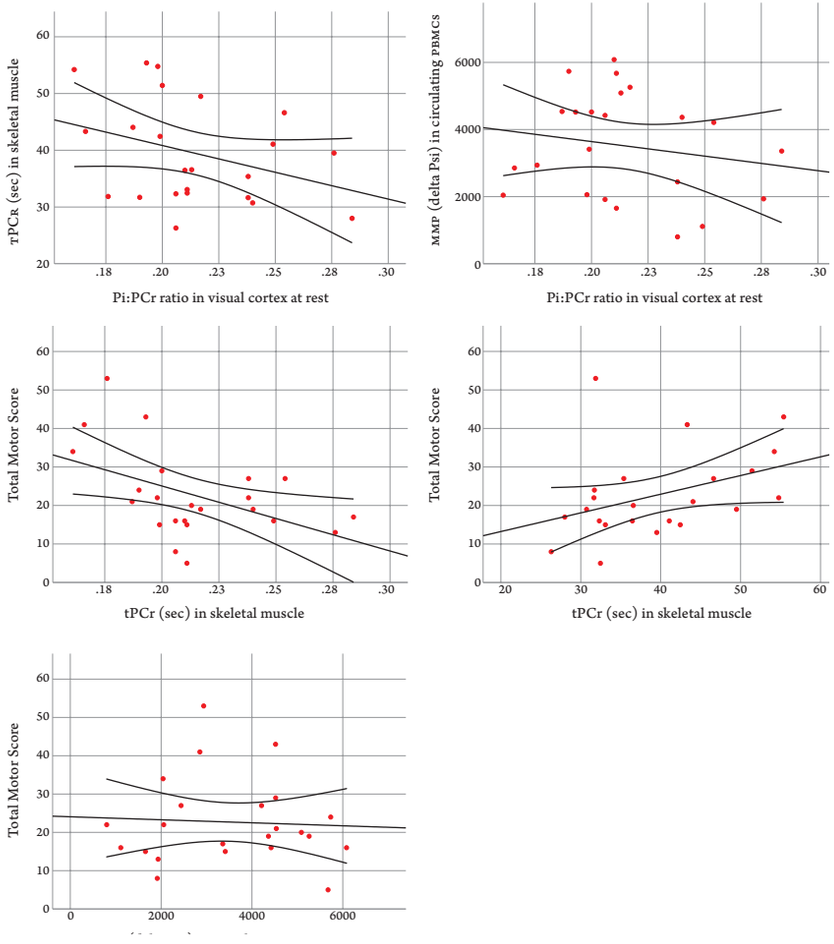
MMP = mitochondrial membrane potential, Pi = inorganic phosphate, ATP = adenosine triphosphate, PCr = phosphocreatine, IC50 = half maximal inhibitory concentration.

FIGURE 1 **Central mitochondrial function.** Central mitochondrial function, assessed by phosphorous MRS of the visual cortex before, during and after visual stimulation. Bio-energetics was expressed as the ratio between phosphate energy metabolites. As expected in HD patients, no significant change was observed between the before and during and during and after ratios.



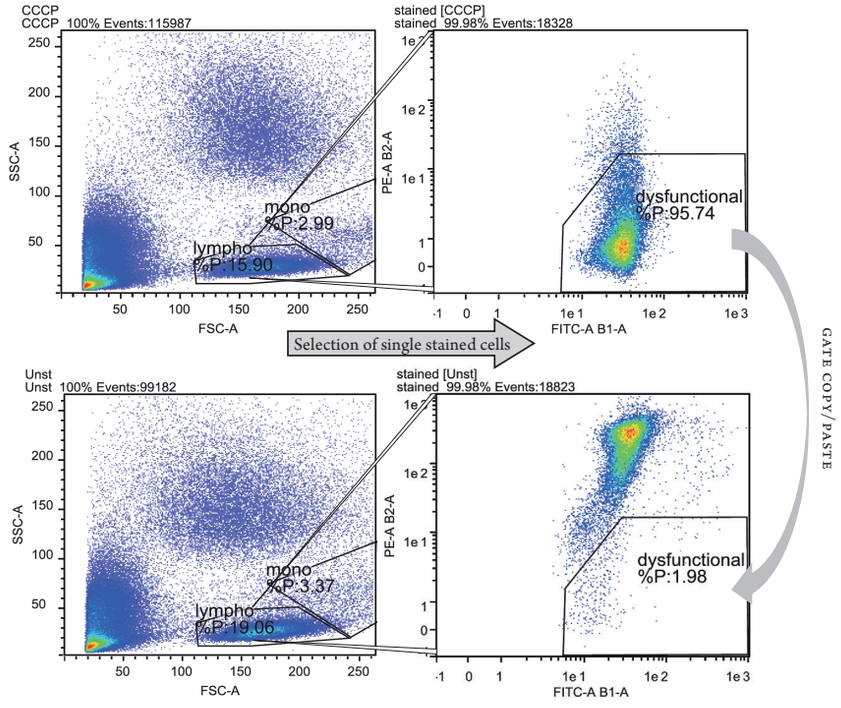
Pi = inorganic phosphate, PCr = phosphocreatine, ATP = adenosine triphosphate.

FIGURE 2 A-E **Pearson correlations.** Scatter plots displaying correlations between A. mitochondrial function in calf muscle and mitochondrial bio-energetics in the visual cortex, B. mitochondrial health in lymphocytes and mitochondrial bio-energetics in the visual cortex, C. Total Motor Score and mitochondrial bio-energetics in the visual cortex, D. Total Motor Score and mitochondrial capacity in calf muscle and E. Total Motor Score and mitochondrial health in lymphocytes.



*$p < 0.05$.

FIGURE 3 **Example of gating of peripheral blood mononuclear cells.** Example of gating of peripheral blood mononuclear cells to determine the percentage of dysfunctional cells. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) causes mitochondria to uncouple and is used as a positive control for mitochondrial dysfunction. This area is then gated and used to estimate the percentage of dysfunctional cells within an unstained population of cells.



CHAPTER VII

MEASUREMENT OF OXYGEN METABOLISM IN VIVO

Published chapter 20 of the book 'Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants'

M.P.J. van Diemen[1] ⋮ R Ubbink[2,3] ⋮ F.M. Münker[3] ⋮ E.G. Mik[2] ⋮ G.J. Groeneveld[1] ⋮⋮⋮ *1. Centre for Human Drug Research, Leiden, NL* ⋮ *2. Erasmus MC, Department of Anesthesiology, Rotterdam, NL* ⋮ *3. Photonics Healthcare B.V., Utrecht, NL*

Introduction – The importance of measuring mitochondrial function in drug trials.

Ever since the discovery of the causal involvement of defective mitochondria in Leber's hereditary optic neuropathy, the roles of mitochondria in cellular physiology have been expanded from merely being an energy producer to a potential driver of apoptosis.[1] Indeed, evidence is increasing that mitochondrial dysfunction plays important roles in many age-related disorders, in some measure because mitochondrial capacity declines with age.

Many different drug classes have toxic effects on mitochondria. Some of these medications are widely utilized so that it is of special interest to elucidate their mechanism of toxicity in various tissues, including muscle.[2] One of such drug class are the statins, with simvastatin being most commonly prescribed.[3-5] Statins induce mitochondrial dysfunction by at least three mechanisms. First, statins inhibit the mevalonate pathway, thereby repressing the biosynthesis of cholesterol and thus lowering plasma levels. However, the biosynthesis of co-enzyme Q10 (Q10), an essential electron carrier in the electron transport system, is also repressed, which can correspondingly repress of ATP production. This a decrease in plasma and muscle Q10 levels after statin administration has been shown[6-9] , although discussion persists as to whether such decreases erode oxidative phosphorylation (OXPHOS).[10] Second, simvastatin significantly inhibits respiratory complexes I, II, III, IV and V using immuno-captured complexes (Nadanaciva et al., Tox & Appl Pharm, 223:277, 2007).[11] Finally, among the statins evaluated, simvastatin most potently uncoupled respiration from phosphorylation, dissipating membrane potential and so forestalling OXPHOS.

Another class of medications that causes mitochondrial toxicity is the biguanides; phenformin, buformin and metformin. The first two were withdrawn from the market because of fatal lactic acidosis, while metformin remains on the market and is associated with lactic acidosis as a persistent adverse event.[12] All three molecules inhibit complex I of the electron transport chain with IC50s in accord with their systemic toxicity.[13-15] Interestingly, metformin has been reported to exert its anti-diabetic effect through this inhibition, which represses diminution of hepatic gluconeogenesis and enhancement of glucose utilization in peripheral tissue as glycolysis accelerates to compensate for reduced OXPHOS.[16]

It is apparent that mitochondrial assessments are needed for development of novel drugs designed to enhance and/or maintain mitochondrial function. Given the growing number of drugs with deleterious effects on mitochondrial function, such assessments should be done early in the drug development program while there is still chemical diversity of the lead screen hits. In addition to avoiding xenobiotic toxicity, the involvement of mitochondrial dysfunction in a wide range of diseases underscores the need for clinical mitochondrial assessments for diagnosis, progression and prognosis. A number of *In vivo* techniques can assess mitochondria in their physiological environment, and we have been focusing on development of non-invasive alternatives to more invasive procedures, such as a muscle biopsy.

Methods – In vivo methods to measure drug effects on mitochondrial function in a clinical setting

The ideal method of measuring mitochondrial function would be inexpensive, and non-invasive, yet able to show multiple parameters of mitochondrial function, such as both oxygen consumption and phosphorous metabolism. *In vivo* assessments reflect mitochondrial function *in situ,* which, because of the complexity of the interaction of mitochondria and intra- and extracellular signals, is arguably most accurate.

Several methods are currently available for both research and clinical settings. The gold standard of *in vivo* mitochondrial assessments relies on monitoring the stable isotope of 31 phosphorus using magnetic resonance spectroscopy (31P-MRS), and has been used in the research lab and clinic. 31P-MRS is not a new technique and its ability to measure phosphorus metabolism, has been used clinically since the early 1980s to study mitochondrial diseases and myopathies.[17-19] One of the parameters, best reflecting mitochondrial function, is the phosphocreatine (PCR) recovery time (τPCR).[20,21] When ATP is consumed, for instance by muscle tissue during exercise, PCR serves as a 'battery', maintaining a constant level of ATP via PCR kinase. By focusing on the nuclear spins of phosphorus and hydrogen, the three phosphates of ATP, and PCR are readily resolved. A typical experimental design entails some sort of stress such as restricting blood flow with a blood pressure cuff, or physical exertion, to deplete PCR and ATP, and then monitor the rate of PCR recovery (τPCR), which has been well validated as directly reflecting mitochondrial status, a contention also corroborated by *in vitro* respirometry.[20-22] Although use of surface coils renders this technique non-invasive, dynamic 31P-MRS measurements are expensive, and require specialized instrumentation and expertise, so it is not yet used for routine measurements in the clinic.

Mitochondrial function can be determined using several less burdensome and cheaper alternatives. For example, systemic mitochondrial dysfunction can

be assessed *ex vivo* by monitoring the mitochondrial membrane potential (MMP) in peripheral blood mononuclear cells (PBMCS). The MMP has long served as a direct reflection of the integrity of the mitochondrial membrane[23] and a direct index of mitochondrial health. The MMP can be disrupted by blockade of the mitochondrial respiratory chain or interference with mitochondria-related death pathways.[24-28] Uncoupling the proton gradient from ATP production, via opening the mitochondrial permeability 'transition pore' or by allowing the protons to flow down the energy gradient bypassing ATP-synthase.[29,30] Fluorescent dyes are typically used to examine the MMP. For example, JC-1.[23] is attracted by the negatively charge in the inner membrane space. JC-1 fluoresces green at low concentrations, but at high concentrations aggregates form that fluoresce red, thereby providing an index of MMP.[23] Measuring MMP as an *ex vivo* assessment can be performed in various types of intact cells or isolated mitochondria and is therefore a usable technique for various diseases and study designs. Perhaps most interestingly is measuring the MMP in peripheral blood mononuclear cells (PBMCS) to assess systemic mitochondrial function. Several studies indeed show a decrease in MMP in circulating PBMCS of patients with neurodegenerative diseases, such as Alzheimer's and Hunting's Disease.[31,32]

Oxygen consumption rate can be determined in skeletal muscle tissue, using Near Infrared Spectroscopy (NIRS), and in skin, using a new technique called Protoporphyrin IX Triplet State Lifetime Technique (PPIX-TSLT). This novel technique makes use of the oxygen-dependent delayed fluorescence of protoporphyrin IX, a precursor protein in the heme-synthesis, which occurs in the mitochondria.

Measuring mitochondrial oxygen consumption with the Protoporphyrin IX Triplet State Lifetime Technique

A novel technique is now available that enables real time mitochondrial oxygen tension in mmHg *in vivo*. Mitochondrial oxygen tension (MitoPO_2) is measured by means of the Protoporphyrin IX–Triplet State Lifetime Technique (PPIX-TSLT) and it is used to assess mitochondrial function in human skin cells in vivo.[33] This is possible because of an oxygen-dependent time of afterglow (triplet state lifetime) of protoporphyrin IX (PPIX), the final precursor of heme in the biosynthetic pathway located in the mitochondria.[34] The human skin has been used to perform the measurements, because of easy access and the non-invasive nature, but the technique is not limited to measurements in the skin. All protoporphyrin IX synthesizing cells are potential measurement locations, for example the kidney and liver.[35,36]

Overproduction of PPIX can be induced in active mitochondria by exogenously providing aminolevulinic acid (ALA), the precursor to PPIX. This bypasses the negative feedback controls in the heme biosynthetic pathway, so that PPIX builds up inside the mitochondria overwhelming the slower reactions converting PPIX to heme.[37,38] This is necessary because, under normal (non-sensitized) conditions, PPIX is present in human skin at very low concentrations and is not detected with the PPIX-TSLT. As a small molecule, ALA penetrates the stratum corneum and topical administration of ALA not only increases enhance PPIX to detectable levels, but also ensures mitochondrial origin of the triplet state fluorescence signal.[35,39,40] After topical administration of ALA on healthy skin, typically for 4 hours or more, PPIX is synthesized. In healthy skin this limits the measurement location and signal origin of the skin sensor to the epidermis with a thickness of about 0,1mm.[41] The recommended measurement location is the skin of the sternum. This provides a central measurement location less influenced by temperature changes, movement and peripheral vasoconstriction.[42]

The optical properties of PPIX make possible its use as a mitochondrial oxygen probe. After excitation with a short-pulsed laser both immediate and triplet state delayed fluorescence can be detected. The delayed fluorescence lifetime is inversely proportional to the amount of oxygen according to the Stern-Volmer equation.[43,44] Local pressure is applied by gently pressing down the the probe, which stops local blood flow, thereby allowing determination of cellular oxygen utilization. In this way, the Oxygen Disappearance Rate (ODR) is determined, as is the recovery rate upon reperfusion.[45] This may become an easily accessible technique to determine mitochondrial function in real time at the bed side. It has been evaluated in this capacity using healthy volunteers and to detect changes in mitochondrial function in rats and volunteers.[33,36,45-47]

Features of a novel COMET measurement system: the first bedside monitor of cellular oxygen metabolism

After introduction of the protoporphyrin IX-triplet state lifetime technique as a new method to measure mitochondrial oxygen tension *in vivo*, the development of a clinical monitor was started. The prototype used in the volunteer trial

has since been further developed by Photonics Healthcare B.V. (Utrecht, The Netherlands). This resulted in the COMET measurement system, an acronym for Cellular Oxygen METabolism. The COMET is a compact medical device approved for clinical use in Europe in 2016. It consists of a monitor and a skin sensor and uses a pulsed-laser to illuminate the measurement site. The delayed fluorescent signal from PPIX accumulated in active mitochondria is projected on a gated red-sensitive photomultiplier tube. Lifetimes of the raw data are calculated on an embedded control board. The thumb-sized Skin Sensor holds optical fibers for excitation and detection. Sensor temperature, used as an approximation of skin temperature, is measured with an electrical resistive sensor.

Clinical trial – Effect of simvastatin on mitochondrial function in vivo in healthy volunteers

The *in vivo* techniques discussed above measure mitochondrial function in different ways and so are complementary. Combining several techniques in a single study could therefore give a more complete and corroborating understanding of how and to what extent a drug might undermine mitochondrial function. To test this notion, we performed a clinical study at the Centre for Human Drug Research (CHDR, Leiden, The Netherlands), in which mitochondrial dysfunction was induced by administering a typical dose of simvastatin (40mg once daily) for 4 weeks to healthy subjects. Mitochondrial function was assessed both *in vivo* and *ex vivo* using 31P-MRS, NIRS, PPIX-TSLT and MMP in PBMCS. After 4 weeks of simvastatin treatment, 31P-MRS revealed that τPCR was significantly prolonged by 15.2% compared to baseline; this is pathognomonic for mitochondrial impairment (from 31.36 to 36.12 seconds, CI95%, 2.0-30.0; $P < 0.05$, Figure 7.1A). Over the same period, measurements by PPIX-TSLT revealed that oxygen consumption within mitochondrial (mitoVO2) increased 13% (CI95%, -0.014-2.716; $P = 0.052$, Figure 7.1B). For all subjects, the mVO_2 showed a trend towards increasing (CI95%, -0.25-3.418; $P = 0.089$, Figure 7.1C), although the differences were not significant. Importantly, the percentage of dysfunctional PBMCS significantly increased from 5.20% at baseline to 14.43% after 4 weeks of simvastatin administration (CI95%, 2.416-16.056; $P= 0.016$, see Figure 7.1D).

Taken together, the data indicate that simvastatin induces detectable mitochondrial dysfunction in as little as 4 weeks. This is in accord with the 31P-MRS results of Wu *et al.,* (2011) who reported that PCR exercise recovery kinetics times doubled (from 28.1 s to 55.4 s) after a 4-week regimen of statin therapy. They used a custom-built calf flexion pedal ergometer that could fit into the magnet and obtained spectra before, during and after exercise.[48]

Both using NIRS and PPIX-TSLT techniques, increased *in vivo* oxygen consumption was revealed, although in the case of muscle oxygen consumption using NIRS this effect was only apparent as a trend. However, the mitochondrial oxygen consumption, measured by PPIX-TSLT, shows to be close to significantly increased. Furthermore, the change in oxygen consumption follows the prolongation in τPCR, measured by 31P-MRS.

The effects of simvastatin on mitochondrial function and mitochondrial respiration can be distinguished between acute and chronic effects. The acute effect of simvastatin seems to be explained by a decrease of mitochondrial function only. Andreux *et al.* showed that simvastatin decreased the basal oxygen consumption rate in *C. Elegans* after 30 hours of administration.[49] The chronic effect of simvastatin, however, might point to an uncoupling effect on mitochondria, causing a decrease in mitochondrial function together with an increase of mitochondrial oxygen consumption. This idea is strengthened by the finding that the mitochondrial membrane potential in PBMC's decreased after 4 weeks of simvastatin administration.

It should be noted in this context that, in an acute exposure model using isolated rat-liver mitochondria, most of the statins, including simvastatin, both inhibit respiration, and also uncouple OXPHOS. Using isolated organelles reveals what could happen in the cell, tissue or patient, but the later are the result of interactions between a host of variables, such as drug metabolism, uptake carriers that increase dose concentrations in a subset of cells, and MMP that can increase mitochondrial exposure. In this way, it's difficult to predict the phenotype of mitochondrial dysfunction. For example, simvastatin not only inhibits Complexes I, II-III, IV, and V, which would eventually repress oxygen consumption and deplete MMP, but it also uncouples OXPHOS, which would increase oxygen consumption to the extent that ETS is inhibited. The data here imply that both inhibition (more dysfunctional PBMC with lower MMPs and slower PCR recharge) and uncoupling (increases in oxygen consumption) are occurring. The approach of combining corroborating assays with different readouts, plus the development of the first bedside monitor of cellular oxygen utilization, provides a more robust assessment and clinically relevant of drug-induced mitochondrial dysfunction.

REFERENCES

1 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.

2 Hargreaves IP, Al Shahrani M, Wainwright L, Heales SJ. Drug-Induced Mitochondrial Toxicity. *Drug safety*. 2016;39(7):661-674.

3 Bouitbir J, Charles AL, Rasseneur L, et al. Atorvastatin treatment reduces exercise capacities in rats: involvement of mitochondrial impairments and oxidative stress. *Journal of applied physiology*. 2011;111(5):1477-1483.

4 Dai YL, Luk TH, Siu CW, et al. Mitochondrial dysfunction induced by statin contributes to endothelial dysfunction in patients with coronary artery disease. *Cardiovascular toxicology*. 2010;10(2):130-138.

5 Diebold BA, Bhagavan NV, Guillory RJ. Influences of lovastatin administration on the respiratory burst of leukocytes and the phosphorylation potential of mitochondria in guinea pigs. *Biochimica et biophysica acta*. 1994;1200(2):100-108.

6 Bleske BE, Willis RA, Anthony M, et al. The effect of pravastatin and atorvastatin on coenzyme Q10. *American heart journal*. 2001;142(2):E2.

7 Deichmann R, Lavie C, Andrews S. Coenzyme Q10 and statin-induced mitochondrial dysfunction. *The Ochsner journal*. 2010;10(1):16-21.

8 Paiva H, Thelen KM, Van Coster R, et al. High-dose statins and skeletal muscle metabolism in humans: a randomized, controlled trial. *Clinical pharmacology and therapeutics*. 2005;78(1):60-68.

9 Lamperti C, Naini AB, Lucchini V, et al. Muscle coenzyme Q10 level in statin-related myopathy. *Archives of neurology*. 2005;62(11):1709-1712.

10 Smith PF, Eydelloth RS, Grossman SJ, et al. HMG-COA reductase inhibitor-induced myopathy in the rat: cyclosporine A interaction and mechanism studies. *The Journal of pharmacology and experimental therapeutics*. 1991;257(3):1225-1235.

11 Schirris TJ, Renkema GH, Ritschel T, et al. Statin-Induced Myopathy Is Associated with Mitochondrial Complex III Inhibition. *Cell metabolism*. 2015;22(3):399-407.

12 Dykens JA, Jamieson J, Marroquin L, Nadanaciva S, Billis PA, Will Y. Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HEPG2 cells and human hepatocytes in vitro. *Toxicology and applied pharmacology*. 2008;233(2):203-210.

13 Ota S, Horigome K, IshII T, et al. Metformin suppresses glucose-6-phosphatase expression by a complex I inhibition and AMPK activation-independent mechanism. *Biochemical and biophysical research communications*. 2009;388(2):311-316.

14 El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *The Journal of biological chemistry*. 2000;275(1):223-228.

15 Andrzejewski S, Gravel SP, Pollak M, St-Pierre J. Metformin directly acts on mitochondria to alter cellular bioenergetics. *Cancer & Metabolism*. 2014;2:12.

16 Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochemical Journal*. 2000;348(Pt 3):607-614.

17 Edwards RH, Dawson MJ, Wilkie DR, Gordon RE, Shaw D. Clinical use of nuclear magnetic resonance in the investigation of myopathy. *Lancet*. 1982;1(8274):725-731.

18 Gadian D, Radda G, Ross B, et al. Examination of a myopathy by phosphorus nuclear magnetic resonance. *Lancet*. 1981;2(8250):774-775.

19 Radda GK, Bore PJ, Rajagopalan B. Clinical aspects on 31P NMR spectroscopy. *British medical bulletin*. 1984;40(2):155-159.

20 Bendahan D, Mattei JP, Guis S, Kozak-Ribbens G, Cozzone PJ. [Non-invasive investigation of muscle function using 31P magnetic resonance spectroscopy and 1H MR imaging]. *Revue neurologique*. 2006;162(4):467-484.

21 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.

22 Layec G, Bringard A, Le Fur Y, et al. Reproducibility assessment of metabolic variables characterizing muscle energetics in vivo: A 31P-MRS study. *Magnetic resonance in medicine: official journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine*. 2009;62(4):840-854.

23 Perelman A, Wachtel C, Cohen M, Haupt S, Shapiro H, Tzur A. JC-1: alternative excitation wavelengths facilitate mitochondrial membrane potential cytometry. *Cell death & disease*. 2012;3:e430.

24 Chen LB. Mitochondrial membrane potential in living cells. *Annual review of cell biology*. 1988;4:155-181.

25 Choi EM, Lee YS. Mitochondrial defects and cytotoxicity by antimycin A on cultured osteoblastic MC3T3-E1 cells. *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association*. 2011;49(9):2459-2463.

26 Isenberg JS, Klaunig JE. Role of the mitochondrial membrane permeability transition (MPT) in rotenone-induced apoptosis in liver cells. *Toxicological sciences: an official journal of the Society of Toxicology*. 2000;53(2):340-351.

27 Kim HA, Blanco FJ. Cell death and apoptosis in osteoarthritic cartilage. *Current drug targets*. 2007;8(2):333-345.

28 You BR, Park WH. The effects of antimycin A on endothelial cells in cell death, reactive oxygen species and GSH levels. *Toxicology in vitro: an international journal published in association with BIBRA*. 2010;24(4):1111-1118.

29 Ding H, Han C, Zhu J, Chen CS, D'Ambrosio SM. Celecoxib derivatives induce apoptosis via the disruption of mitochondrial membrane potential and activation of caspase 9. *International journal of cancer*. 2005;113(5):803-810.

30 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.

31 Lunnon K, Ibrahim Z, Proitsi P, et al. Mitochondrial dysfunction and immune activation are detectable in early Alzheimer's disease blood. *Journal of Alzheimer's disease: JAD*. 2012;30(3):685-710.

32 Panov AV, Gutekunst CA, Leavitt BR, et al. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nat Neurosci*. 2002;5(8):731-736.

33 Harms F, Stolker RJ, Mik E. Cutaneous Respirometry as Novel Technique to Monitor Mitochondrial Function: A Feasibility Study in Healthy Volunteers. *PloS one*. 2016;11(7).

34 Poulson R. The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX in mammalian mitochondria. *The Journal of biological chemistry*. 1976;251(12):3730-3733.

35 Mik EG, Johannes T, Zuurbier CJ, et al. In vivo mitochondrial oxygen tension measured by a delayed fluorescence lifetime technique. *Biophysical journal*. 2008;95(8):3977-3990.

36 Harms FA, Bodmer SI, Raat NJ, Stolker RJ, Mik EG. Validation of the protoporphyrin IX-triplet state lifetime technique for mitochondrial oxygen measurements in the skin. *Optics letters*. 2012;37(13):2625-2627.

37 Malik Z, Kostenich G, Roitman L, Ehrenberg B, Orenstein A. Topical application of 5-aminolevulinic acid, DMSO and EDTA: protoporphyrin IX accumulation in skin and tumours of mice. *Journal of photochemistry and photobiology B, Biology*. 1995;28(3):213-218.

38 Fukuda H, Casas A, Batlle A. Aminolevulinic acid: from its unique biological function to its star role in photodynamic therapy. *The international journal of biochemistry & cell biology*. 2005;37(2):272-276.

39 Kennedy JC, Pottier RH. Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. *Journal of photochemistry and photobiology B, Biology*. 1992;14(4):275-292.

40 Mik EG, Ince C, Eerbeek O, et al. Mitochondrial oxygen tension within the heart. *Journal of molecular and cellular cardiology*. 2009;46(6):943-951.

41 Sandby-Moller J, Poulsen T, Wulf HC. Epidermal thickness at different body sites: relationship to age, gender, pigmentation, blood content, skin type and smoking habits. *Acta dermato-venereologica*. 2003;83(6):410-413.

42 Campbell I. Body temperature and its regulation. *Anaesthesia & Intensive Care Medicine*.9(6):259-263.

43 Mik EG. Special article: measuring mitochondrial oxygen tension: from basic principles to application in humans. *Anesthesia and analgesia*. 2013;117(4):834-846.

44 Mik EG, Donkersloot C, Raat NJ, Ince C. Excitation pulse deconvolution in luminescence lifetime analysis for oxygen measurements in vivo. *Photochemistry and photobiology*. 2002;76(1):12-21.

45 Harms FA, Voorbeijtel WJ, Bodmer SI, Raat NJ, Mik EG. Cutaneous respirometry by dynamic measurement of mitochondrial oxygen tension for monitoring mitochondrial function in vivo. *Mitochondrion*. 2013;13(5):507-514.

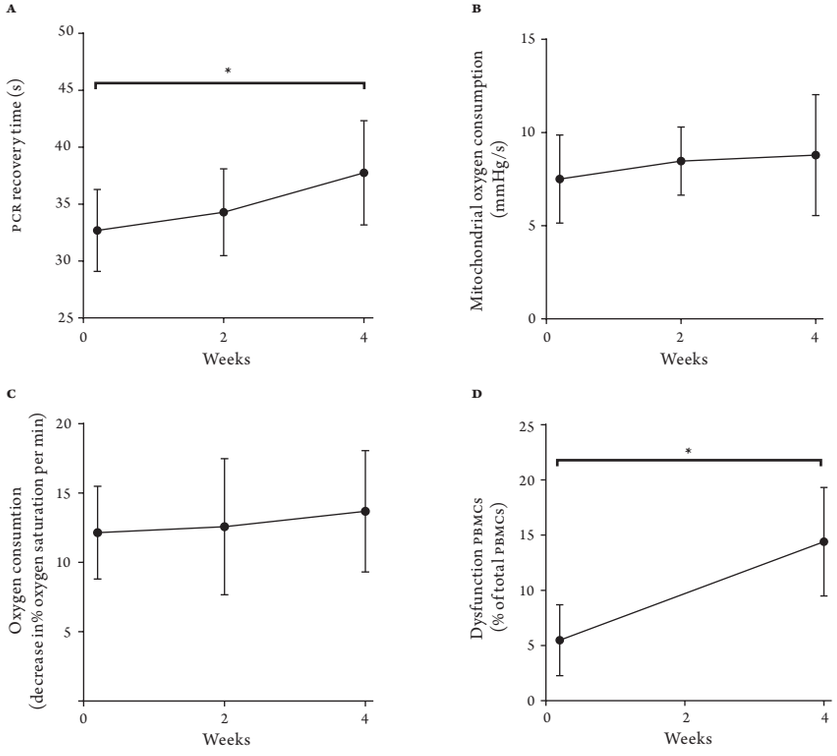
46 Harms FA, Bodmer SI, Raat NJ, Mik EG. Non-invasive monitoring of mitochondrial oxygenation and respiration in critical illness using a novel technique. *Critical care (London, England)*. 2015;19:343.

47 Harms FA, de Boon WM, Balestra GM, et al. Oxygen-dependent delayed fluorescence measured in skin after topical application of 5-aminolevulinic acid. *Journal of biophotonics*. 2011;4(10):731-739.

48 Wu JS, Buettner C, Smithline H, Ngo LH, Greenman RL. Evaluation of skeletal muscle during calf exercise by 31-phosphorus magnetic resonance spectroscopy in patients on statin medications. *Muscle & nerve*. 2011;43(1):76-81.

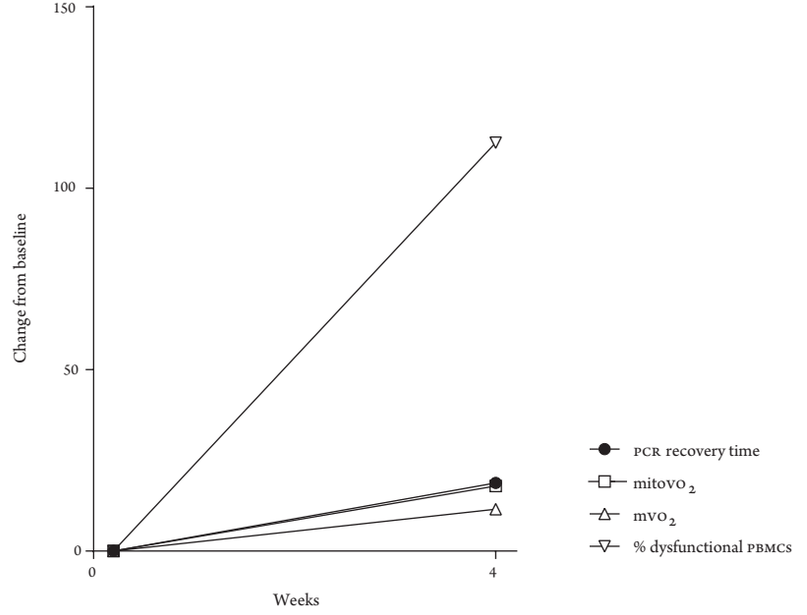
49 Andreux PA, Mouchiroud L, Wang X, et al. A method to identify and validate mitochondrial modulators using mammalian cells and the worm C. elegans. *Scientific reports*. 2014;4:5285.

FIGURE 1 **Mitochondrial function measurements.** Different methods to measure mitochondrial function in vivo or ex vivo. A. phosphorous MRS, B. Protoporphyrin 9 Triplet State Lifetime Technique, C. Near Infrared Spectroscopy and D. Mitochondrial Membrane Potential.



*$p<0.05$.

FIGURE 2 **Change from baseline.** Change from baseline of different methods.



CHAPTER VIII

GENERAL DISCUSSION

GENES, ANATOMY AND…ENERGY

Disease in Western medicine has been based on two fundamental principles: the anatomy of the human body and genetic inheritance. In 1543 Andries van Wesel (*1516-†1564), better known as Andreas Vesalius, described the human body as a corporeal structure filled with distinct and essential organs, arranged in a three-dimensional space. His groundbreaking, but for the time utmost controversial, work *De humani corporis fabrica,* is widely regarded as the foundation of modern anatomy and has led to the belief that tissue specific symptoms must be due to a tissue specific defect. A while later, Gregor Johann Mendel (1822-1884) established the ways of heredity around 1863, which would later be known as Mendelian inheritance of genetic information. Eventually, the theory was extended with genes and chromosomes and together this explained change in anatomy (either healthy or pathological variation) as being due to Mendelian inheritance and thus chromosomal. This also meant that any change, which could not be explained by chromosomal genetics, must be due to the environment.

Mendelian/anatomical medicine has been successful in explaining, diagnosing and treating acute diseases, but has failed to cure most of the chronic (age-related) diseases, which have become an ever-increasing burden on our global, aging society. The reason for this is that anatomy (structure) and genes (information) are only two out of three ingredients of life, the third being energy. Mitochondria provide over 90% of the cell's energy need and are required for everything we do (including writing this thesis). Mitochondria have their own DNA containing a set of genes encoding electron transport chain (ECT) proteins, which puts the mitochondria themselves in charge of our energy. Doug Wallace has been the pioneer in showing the importance of energy on disease. Looking from a bioenergetic point of view, with mitochondrial function at the center, all complex diseases and aging can be understood via the common pathophysiological mechanism 'mitochondrial dysfunction', the severity of which varies with the severity of the resulting disease.[1] Critical dysfunction of mitochondria is fatal at birth or during infancy while the natural course of accumulation of mitochondrial DNA mutations and the resulting gradual decrease in mitochondrial function is at least partly responsible for aging. A partial energy defect can be expected to specifically target organs with the highest energy demand. The brain only weighs 2% of total bodyweight, but uses 20% of the energy. Involvement of mitochondrial dysfunction in neurodegenerative and other age-related diseases is therefore not coincidental.

The central theme of this thesis was mitochondrial (dys)function of muscle and brain in aging and neurodegenerative disorders with the aim to explore several fundamental aspects of pharmacological treatment. Mitochondria are increasingly being investigated as a drug target[2-5] , however the translation of compounds from pre-clinical models into clinical trials is often unsuccessful.

Sarcopenia

> *'Last scene of all, That ends this strange eventful history, second childishness and mere oblivion; Sans teeth, sans eyes, sans taste, sans everything,'*

A common age-related disease is sarcopenia, or the decline in muscle mass and strength in the elderly population, which is part of the frailty syndrome. Sarcopenic elderly are prone to falling, often leading to a hip fracture and high mortality [6] . Mitochondria in muscle of sarcopenic elderly are dysfunctional and it is thought that mitochondrial dysfunction is a driver of the pathophysiology.[7-9] In **Chapter 2**, we explored mitochondrial function in pre-frail, sedentary elderly with the aim to further elucidate the etiological role of mitochondrial dysfunction in sarcopenia in order to identify strategies for prevention. We found that mitochondrial function was impaired in pre-frail elderly, when compared to healthy, active elderly, at multiple sites within the mitochondria and when measured *ex vivo* in muscle tissue and *in vivo* by phosphorous magnetic spectroscopy (31P-MRS) in the calf muscles. The findings confirm that mitochondrial dysfunction is a hallmark of pre-frailty and development of frailty. The results are important, first because such a comprehensive evaluation in the pre-frail elderly population was lacking and second because the results show that mitochondrial dysfunction precedes the frailty stage, during which (pharmacological) therapy has proven to be difficult.[10] As prevention is the best treatment, additional research should be performed to clinically evaluate the disease progression after early treatment of pre-frail elderly with interventions aimed at restoring mitochondrial function in the muscle.

An interesting find was that handgrip strength strongly correlated with the mitochondrial ECT complex activities measured in the muscle biopsy (see Table 8.1). Handgrip strength correlated even better to ECT complex activities than gold standard 31P-MRS of the calf muscle or quadriceps strength, even though this latter measurement was performed in the same leg as the muscle biopsy. Handgrip strength measurement can be easily performed in the outpatient clinic and is

thought to reflect the general condition of a person.[11-13] It has also been shown to predict outcome after several surgical procedures [11] and correlates to cognitive functioning in elderly.[14] These correlations are in accordance with the theory that disease and aging can be seen as a bodywide disturbance in bioenergetic status.[1]

There has been much speculation regarding the origin of the mitochondrial dysfunction in age-related diseases, including sarcopenia and frailty.[15] In our study, we used physical activity as a criterium amongst others to select pre-frail elderly subjects and their matched elderly controls. The pre-frail group had a mean energy expenditure of 392 metabolic equivalent (MET) minutes per week, which corresponds to less than 20 minutes of walking per day. In comparison, the mean energy expenditure in the active group was 6,508 MET minutes per week, which corresponds to 1 hour of vigorous exercise per day. This raises suspicion that a sedentary lifestyle could by itself cause mitochondrial dysfunction in skeletal muscle. This thought is supported by the fact that (resistance) exercise improves mitochondrial function in sarcopenia and frailty.[16] Even in neurodegenerative disorders, the effects on cognition are beneficial.[17] With obesity and an increasingly sedentary lifestyle on the rise, exercise appears to be more important than ever.

In **Chapter 3**, we established a model to predict recovery in mobility after a total knee arthroplasty (TKA) based on several pre-surgery functional measurements. Using wearable activity trackers to monitor patient's recovery after TKA, A multivariate regression analysis led to a positive correlation between the increase of the daily number of steps after TKA and baseline mitochondrial function (i.e. complex 5 abundancy in skeletal muscle), baseline activity (daily number of steps) and baseline grip strength. Combining these results, we formulated the following algorithm to predict the rate of recovery after TKA, based on baseline measurements:

Increase in daily number of steps = -112 + (0.02 × [activity before surgery]) + (0.2 x [CP5 abundancy]) + (3 × [grip strength])

Predicting and monitoring recovery after TKA is clinically important, because despite advances in technology and patient care, an estimated 20-25% of procedures have unsatisfactory results, with dissatisfaction in functional outcome ranging from 16-30%.[18-21] Having a better estimation of recovery might inform surgeons in how intense physical therapy should be and better manage patient's expectations. Measuring grip strength and the daily number of steps are easy to perform in a clinical setting with very low patient burden. Due to the increasing availability of modern smartphones, monitoring a patient's activity as part of postoperative recovery is now readily available. The built-in accelerometer and GPS are able to measure not only activity, but also the traveled distance and quality of walking (e.g. slow, fast and sitting), accurately. The data can automatically be shared online with the treating surgeon, providing objective data on mobility and still minimizing patient burden.

In **Chapter 4**, we demonstrated that simvastatin, a cholesterol lowering drug, can be used to sub-clinically lower mitochondrial function in a group of healthy middle-aged subjects, which can subsequently be reversed by simultaneous suppletion of ubiquinol, the reduced form of coenzyme Q10 (CoQ10). The purpose of the study described in Chapter 4 was to validate a human pharmacological challenge model which can be used to evaluate and prove the pharmacology of novel mitochondrial function enhancing compounds in the healthy subject stage of clinical drug development. In our study we managed to partially reverse the induced dysfunction. The reason for this is that simvastatin inhibits the biosynthesis of CoQ10, which passes electrons from ECT complex I and II to complex III but also directly inhibits complex III (explaining the partial reverse by CoQ10 suppletion). We believe that this makes the model potentially suitable for studies with future compounds that act on ECT complex I, II and III. This requires knowledge of the pharmacological mechanism of the compound beforehand in order to decide, whether the simvastatin POP model is a suitable model of efficacy. More POP models, targeting mitochondrial function in different ways, should be available for clinical studies. Although there are many known mitotoxic drugs like simvastatin, the problem is that for the model to work, the mitotoxic effects must also allow reversal by administration of another compound, like ubiquinol or the new drug under investigation. Unfortunately, most other candidates are too toxic. For instance, a small dose of cyanide is an excellent way to uncouple oxidative phosphorylation and to induce a significant amount of mitochondrial dysfunction, but not very suitable.[22]

In **Chapter 5**, we conducted a clinical trial with the novel mitochondrial function enhancing compound SBT-020 in a group of patients with mild to moderate Huntington's Disease (HD). SBT-020 optimizes the electron flow within the mitochondrial ECT by protecting cardiolipin from oxidation by radical oxygen species, similar to the related compound SS-31 (elamipretide).[2] Mitochondrial capacity was measured peripherally (in the calf muscle and in peripheral blood mononuclear cells (PBMCS)) and in the central nervous system (bio-energetic state in the visual cortex). Although the compound was safe during the multiple

ascending dose part (1 week) and the subsequent longer multiple dose part (4 weeks) and behaved well from a pharmacokinetic point of view, we did not observe clear pharmacological effects on mitochondrial function. HD is a complex and devastating disease, affecting motor, cognitive and psychiatric functioning.[23] There are strong indications in animals and man that mitochondrial dysfunction plays an important role in the pathophysiology of HD, induced via toxic accumulations of misfolded mutant Huntingtin (HTT) protein.[24] When the mitotoxic compound 3-nitropropionic acid is administered to mice, mitochondrial dysfunction is induced, and the animals start showing symptoms, typical for HD.[25] The same happens when mitochondrial function is chronically impaired in non-human primates.[26] In human HD patients, a disbalance in mitochondrial bio-energetics has been reported in the central nervous system (CNS), in the skeletal muscle and in circulating white blood cells.[27-29] Additionally, mutant HTT has been described to localize near mitochondria[30-32] and interact with its proteins.[33] Although mitochondrial dysfunction thus seems to be omnipresent in HD, the source of the symptoms clearly comes from atrophy of the striatum.[23] The striatum is a structure in the brain that is particularly energy demanding[34] and its cells are vulnerable to mitochondrial dysfunction, putting the bioenergetic theory of disease (discussed earlier) in practice. Thus, the reason for the lack of activity of this compound remains unclear

In **Chapter 6** we subsequently explored correlation between peripheral mitochondrial capacity (31P-MRS of the calf muscle), mitochondrial health in PBMCS (mitochondrial membrane potential ($\Delta\Psi m$)) and central mitochondrial bio-energetic state (31P-MRS of the visual cortex) within the same HD patients. We could not demonstrate a correlation between the peripheral and central variables or between the peripheral variables and clinical function, measured as the Unified Huntington's Disease Rating Scale (UHDRS) Total Motor Score (TMS). By contrast, the central mitochondrial bio-energetic state did show a significant correlation ($R = 0.48$, $p = 0.02$) to the TMS. This may be explained by the fact that mitochondria in skeletal muscle are known to have a higher reserve capacity than mitochondria in the striatum.[35] It has been shown that mutant HTT not only accumulates in the striatum, but also in skeletal muscle[27] , but the lack of correlation indicates that mitochondria in skeletal muscle and striatum are not affected to the same degree, even though both tissues are high in energy demand.[34,36] Mitochondrial function in skeletal muscle is also strongly improved by physical exercise such as walking or running, whereas striatal mitochondria are not likely to be influenced by any activity, physical or mental.[37]

Because the dysfunctional mitochondrion has been identified as a target in clinical trials for HD, it is relevant to use a method for mitochondrial function measurements that correlates to clinical function. Although *in vivo* 31P-MRS of the calf muscle has been proposed in other studies as a suitable marker for pharmacodynamic effects[27] , they do not appear to reflect clinical function. Repeat neurocognitive and motor testing (including the Stroop test, Single Digit Modalities Test and Tapping test) has shown to be sensitive to pick up changes over time that correlate with disease progression and thus offer greater value as pharmacodynamic measurements[38,39] , but are not a direct measurement of mitochondrial function. The central bio-energetic state of the visual cortex might be especially useful, because it reflects the bio-energetic state of neuronal mitochondria and correlates to clinical function.

The function of mitochondria within circulating PBMCS did also not correlate to the mitochondrial function in other tissues or clinical function. In the literature, PBMCS of HD patients have been observed to be dysfunctional and with a lower $\Delta\Psi m$ when compared to healthy volunteers.[40,41] However, we did not observe a significant difference between the patients and a group of healthy volunteers (data not published).

Due to the intravascular location, PBMCS have been evaluated as a tool for toxicology of existing and novel medications.[42] In **Chapter 7** we discussed mitotoxicity of commonly prescribed medications and methods to measure mitochondrial function *in vivo*. Mitotoxicity has only recently been recognized as an important mechanism of adverse drug effects and though to be the cause for withdrawal of previously approved medications, such as phenformin and buformin.[43] This has led more and more pharmaceutical companies to screen for mitotoxicity in addition to the standard toxicology screening. In preclinical studies, this is mainly done *in vitro*, using the SeaHorse or Oroboros respirometry devices. These devices assess the activity of the different ECT complexes by measuring the oxygen disappearance rate after adding a series of substrates, used by the complexes.[44] In clinical trials, however, *in vivo* or *ex vivo* methods are preferred due to the complexity of intra- and extracellular signals. The gold standard for measuring mitochondrial function *in vivo* has been 31P-MRS.[45] Although its primary outcome (the phosphocreatine recovery rate) is very reliable, the method requires specialized equipment and staff to operate. Several other (less burdensome and cheaper) techniques are available to measure mitochondrial function in the clinical setting, which we exploratorily used during the study with simvastatin in healthy volunteers (**Chapter 4**): the $\Delta\Psi m$ in PBMCS and oxygen consumption rate in thenar

muscle (using near-infrared spectroscopy (NIRS)) and the novel Protoporphyrin IX Triple State Lifetime Technique (PPIX-TSLT) technique which measures oxygen consumption in the skin. The PPIX-TSLT, a novel technique, makes use of the oxygen-dependent delayed fluorescence of protoporphyrin IX, a precursor protein in the heme-synthesis, which occurs within the mitochondria.[46] The oxygen disappearance rate indicates mitochondrial function.

The *ex vivo* measurement of the $\Delta\Psi m$ in PBMCS could only be performed in 8 subjects, but in this limited number did show an increase of the percentage of dysfunctional cells from 5.20% at baseline to 14.43% after 4 weeks of simvastatin use (CI95%, 2.416–16.056; p = 0.016). These results are consistent with previously observed effects from other mitotoxic medications, such as anti-retroviral medications, and highly useful as a screening tool for mitotoxicity in the clinical setting.[42]

Measuring oxygen consumption *in vivo,* both by NIRS and PPIX-TSLT, did not show an effect from simvastatin. Whereas NIRS measures the oxygen disappearance rate within the capillaries, which is not quite sensitive for mitochondrial dysfunction per se (ischemia triggers it as well), the PPIX-TSLT determines oxygen consumption only within the mitochondria.[46] PPIX-TSLT has been able to accurately measure the uncoupling of oxidative phosphorylation by cyanide when applied in low concentration to the skin[47] , providing a novel way to assess mitotoxicity with minimal exposure for the subject. The COMET is the latest evolution of the technique and a portable device for the clinical setting.

Future perspectives: development of mitochondrial function enhancing drugs

The future of pharmacological enhancement of mitochondria looks bright. More and more pharmacological companies have made mitotoxicity part of the pre-clinical toxicology screening and are investigating mitochondrial function enhancing compounds. The majority of the current clinical trials – listed on clinincaltrials.gov – are exploring the effect of existing medications in treatment of mitochondrial diseases or dysfunction, but also novel compounds are investigated (see Box 1). These developments are hopeful, but the translation of compounds from the pre-clinical phase into clinical trials is still largely based on clinical noticeable effects, such as disease severity. These outcomes are difficult to achieve even when the compound is pharmacologically active, due to the limited treatment time in early phase clinical trials. This is where the POP model with simvastatin will be useful. Mitochondria play an important role in the function of nearly all cells and unexpected or undesired pharmacology could therefore be potentially be severe. This requires early stage clinical trials with novel compounds to be conducted in healthy volunteers, instead of patient populations, to optimize safety. Proving the desired pharmacology during the same study using the POP model could provide a rational to further develop the compound or to end it, saving time and effort. Simvastatin affects mitochondrial function at different sites within the mitochondrial ETC, which makes it suitable for a broad range of mitochondrial function enhancing compounds (acting on complex I, II and III).[48,49] It is difficult to predict whether the POP model could be improved. By increasing the daily dose of simvastatin (for instance to a daily dose of 80mg), a more severe degree of mitochondrial dysfunction could be induced. This could provide more room for the novel medicine to reverse the dysfunction, but at the same time it does not reflect the low degree of mitochondrial dysfunction in age-related diseases and also would probably cause more muscle related adverse events in the healthy volunteers. However, prolonging the simvastatin administration period (for instance to 2 months) could maybe induce a more chronic form of mitochondrial dysfunction, more in line with age-related diseases.

Box 1 – Novel mitochondrial function enhancing compounds and their mechanism of action:

- Novel anti-oxidants: **EPI589, EPI-743, RP103, KH176,**
- Mitochondrial biogenesis: **RTA408, KL1333, SRT2104, TAK831**
- Improving mitophagy: **AMAZ02**
- Inhibiting NF-kappaB: **RG2133**
- Gene therapy: **GS010**

Future perspectives: mitochondrial dysfunction in neurodegenerative diseases

It has become clear that neuronal cells are influenced by a decrease in bio-energetic state resulting from mitochondrial dysfunction, proven by the fact that mitochondrial dysfunction is a common phenomenon in most of the neurodegenerative disorders. The question is therefore not if, but when efficacy of a mitochondrial function enhancing therapy in neurodegenerative disorders will be shown. Until recently, clinical outcome measures have been used to evaluate efficacy, which are notoriously difficult to influence in early stage clinical trials. The availability of *in*

vivo mitochondrial function specific biomarkers should make it easier to show efficacy. 31P-MRS of the visual cortex was an important first step, but contrary to 31P-MRS of the calf muscles, it merely evaluates the bio-energetic state and is not a robust reflection of true mitochondrial function. Due to the secluded nature of brain tissue, other techniques, such as the Near Infrared Spectroscopy and the Protoporphyrin 9 Triple State Lifetime Technique, are difficult to use. The future could lay in a specific biomarker in cerebrospinal fluid (CSF). A very recent mouse study showed the correlation between degenerative changes in the brain and FGF-21, which is a marker for mitochondrial stress, in CSF.[50] FGF-21 has previously been proposed to be a systemic marker for mitochondrial dysfunction[51-54] , and its role in human degenerative disorders should therefore be further explored.

Future perspectives: targeting mitochondria in skeletal muscle to influence body-wide metabolism

Just as mitochondria can no longer be regarded as solely powerhouses of the cell, skeletal muscle can no longer be seen solely as an organ to only grant us mobility. Apart from being able to move things through muscle contractions, skeletal muscle is a secretory organ and communicates with other organs – such as the liver, adipose tissue and the brain – through cytokines.[55] Termed myokines, these cytokines are released by myocytes on muscle contraction and play a role in the body-wide metabolism, including counteracting the pro-inflammatory effect of adipokines (cytokines secreted by adipocytes).[56,57] Exercise (aerobic or non-aerobic) results in a healthy adipokine-myokine balance, whereas a sedentary lifestyle results in the opposite.[56] The effects of exercise (or the lack of) also become clear in a clinical setting: an active lifestyle with plenty of exercise significantly reduces the chance on medical conditions such as cardiovascular pathologies, diabetes, certain types of cancer, depression, neurological disorders or stroke.[58-60] A sedentary lifestyle increases the chance on such conditions.[61-64] Combine this with an excessive calorie intake and the odds are exacerbated by the resulting obesity.[65,66] One could actually argue that doctors should have the option to prescribe physical activity with the same ease as medications. A declining muscle mass due to physical inactivity (eventually leading to sarcopenia) might therefore have influences on the beneficial effects of skeletal muscle on metabolism due to a myokine – adipokine disbalance. Recently, a low level of the circulating myokine irsin was proposed to be a predictive biomarker for sarcopenia.[67] Given the close relationship between inactivity, sarcopenia and skeletal muscle mitochondrial dysfunction, the effects of improving mitochondrial function go further than local effects in skeletal muscle alone. Again, physical activity will play an important role, but it is not always feasible or even possible to pursue an active lifestyle, as is obvious in for instance the recovery period after surgery or during hospitalization. Therefore, pharmacologically improving mitochondrial function in skeletal muscle to induce the beneficial effect on metabolism could be an alternative to physical exercise. In other words, improving mitochondrial function in sarcopenic elderly should thus aim to improve the myokine-adipokine balance, leading to a general improvement in body-wide metabolism and bio-energetic state. Future clinical studies in this field should thus expand the scope from focusing on separate organs to a holistic view of the body in terms of bio-energetics. *In vivo* methods to measure mitochondrial function, such as 31P-MRS, will be of essence. Also, the correlation between myokines and mitochondrial function in different organs should be studied. Skeletal muscle makes up about 30-40% of our body weight, so let's put it to work!

REFERENCES

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

2 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.

3 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.

4 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.

5 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.

6 Schnell S, Friedman SM, Mendelson DA, Bingham KW, Kates SL. The 1-Year Mortality of Patients Treated in a Hip Fracture Program for Elders. *Geriatric Orthopaedic Surgery & Rehabilitation.* 2010;1(1):6-14.

7 Joseph AM, Adhihetty PJ, Buford TW, et al. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. *Aging cell.* 2012;11(5):801-809.

8 Short KR, Bigelow ML, Kahl J, et al. Decline in skeletal muscle mitochondrial function with aging in humans. *Proceedings of the National Academy of Sciences of the United States of America.* 2005;102(15):5618-5623.

9 Alway SE, Mohamed JS, Myers MJ. Mitochondria Initiate and Regulate Sarcopenia. *Exercise and sport sciences reviews.* 2017;45(2):58-69.

10 Marzetti E, Calvani R, Tosato M, et al. Sarcopenia: an overview. *Aging clinical and experimental research.* 2017;29(1):11-17.

11 Allard JP, Keller H, Teterina A, et al. Lower handgrip strength at discharge from acute care hospitals is associated with 30-day readmission: A prospective cohort study. *Clinical nutrition (Edinburgh, Scotland).* 2016.

12 Davies CW, Jones DM, Shearer JR. Hand grip–a simple test for morbidity after fracture of the neck of femur. *Journal of the Royal Society of Medicine.* 1984;77(10):833-836.

13 Shyam Kumar AJ, Beresford-Cleary N, Kumar P, et al. Preoperative grip strength measurement and duration of hospital stay in patients undergoing total hip and knee arthroplasty. *European journal of orthopaedic surgery & traumatology: orthopedie traumatologie.* 2013;23(5):553-556.

14 Firth J, Stubbs B, Vancampfort D, et al. Grip Strength Is Associated With Cognitive Performance in Schizophrenia and the General Population: A UK Biobank Study of 476559 Participants. *Schizophrenia bulletin.* 2018;44(4):728-736.

15 Srivastava S. The Mitochondrial Basis of Aging and Age-Related Disorders. *Genes.* 2017;8(12).

16 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.

17 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.

18 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.

19 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.

20 Dunbar MJ, Richardson G, Robertsson O. I can't get no satisfaction after my total knee replacement: rhymes and reasons. *The bone & joint journal.* 2013;95-b(11 Suppl A):148-152.

21 van de Water RB, Leichtenberg CS, Nelissen R, et al. Preoperative Radiographic Osteoarthritis Severity Modifies the Effect of Preoperative Pain on Pain/Function After Total Knee Arthroplasty: Results at 1 and 2 Years Postoperatively. *The Journal of bone and joint surgery American volume.* 2019;101(10):879-887.

22 Goldsby RA, Heytler PG. Uncoupling of oxidative phosphorylation by carbonyl cyanide phenylhydrazones. II. Effects of carbonyl cyanide m-chlorophenylhydrazone on mitochondrial respiration. *Biochemistry.* 1963;2:1142-1147.

23 A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell.* 1993;72(6):971-983.

24 Quintanilla RA, Johnson GVW. Role of Mitochondrial Dysfunction in the Pathogenesis of Huntington's Disease. *Brain research bulletin.* 2009;80(4-5):242-247.

25 Beal MF, Brouillet E, Jenkins BG, et al. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* 1993;13(10):4181-4192.

26 Brouillet E, Hantraye P, Ferrante RJ, et al. Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. *Proceedings of the National Academy of Sciences of the United States of America.* 1995;92(15):7105-7109.

27 Saft C, Zange J, Andrich J, et al. Mitochondrial impairment in patients and asymptomatic mutation carriers of Huntington's disease. *Movement disorders: official journal of the Movement Disorder Society.* 2005;20(6):674-679.

28 Mochel F, N'Guyen TM, Deelchand D, et al. Abnormal response to cortical activation in early stages of Huntington disease. *Movement disorders: official journal of the Movement Disorder Society.* 2012;27(7):907-910.

29 Finsterer J, Zarrouk Mahjoub S. Mitochondrial toxicity of antiepileptic drugs and their tolerability in mitochondrial disorders. *Expert opinion on drug metabolism & toxicology.* 2012;8:71-79.

30 Orr AL, Li S, Wang CE, et al. N-terminal mutant huntingtin associates with mitochondria and impairs mitochondrial trafficking. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* 2008;28(11):2783-2792.

31 Yu ZX, Li SH, Evans J, Pillarisetti A, Li H, Li XJ. Mutant huntingtin causes context-dependent neurodegeneration in mice with Huntington's disease. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* 2003;23(6):2193-2202.

32 Song W, Chen J, Petrilli A, et al. Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity. *Nature medicine.* 2011;17(3):377-382.

33 Yano H, Baranov SV, Baranova OV, et al. Inhibition of mitochondrial protein import by mutant huntingtin. *Nat Neurosci.* 2014;17(6):822-831.

34 Sauerbeck A, Pandya J, Singh I, et al. Analysis of regional brain mitochondrial bioenergetics and susceptibility to mitochondrial inhibition utilizing a microplate based system. *Journal of neuroscience methods.* 2011;198(1):36-43.

35 Li H, Kumar Sharma L, Li Y, et al. Comparative bioenergetic study of neuronal and muscle mitochondria during aging. *Free radical biology & medicine.* 2013;63:30-40.

36 Iuso A, Repp B, Biagosch C, Terrile C, Prokisch H. Assessing Mitochondrial Bioenergetics in Isolated Mitochondria from Various Mouse Tissues Using Seahorse XF96 Analyzer. *Methods in molecular biology (Clifton, NJ).* 2017;1567:217-230.

37 Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE, Goodpaster BH. Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *The journals of gerontology Series A, Biological sciences and medical sciences.* 2006;61(6):534-540.

38 Stout JC, Jones R, Labuschagne I, et al. Evaluation of longitudinal 12 and 24 month cognitive outcomes in premanifest and early Huntington's disease. *Journal of neurology, neurosurgery, and psychiatry.* 2012;83(7):687-694.

39 Tabrizi SJ, Scahill RI, Durr A, et al. Biological and clinical changes in premanifest and early stage Huntington's disease in the TRACK-HD study: the 12-month longitudinal analysis. *Lancet neurology.* 2011;10(1):31-42.

40 Sawa A, Wiegand GW, Cooper J, et al. Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. *Nature medicine.* 1999;5(10):1194-1198.

41 Panov AV, Gutekunst C-A, Leavitt BR, et al. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nature Neuroscience.* 2002:731-736.

42 Sternfeld T, Schmid M, Tischleder A, et al. The influence of HIV infection and antiretroviral therapy on the mitochondrial membrane potential of peripheral mononuclear cells. *Antiviral therapy.* 2007;12(5):769-778.

43 Dykens JA, Jamieson J, Marroquin L, Nadanaciva S, Billis PA, Will Y. Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HEPG2 cells and human hepatocytes in vitro. *Toxicology and applied pharmacology.* 2008;233(2):203-210.

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

45 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.

46 Mik EG, Johannes T, Zuurbier CJ, et al. In vivo mitochondrial oxygen tension measured by a delayed fluorescence lifetime technique. *Biophysical journal.* 2008;95(8):3977-3990.

47 Harms F, Stolker RJ, Mik E. Cutaneous Respirometry as Novel Technique to Monitor Mitochondrial Function: A Feasibility Study in Healthy Volunteers. *PloS one.* 2016;11(7).

48 Schirris TJ, Renkema GH, Ritschel T, et al. Statin-Induced Myopathy Is Associated with Mitochondrial Complex III Inhibition. *Cell metabolism.* 2015;22(3):399-407.

49 van Diemen MPJ, Berends CL, Akram N, et al. Validation of a pharmacological model for mitochondrial dysfunction in healthy subjects using simvastatin: A randomized placebo-controlled proof-of-pharmacology study. *European journal of pharmacology.* 2017;815:290-297.

50 Restelli LM, Oettinghaus B, Halliday M, et al. Neuronal Mitochondrial Dysfunction Activates the Integrated Stress Response to Induce Fibroblast Growth Factor 21. *Cell reports.* 2018;24(6):1407-1414.

51 Davis RL, Liang C, Edema-Hildebrand F, Riley C, Needham M, Sue CM. Fibroblast growth factor 21 is a sensitive biomarker of mitochondrial disease. *Neurology.* 2013;81(21):1819-1826.

52 Kim KH, Jeong YT, Oh H, et al. Autophagy deficiency leads to protection from obesity and insulin resistance by inducing Fgf21 as a mitokine. *Nature medicine.* 2013;19(1):83-92.

53 Suomalainen A, Elo JM, Pietilainen KH, et al. FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies: a diagnostic study. *Lancet neurology.* 2011;10(9):806-818.

54 Woo YC, Xu A, Wang Y, Lam KS. Fibroblast growth factor 21 as an emerging metabolic regulator: clinical perspectives. *Clinical endocrinology.* 2013;78(4):489-496.

55 Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nature reviews Endocrinology.* 2012;8(8):457-465.

56 Gorgens SW, Eckardt K, Jensen J, Drevon CA, Eckel J. Exercise and Regulation of Adipokine and Myokine Production. *Progress in molecular biology and translational science.* 2015;135:313-336.

57 Fasshauer M, Bluher M. Adipokines in health and disease. *Trends in pharmacological sciences.* 2015;36(7):461-470.

58 Ekelund U, Steene-Johannessen J, Brown WJ, et al. Does physical activity attenuate, or even eliminate, the detrimental association of sitting time with mortality? A harmonised meta-analysis of data from more than 1 million men and women. *Lancet.* 2016;388(10051):1302-1310.

59 Handschin C, Spiegelman BM. The role of exercise and PGC1alpha in inflammation and chronic disease. *Nature.* 2008;454(7203):463-469.

60 Warburton DE, Nicol CW, Bredin SS. Health benefits of physical activity: the evidence. *CMAJ: Canadian Medical Association journal = journal de l'Association medicale canadienne.* 2006;174(6):801-809.

61 Ding D, Lawson KD, Kolbe-Alexander TL, et al. The economic burden of physical inactivity: a global analysis of major non-communicable diseases. *Lancet.* 2016;388(10051):1311-1324.

62 Lobelo F, Stoutenberg M, Hutber A. The Exercise is Medicine Global Health Initiative: a 2014 update. *British journal of sports medicine.* 2014;48(22):1627-1633.

63 Pedersen BK, Saltin B. Exercise as medicine – evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scandinavian journal of medicine & science in sports.* 2015;25 Suppl 3:1-72.

64 Sallis RE. Exercise is medicine and physicians need to prescribe it! *British journal of sports medicine.* 2009;43(1):3-4.

65 Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *Lancet.* 2017;390(10113):2627-2642.

66 Lobstein T, Baur L, Uauy R. Obesity in children and young people: a crisis in public health. *Obesity reviews: an official journal of the International Association for the Study of Obesity.* 2004;5 Suppl 1:4-104.

67 Chang JS, Kim TH, Nguyen TT, Park KS, Kim N, Kong ID. Circulating irisin levels as a predictive biomarker for sarcopenia: A cross-sectional community-based study. *Geriatrics & gerontology international.* 2017;17(11):2266-2273.

TABLE 1 **Pearson correlations.** Correlations between mitochondrial function and clinical function measurements in pre-frail and active elderly.

	Handgrip	31P-MRS	Quadriceps strength	IPAQ
Complex 1	R = 0.64 P = 0.002	R = – 0.44 P = 0.04	R = 0.52 P = 0.01	R = 0.66 P = 0.001
Complex 2	R = 0.42 P = 0.06	R = 0.16 P = 0.49	R = 0.34 P = 0.13	R = – 0.06 P = 0.78
Complex 4	R = 0.62 P = 0.003	R = – 0.48 P = 0.03	R = 0.47 P = 0.03	R = 0.56 P = 0.008
Complex 5	R = 0.66 P = 0.001	R = – 0.44 P = 0.05	R = 0.64 P = 0.002	R = 0.57 P = 0.007

NEDERLANDSE SAMENVATTING

ANATOMIE, GENEN EN... ENERGIE

Andries Vesalius (1516-1564), beter gekend als Andreas Vesalius, heeft in 1543 de fundamenten gelegd voor de Westerse geneeskunde door de mens anatomisch in te delen in verschillende organen. Ziektes konden hierdoor verklaard worden door een probleem in een specifiek orgaan. Later, ontdekte Gregor Johann Mendel (1822-1884) omstreeks 1863 erfelijkheid, wat Mendeliaanse overerving zou gaan heten. Deze theorie werd aangevuld met genetica en leidde tot het fenomeen van genetische erfelijkheid van ziekte. Als ziekte niet hierdoor kon worden verklaard, dan kwam het door invloeden vanuit de omgeving.

Met Mendeliaanse geneeskunde kunnen acute klachten nu met succes worden gediagnosticeerd en behandeld, maar dit geldt niet voor de meeste ouderdomsziektes. Dit komt, omdat naast anatomie en genetica een derde ingrediënt niet in acht wordt genomen: energie. Mitochondriën, de energiefabriekjes binnen de cellen, zorgen voor 90% van deze energiebehoefte en worden voor alles wat het lichaam doet ingezet (ook voor het lezen van dit proefschrift). Mitochondriën hebben een eigen set van genen, die coderen voor de bouwstenen van de energieketen, waarmee mitochondriën via energievoorziening dus een grote invloed uitoefenen op de rest van het lichaam. Douglas Wallace heeft als een van de eersten aangetoond dat dit inderdaad het geval is bij bepaalde ziektes. Ouderdomsziektes en veroudering kunnen worden verklaard vanuit het niet goed functioneren van mitochondriën. Helemaal niet werkende mitochondriën zorgen voor sterfte na de geboorte of vroeg in de kindertijd, terwijl het verslijten van mitochondriën tijdens het leven zorgt voor veroudering. Weefsels met de hoogste energiebehoefte zijn de eerste die hier last van krijgen. De hersenen zijn grootverbruikers van energie en het is daarom niet vreemd dat mitochondriële dysfunctie voorkomt bij de meeste degeneratieve hersenaandoeningen.

Het centrale thema van dit proefschrift was mitochondriële dysfunctie bij veroudering van spieren en hersenen en de rol binnen klinische farmacologie. Mitochondriën worden steeds vaker gezien als een aangrijpingspunt voor geneesmiddelen, maar het lukt nog niet goed om de goede effecten bij proefdieren ook bij mensen te laten zien.

Sarcopenie is de term voor veroudering van spieren, waarbij kracht en spiermassa dramatisch afneemt. Veel sarcopenische ouderen vallen hierdoor, wat vaak leidt tot een gebroken heup en sterfte. Het is aangetoond dat mitochondriën in sarcopenische spieren niet goed werken en er wordt ook gedacht dat spieren juist door mitochondriële dysfunctie aftakelen. In **Hoofdstuk 2** van dit proefschrift hebben we mitochondriële functie binnen sarcopenische ouderen onderzocht met een gespecialiseerde MRI scanner om te zien hoe sarcopenie ontstaat en wat er gedaan kan worden ter preventie. We kwamen erachter dat mitochondriën veel minder goed werkten bij zwakkere ouderen met weinig lichaamsbeweging in de fase voor sarcopenie dan bij gezonde ouderen met veel lichaamsbeweging. Dit betekent ten eerste dat mitochondriële dysfunctie een grote rol speelt in het ontstaan van sarcopenie en ten tweede dat sarcopenie wellicht voorkomen kan worden door mitochondriën gezond te houden met een geneesmiddel. Verder onderzoek moet dit uitwijzen. Interessant was dat knijpkracht zelfs beter correleerde met mitochondriële functie dan de MRI scanner, wat overeenkomt met de gedachte dat mitochondriële dysfunctie in het hele lichaam voorkomt en knijpkracht een eenvoudige, maar waardevolle meting maakt voor algemeen spierverval binnen ouderen. Het tekort aan lichaamsbeweging bij de zwakkere ouderen zorgt waarschijnlijk voor het slechter werken van mitochondriën. Dit maakt lichaamsbeweging een belangrijke factor in het voorkomen van mitochondriële dysfunctie.

In **Hoofdstuk 3** hebben we gekeken of mitochondriële functie een rol speelt bij het herstel na een knievervanging. We maakten hierbij gebruik van stappentellers en zagen dat het herstel in dagelijkse stappen na de operatie kon worden voorspeld door het combineren van mitochondriële functie, knijpkracht en lichaamsbeweging voor de operatie. Het voorspellen en meten van herstel na een knievervanging is erg belangrijk, omdat een groot deel van de patiënten niet tevreden is na de operatie en ook veel patiënten minder actief zijn dan verwacht. Als orthopedisch chirurgen van tevoren kunnen voorspellen hoe een patiënt het na de operatie gaat doen, dan kunnen ze het revalidatieproces aanpassen met meer of minder fysiotherapie en kunnen patiënten een beter beeld krijgen wat te verwachten. Knijpkracht en dagelijkse stappen kunnen makkelijk gemeten worden, aangezien tegenwoordig bijna alle (oudere) patiënten een smartphone met GPS hebben.

In **Hoofdstuk 4** hebben we aangetoond dat mitochondriële functie kan worden verlaagd met simvastatine, een veelgebruikte cholesterolremmer, en dat dit effect weer deels omgekeerd kan worden met ubiquinol, een belangrijke stof voor de werking van de energieketen binnen mitochondriën. Het doel van het onderzoek was om bij gezonde proefpersonen de mitochondriële functie wat te verlagen, zodat nieuwe geneesmiddelen voor mitochondriële dysfunctie effectief en veilig kunnen worden uitgetest in mensen. Simvastatine beinvloed niet alle eiwitten binnen de mitochondriële energieketen, waardoor kennis over de werking van toekomstige geneesmiddelen nodig is voordat ze in dit model uit te testen.

Het mooie aan dit model is dat mitochondriën voldoende worden beïnvloed om geneesmiddelen te testen, maar dat patiënten er bijna geen bijwerkingen aan ondervinden. Cyanide is bijvoorbeeld ook een goed middel om mitochondriële dysfunctie in induceren, maar is om deze reden levensgevaarlijk.

In **Hoofdstuk 5** hebben we een geneesmiddelonderzoek uitgevoerd met het nieuwe middel SBT-020 bij een groep patiënten met de ziekte van Huntington, een slopende ziekte, die zorgt voor dementie, bewegingsstoornissen en depressie. SBT-020 werkt door de energieketen binnen de mitochondriën te stabiliseren en heeft bij proefdieren al veel goede resultaten opgeleverd. Om de juiste dosis te vinden hebben we eerst gedurende een week het middel getest en daarna gedurende 4 weken om een goed beeld te krijgen van het effect. De mitochondriële functie werd met een MRI scanner gemeten in kuitspieren en in de hersenen en in witte bloedcellen. SBT-020 was een veilig middel met voornamelijk bijwerken tijdens het injecteren onder de huid, maar zorgde niet voor een verbetering in mitochondriële functie.

In **Hoofdstuk 6** hebben we de uitkomsten van mitochondriële functie in kuitspier, in witte bloedcellen en in de hersenen - van dezelfde groep patiënten met de ziekte van Huntington - met elkaar vergeleken om te onderzoeken of mitochondriële dysfunctie in dezelfde mate voorkomt binnen verschillende weefsels. We vonden geen correlatie tussen mitochondriële functie in verschillende weefsels, maar wel dat mitochondriële in de hersenen een significante correlatie had met klinische functie. Alhoewel SBT-020 geen effect had op mitochondriële functie, speelt mitochondriële functie binnen patiënten met de ziekte van Huntington toch een veelbelovende rol in een toekomstige behandeling.

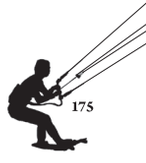
In **Hoofdstuk 7** bespraken we een aantal veel voorgeschreven medicijnen en de bijwerkingen op de mitochondriën hiervan (mitotoxiciteit). Ook bespraken hoe we mitochondriële functie kunnen meten in de levende proefpersoon. Mitotoxiciteit is sinds kort een begrip binnen de farmaceutische industrie en heeft er al voor gezorgd dat bekende geneesmiddelen (zoals fenformin en buformine) van de markt werden gehaald. Tegenwoordig wordt er al routinematig in het lab getest op mitotoxiciteit. In de klinische fase, als de middelen worden getest in mensen, wordt gebruik gemaakt van een gespecialiseerde MRI scanner(31P-MRS) om mitochondriële functie te meten. Andere methoden, waar wij veel metingen mee hebben gedaan zijn de mitochondriële membraan spanning ($\Delta\Psi m$) in witte bloedcellen, zuurstof verbruik in spier (NIRS) en zuurstofverbruik direct in mitochondriën (PPIX-TSLT). PPIX-TSLT maakt gebruik van het terugkaatsen van licht door protoporfyrine, waarvan de snelheid zuurstof afhankelijk is. Door gebruik te maken van een laser en het terugkaatsen hiervan te meten kan men het zuurstof verbruik meten. Tijdens de studie met simvastatine bij gezonde proefpersonen kon mitochondriële dysfunctie goed worden gemeten via de mitochondriële membraan spanning in witte bloedcellen, maar niet via zuurstof verbruik in spier of mitochondriën.

Toekomstperspectief: ontwikkeling van geneesmiddelen voor een betere mitochondriële functie

De ontwikkeling van geneesmiddelen met een positief effect op mitochondriële functie is in volle gang en er zijn al meerdere middelen, die op celculturen in het lab en bij proefdier-modellen goede effecten tonen. Het aantonen van effecten in mensen is echter nog moeilijk, omdat het gewenste effect (de ernst van ziekte verlagen) erg moeilijk is in ziektes waarbij mitochondriële dysfunctie ene rol speelt. Om toch aan te kunnen tonen dat een kandidaatsmiddel een gunstig effect heeft kunnen daarom proof-of-pharmacology modellen in gezonde vrijwilligers, zoals het simvastatine-ubiquinol model (beschreven in Hoofdstuk 4) worden ingezet. In dit model kan de ernst van mitochondriële dysfunctie worden versterkt door de dosis simvastatine te verhogen (naar 80mg ipv 40mg) of om de behandelduur van simvastatine te verlengen naar 2 maanden om zo een meer chronische (ouderdoms) aandoening na te bootsen.

Toekomstperspectief: mitochondriële dysfunctie binnen neurodegeneratieve ziektes

Mitochondriële dysfunctie is een gemeenschappelijk kenmerk binnen de verschillende neurodegeneratieve ziektes (Ziekte van Alzheimer/Parkinson/Huntington etc.) en vormt een belangrijke strategie voor farmacologische therapie. Het aantonen van een gunstig effect op klinische functie is echter lastig, omdat gedane schade vaak niet kan worden teruggedraaid en het remmen van verdere schade pas merkbaar wordt na een lange tijd behandelen. Het normaliseren van mitochondriele functie zou veel sneller kunnen gaan, waardoor het monitoren van mitochondriële functie een betere strategie is. Het meten van mitochondriële functie binnen de hersenen is momenteel nog beperkt tot gespecialiseerde MRI van de visuele cortex, maar geeft niet een hele robuuste meting zoals in kuitspieren. Daarom zou er gezocht moeten worden naar specieke biomarkers in hersenvocht, zoals FGF-21, een marker voor mitochondriële stress.



Toekomstperspectief: mitochondriën in spier behandelen om metabolisme te beïnvloeden

Spierweefsel is als een orgaan niet enkel belangrijk voor beweging maar is ook hormonaal actief in communicatie naar de lever en vetweefsel via myokines (spier-specifieke cytokines). Myokines hebben een sturende rol binnen het metabolisme en zijn een tegenhanger van adipokines (vet-specifieke cytokines). Lichaamsbeweging zorgt voor een gezonde balans tussen de twee en een sedentaire levensstijl zorgt juist voor een disbalans. Het is al uitgebreid bewezen dat een actieve levensstijl een gunstig effect heeft op veel verschillende ziekten zoals diabetes, depressie, bepaalde types kanker en neurodegeneratieve aandoeningen. Een sedentaire levensstijl zorgt juist voor een toename van die ziektes. Het zou daarom geen gek idee zijn dat artsen lichaamsbeweging zouden kunnen voorschrijven aan patiënten. Een afname van spiermassa (sarcopenie) kan ervoor zorgen dat de adipokine-myokine wordt verstoord, met een beinvloeding van het metabolisme. Lichaamsbeweging is niet altijd mogelijk – bijvoorbeeld tijdens een ziekenhuisopname – waardoor er een rol bestaat voor het farmacologisch verbeteren van mitochondriële functie in spier. De link tussen mitochondriële functie en metabolisme nodigt uit tot een holistische blik op het lichaam en leidt weg van het denken in verschillende organen. Toekomstig onderzoek zou zich moeten focussen op andere invloeden van myokines en het gebruik ervan in behandelen van complexe en ouderdomsziektes.

List of Publications

VAN DIEMEN MPJ, HART EP, HAMEETEMAN PW, COPPEN EM, WINDER JY, DEN HEIJER J, MOERLAND M, KAN H, VAN DER GROND J, WEBB A, ROOS RAC, GROENEVELD GJ. *Brain Bio-Energetic State Does Not Correlate to Muscle Mitochondrial Function in Huntington's Disease.* J Huntingtons Dis. 2020;9(4):335-344.

VAN DIEMEN MPJ, HART EP, ABBRUSCATO A, MEAD L, VAN BEELEN I, BERGHEANU SC, HAMEETEMAN PW, COPPEN E, WINDER JY, MOERLAND M, KAN H, VAN DER GROND J, WEBB A, ROOS RAC, GROENEVELD GJ. *Safety, Pharmacokinetics and Pharmacodynamics of SBT-020 in Patients with Early Stage Huntington's Disease, a two-part study.* Br J Clin Pharmacol. 2020 Nov 16.

VAN DIEMEN MPJ, ANDREUX PA, HEEZEN MR, AUWERX J, RINSCH C, GROENEVELD GJ, SINGH A. *Mitochondrial function is impaired in the skeletal muscle of pre-frail elderly*. Sci Rep. 2018 Jun 4

MPJ VAN DIEMEN, R UBBINK, FM MÜNKER, EG MIK, GJ GROENEVELD. Measurement of Oxygen Metabolism In Vivo. p.315-322. Chapter 20 of Will Y, Dykens J.A. (Editors). *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants.* Wiley 2018. ISBN 978-1-119-32974-9

VAN DIEMEN MPJ, BERENDS CL, AKRAM N, WEZEL J, TEEUWISSE WM, MIK BG, KAN HE, WEBB A, BEENAKKER JWM, GROENEVELD GJ. *Validation of a pharmacological model for mitochondrial dysfunction in healthy subjects using simvastatin: A randomized placebo-controlled proof-of-pharmacology study. Eur J Pharmacol. 2017 Nov 15*

Curriculum Vitae

Marcus Peter Johannes van Diemen, born in Haarlem on 27 November 1986, knew from a young age that he wanted to become a physician. After graduating from College Hageveld in Heemstede in 2005, Marcus went to Belgium to study Medicine at the Katholieke Universiteit Leuven, from which he graduated in 2012. Immediately after, he returned to the Netherlands and went to pursue a PhD career at the Centre for Human Drug Research under supervision of prof. dr. Adam Cohen and prof.dr. Geert Jan Groeneveld as a research physician and project leader at the Neurology department. From 2012 to 2018, he gradually focused on mitochondrial (dys)function in healthy volunteers and various patient populations. Whilst working at CHDR, he also certified as a clinical pharmacologist.

After CHDR, Marcus pursued a clinical career in orthopedics and surgery. During the first wave of the COVID-19 pandemic crisis, when orthopedic care was greatly reduced, he worked at the Intensive Care Unit of the IJsselland Hospital (Capelle aan de IJssel), where he developed an interest for anesthesiology and Critical Care Medicine. Marcus is currently working as a resident at the Intensive Care of the Amphia hospital (Breda) and wants to pursue a career in anesthesiology.

Acknowledgments

'And the stone that sits up on the very top
of the mountain's mighty face,
does it think that it's more important
than the stones that form the base?'

(Through Heaven's Eyes from Disney's *The Prince of Egypt)*

In front of you lies the end product of a 6-year long journey on the High Seas of clinical pharmacology and mitochondrial medicine. In September 2012 I boarded the CHDR and embarked my Odyssey, during which I have developed myself scientifically and as a person. Smooth sailing was frequently alternated by riding rough seas, but the ship has finally reached port. I am extremely proud of my achievements, but could not have done this alone. I want to thank my fellow voyagers, who inspired and supported me during this journey.

First of all, I want to thank my mum, Marja, for being one of the most important persons in my life. Your everlasting love and support has laid the early foundation of my path. You have taught me to never give up and to be a warm person. These essential skills have proven to be invaluable during my work as a physician. Thank you for being you.

I want to thank all my dear colleagues at CHDR. You have made me feel at home at CHDR for a good 6 years. Some say CHDR is a big family and it truly felt like that. From Christmas party madness to late-hour shots at the office, you gave color to the rather straight forward world of science.

I want to thank my paranymphs Marije and Martin for standing by my side during my trial. Marije, as my sister you have always been one of the closest persons in my life. We have shared good and sad times and grown more closely over the years. I have always admired your adventurous mind and desire to travel the world. Wherever you go, know that you are in my heart. And Martin, my good friend, Leuven would not have been the same without you. The great times at Doc's Bar, the Oude Markt and all the beer cantusses are too numerous to count and cheers to all that the future will bring.

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And last but not least, I want to thank Geert Jan for being my primary mentor at CHDR. It was you who introduced me into the world of mitochondria and therefore shaped my scientific path. Thank you for always being there to consult when I needed support. You taught me to think in a critical way and to push forward when things were difficult. I will use this knowledge to continue my path in medicine and science.