Clinical pharmacological aspects of mitochondrial function in muscle

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CHAPTER IV

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

Published Eur J Pharmacol. 2017 Nov 15;815:290-297

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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.

Acknowledgements

We thank the study volunteers for participating in the study and clinical staff at CHDR for facilitating it.

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

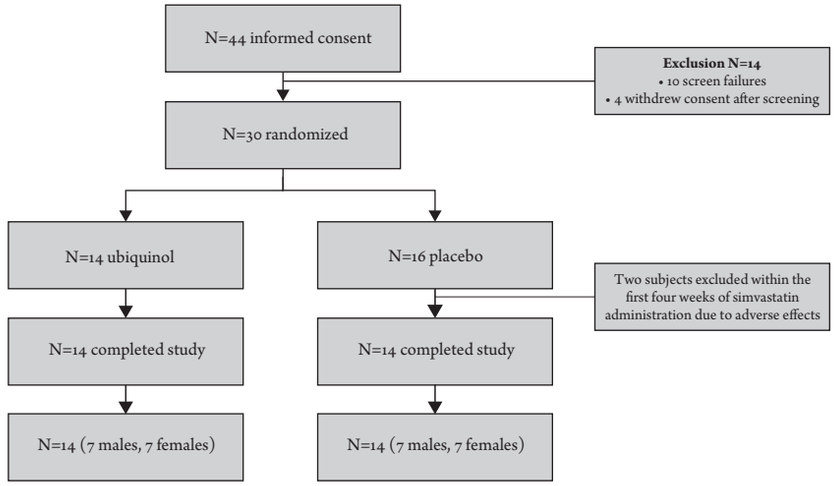
	Ubiquinol	Placebo
Age (years)	61.36 (range 40 – 66)	55.92 (range 44 – 70)
Male (n)	7 (50%)	7 (50%)
Female (n)	7	7
Body mass index (kg/m^2)	25.80 (range 18.29 – 30.37)	25.70 (range 21.13 – 30.98)

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.

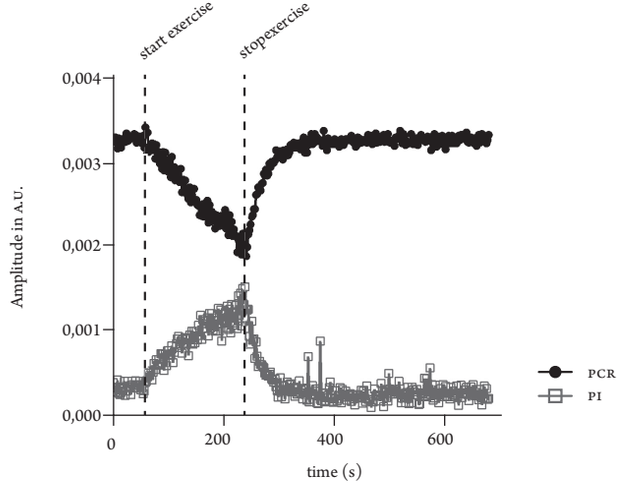
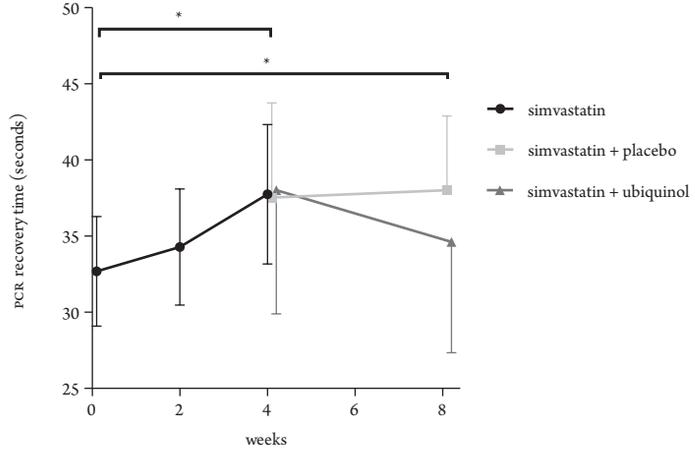


FIGURE 3 **Phosphocreatine recovery time.** τ-PCr after 4 weeks of simvastatin treatment was significantly prolonged compared to baseline by 15.2%. After 8 weeks, the τ-PCr had further prolonged compared to baseline to 18.5% in the placebo group. In the ubiquinol group, however, the τ-PCr prolongation 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.

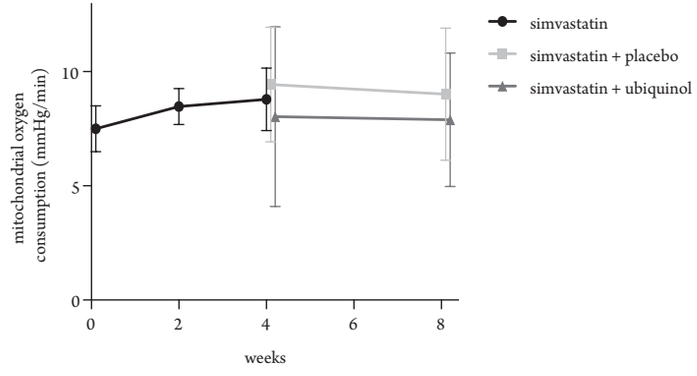


FIGURE 5 Muscular oxygen consumption. Decrease in oxygen saturation (mVO_2) in the thenar muscle. Error bars depict 95% confidence interval. No differences were noted between groups or days.

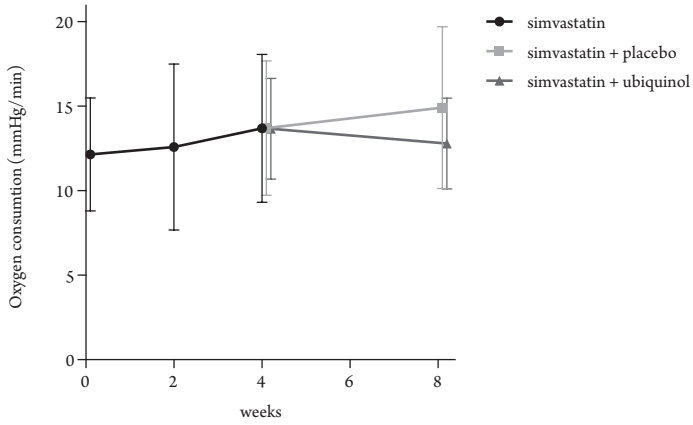
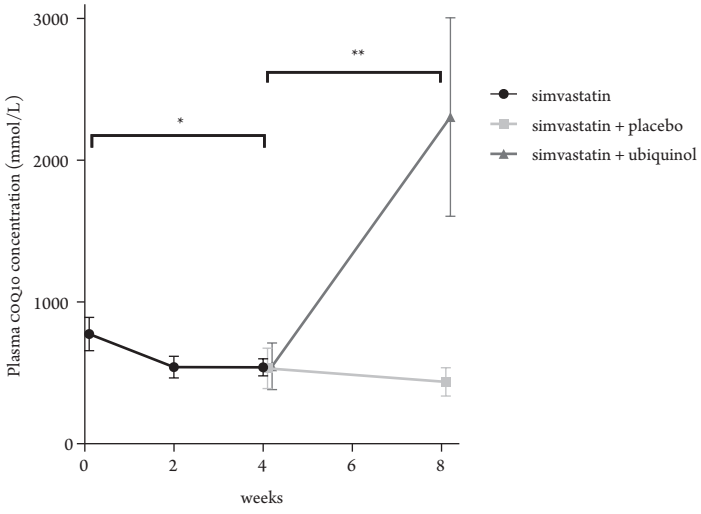


FIGURE 6 Plasma CoQ10 concentration. Plasma CoQ10 concentration over time. Triangles: first 4 weeks of simvastatin treatment; dots: simvastatin + placebo treatment; squares: simvastatin + ubiquinol treatment. Concentration significantly increased in the ubiquinol group and decreased further in the placebo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



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