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The impact of 60 days of −6° head down tilt bed rest on mitochondrial content, respiration and regulators of mitochondrial dynamics

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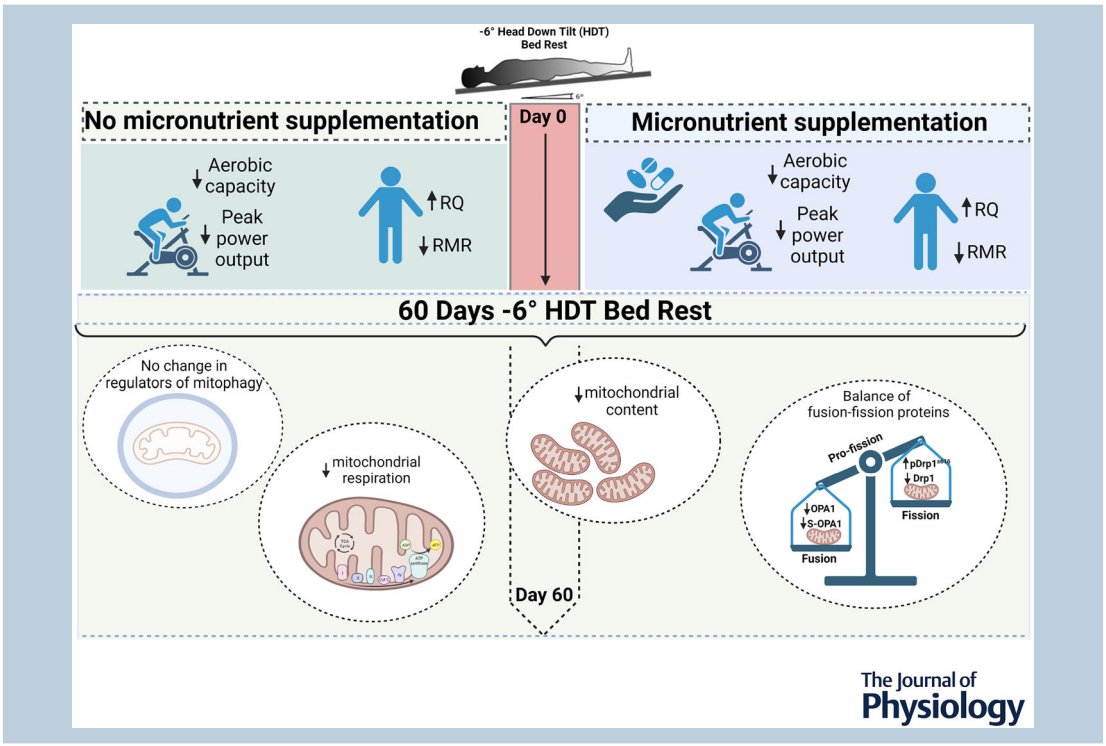
Handling Editors: Paul Greenhaff & Karyn Hamilton
The peer review history is available in the Supporting Information section of this article (https://doi.org/10.1113/JP284734#support-information-section).



Clinical trial.gov Identifier: NCT03594799

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DOI: 10.1113/JP284734

The Journal of Physiology

Abstract It is unclear how skeletal muscle metabolism and mitochondrial function adapt to long duration bed rest and whether changes can be prevented by nutritional intervention. The present study aimed (1) to assess the effect of prolonged bed rest on skeletal muscle mitochondrial function and dynamics and (2) to determine whether micronutrient supplementation would mitigate the adverse metabolic effect of bed rest. Participants were maintained in energy balance throughout 60 days of bed rest with micronutrient supplementation (INT) (body mass index: 23.747 ± 1.877 kg m^{-2}; 34.80 ± 7.451 years; $n = 10$) or without (control) (body mass index: 24.087 ± 2.088 kg m^{-2}; 33.50 ± 8.541 years; $n = 10$). Indirect calorimetry and dual-energy x-ray absorptiometry were used for measures of energy expenditure, exercise capacity and body composition. Mitochondrial respiration was determined by high-resolution respirometry in permeabilized muscle fibre bundles from vastus lateralis biopsies. Protein and mRNA analysis further examined the metabolic changes relating to regulators of mitochondrial dynamics induced by bed rest. INT was not sufficient in preserving whole body metabolic changes conducive of a decrease in body mass, fat-free mass and exercise capacity within both groups. Mitochondrial respiration, OPA1 and Drp1 protein expression decreased with bed rest, with an increase $pDrp1^{s616}$. This reduction in mitochondrial respiration was explained through an observed decrease in mitochondrial content (mtDNA:nDNA). Changes in regulators of mitochondrial dynamics indicate an increase in mitochondrial fission driven by a decrease in inner mitochondrial membrane fusion (OPA1) and increased $pDrp1^{s616}$.

(Received 24 March 2023; accepted after revision 1 November 2023; first published online 30 November 2023)
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Abstract figure legend Reductions in skeletal muscle mitochondrial respiration following 60 days of bed rest explained by a decrease in mitochondrial content, but resulting in an increase in OPA1 and Drp1 mediated mitochondrial fission.

Key points

- Sixty days of −6° head down tilt bed rest leads to significant changes in body composition, exercise capacity and whole-body substrate metabolism.
- Micronutrient supplementation throughout bed rest did not preserve whole body metabolic changes.
- Bed rest results in a decrease in skeletal muscle mitochondrial respiratory capacity, mainly as a result of an observed decrease in mitochondrial content.
- Prolonged bed rest ensues changes in key regulators of mitochondrial dynamics.
- OPA1 and Drp1 are significantly reduced, with an increase in $pDrp1^{s616}$ following bed rest indicative of an increase in mitochondrial fission.
- Given the reduction in mitochondrial content following 60 days of bed rest, the maintenance of regulators of mitophagy in line with the increase in regulators of mitochondrial fission may act to maintain mitochondrial respiration to meet energy demands.

John Noone obtained his PhD at Dublin City University, Ireland, under the supervision of Dr Donal J. O'Gorman studying skeletal muscle metabolic adaptations to inactivity in collaboration with the European Space Agency. He subsequently completed a postdoctoral fellowship at Trinity College Dublin under the guidance of Dr Richard K. Porter studying pharmacological approaches to treat cancer cachexia. Currently, John is a postdoctoral fellow at the Translational Research Institute, AdventHealth, Orlando, Florida, under the mentorship of Dr Bret H. Goodpaster. His research investigates skeletal muscle metabolic responses to inactivity, exercise and disease with a focus on mitochondrial capacity and dynamic responses.

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Introduction

Bed rest results in several metabolic changes, including a decrease in resting metabolic rate (RMR) and metabolic flexibility (i.e. ability of the body to adjust substrates use to changes in substrates availability), culminating in a reduction in skeletal muscle mass (Belavý et al., 2011; Bergouignan et al., 2006). When energy balance is not maintained, there is also an accumulation of visceral and s.c. adipose tissue. The regulation of metabolic processes and the mechanisms underpinning these adaptations in skeletal muscle are not fully understood. The −6° head down tilt (HDT) bed rest model is used as an analogue for space flight adaptations and, as such, has been used to further our understanding of the impact of microgravity and decreased physical activity on physiological processes in the body (Bergouignan et al., 2011; Kenny et al., 2017, 2020; Le Roux et al., 2021; Rudwill et al., 2018).

The contribution of skeletal muscle mitochondria to these metabolic changes during bed rest has been investigated recently. We previously reported a decrease in mitochondrial respiration following 21 days of bed rest; a reduction predominantly explained by a decrease in mitochondrial content (Kenny et al., 2017). However, short duration bed rest studies of 5–10 days have not reported such changes in mitochondrial respiration or mitochondrial content (Marshall et al., 2022), suggesting a time-course effect on the impact of bed rest. There are no studies at present measuring the changes in mitochondrial respiration for greater than 21 days in bed, but it appears the regulation of mitochondrial content is a key mechanism in the adaptation to bed rest.

Mitochondrial dynamics, namey the balance between fission and fusion, has been shown to play an important role in the maintenance of overall mitochondrial function, size and content. An increase in mitochondrial fusion is correlated with a greater capacity to generate energy through oxidative phosphorylation and β-oxidation (Dai & Jiang, 2019; Parra et al., 2014; Pich et al., 2005; Yao et al., 2019; Zanna et al., 2008), whereas increased fission is the division of larger mitochondrial reticula into smaller reticula (Rudwill et al., 2018; Wai & Langer, 2016). Inner mitochondrial membrane fusion is regulated by the actions of optic atrophy 1 (OPA1), whereas outer mitochondrial membrane (OMM) fusion is governed by the actions of mitofusin 1/2 (Mfn1/2) among others (Youle & Van Der Bliek, 2012). Mitochondrial fission is regulated by the phosphorylation and expression of dynamin-related protein 1 (Drp1) and fission protein 1 (Fis1) in particular (Pfluger et al., 2015; Tandler et al., 2018). An increase in pDrp1^{s616} results in an increased mitochondrial abscission followed by fission, whereas an increase in pDrp1^{s637} leads to a reduction in Drp1-mediated fission. However, evidence is emerging that other proteins are also involved in these processes (Han et al., 2008; Zorzano et al., 2010). This phenotypic change is proposed to occur in unison with an increase in phosphatase and tensin homolog-induced kinase 1-Parkin mediated mitochondrial breakdown (mitophagy), thereby acting to mitigate against excessive mitochondrial calcium transfer resulting in a healthier mitochondrial environment (De Brito & Scorrano, 2008).

Nutritional interventions to counteract adverse bed rest induced adaptations are of particular interest because of their motility and benefit to our overall metabolic health (Grune et al., 2003; McGlory et al., 2019; Powers, 2014; Powers et al., 2016). However, few studies have examined nutritional interventions in bed rest (English et al., 2016), with much of our understanding limited to animal models of hind limb suspension (Bennett et al., 2013; Momken et al., 2011). These nutritional benefits are considered to be a result of the impact that dietary supplementation has on multiple targets in throughout the body, particularly with respect to whole-body metabolism. Using an omega-3 containing micronutrient supplement, we have shown that fatty acid oxidation and type IIa muscle fibre cross-sectional area were preserved following 20 days of reduced physical activity in healthy male adults (Damiot et al., 2019). The present study aimed to determine whether a micronutrient supplement, similar in its composition, could mitigate changes in whole-body substrate utilization during 60 days of bed rest. We also investigated the impact that this long duration bed rest had on mitochondrial respiration and determined whether such an environment impacted upon regulators of mitochondrial dynamics and breakdown to preserve mitochondrial function.

Methods

Ethical approval

This study conformed to the standards set by the latest revision of the *Declaration of the Helsinki* and all procedures were approved by the Comité de Protection des Personnes/CPP Sud–Ouest Outre-Mer I and the French Health Authorities (Agence Française de Sécurité Sanitaire des Produits de Santé) (ID RCB: 2016-A00401-50).

Participants

In total, 20 healthy active males (age 34.15 ± 1.75 years; height: 176 ± 5 cm; weight: 73.5 ± 6.1 kg; body mass index: 23.92 ± 0.43 kg m^{-2}) volunteered to participate in this bed rest study. All participants provided their written informed consent and underwent a comprehensive screening protocol prior to selection. Participants were free of any history of diabetes mellitus, hyperlipidaemia, hyperglycaemia, hyperthyroidism or hypothyroidism,

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and were non-smokers, with no excessive alcohol or drug consumption. All experiments were conducted at the Space Clinic of the Institute of Space Medicine and Physiology (Medes-IMPS, Rangueil Hospital) in Toulouse, France, and were sponsored by the European Space Agency (ESA) and the French National Space Agency (CNES).

Study design

This was a randomly assigned between-subject study. All volunteers completed 60 days of a control (CON) (body mass index: 24.09 ± 0.66 kg m^{-2}; 33.5 ± 2.7 years; $n = 10$) or dietary intervention (INT) (body mass index: 23.75 ± 0.59 kg m^{-2}; 34.8 ± 2.36 years; $n = 10$) trial. In both trials, volunteers remained supine with at least one shoulder on the bed at all times. In the INT trial, volunteers were given a combination of anti-oxidant and anti-inflammatory oral tablets three times per day taken at mealtime. As a result of difficulties in finding a neutral oil to encapsulate for a placebo to mask the fish oil odour of the supplement, a placebo was not given to control subjects. Participants resided at the testing facility for 14 days before the bed rest period to standardize preparation and allow baseline data to be collected (baseline data collection, BDC). To maintain subjects' typical physical activity level during the BDC period, subjects' habitual physical activity was objectively measured in free-living conditions over a 10 day period using both self-reported questionnaires and hip-worn triaxial accelerometers (Actigraph GT3x+; Actigraph LLC, Pensacola, FL, USA). Together, these aided in the calculation of subject's free-living mean daily metabolic equivalents score, which was used to develop a personalized physical activity program focused at maintaining subject habitual physical activity level during the BDC period. Room lighting was on between 07:00 h and 23:00 h. Because this study is part of a European Space Agency (ESA) project, the volunteers stayed in a −6° HDT position for the duration of bed rest to preserve simulated microgravity effects. To ensure participants optimally recovered from this intervention, 14 days of in-clinic post-study support and care (Recovery I+1 to +14) was provided to all participants following the 60 days of −6° HDT bed rest. This recovery period was followed by in-clinic check-ups on R+30, +360 and +720. The focus was on returning measures of exercise capacity, muscle mass and energy intake to a pre-trial state. A summary of the study design is provided in Fig. 1.

Diet and energy expenditure

Diet was tightly controlled during the study by registered dieticians and provided by the clinical team. The composition of the diet was the same for all subjects, adapted to their energy expenditure, obtained through indirect calorimetry (the dietary composition can be found within the Supporting information, Table S1 S3), epitomizing no difference between the micronutrient content consumed from the meals provided in each group. For all ESA bed rest studies, the aim is to maintain fat mass at the same time as accepting that the fat-free mass (FFM) will decrease. Fat mass was clamped by ensuring that the total energy intake matches energy expenditure. This was achieved by measuring RMR and body composition and adjusting the diet accordingly. Participants were fed conventional foods calculated to provide 1.2 g kg^{-1} day^{-1} protein, 35% of energy as fat and the rest as carbohydrates. Energy intake was calculated to provide 1.67 times the RMR during the BDC and recovery periods and 1.33 during the best rest. Food ingested was weighted and energy consumption was calculated using NUTRILOG, version 3.11b (https://nutrilog.com). No extra food was allowed between the three daily meals taken at set times. All leftovers on the tray were weighed by the staff of the metabolic kitchen.

Body mass, composition and aerobic fitness

Body mass and composition were measured using dual-energy x-ray absorptiometry and RMR and respiratory quotient (RQ) were measured at various intervals throughout this study. Peak oxygen consumption ($\dot{V}_{O_2peak}$), peak power and peak heart rate during aerobic exercise were measured using standard protocols on a cycle ergometer (Lode, B.V., Groningen, The Netherlands) before and after bed rest.

Micronutrient cocktail composition

The micronutrient cocktail composition was based on a previously published study focused on a reduction in physical activity (Damiot et al., 2019), yet differing slightly in polyphenol concentration per day because of the pronounced effect that bed rest has been shown to have on the body in comparison with reduced physical activity. This cocktail of antioxidant and anti-inflammatory agents is referred to as XXS-2A-BR2 and was designed by Spiral Company (Dijon, France). The daily dose was achieved by the ingestion of six pills (two at breakfast, lunch and dinner) to reach a total dose of 741 mg day^{-1} of polyphenols composed of 323.4 mg day^{-1} flavonols (including quercetin 135 mg), 45.6 mg day^{-1} phenylpropanoides, 78.0 mg day^{-1} oligostilbens (including resveratrol 21 mg), 50.4 mg day^{-1} hydroxycinnamic acids (including 42 mg chlorogenic acid), 135.6 mg day^{-1} flavanols (including epigallocatechin gallate 60 mg) and 108.0 mg day^{-1} flavanones (including naringin 30 mg). This was combined with a 2.1 g daily dose of ω-3 fatty acids (Omacor, Pierre Fabre Laboratories, Toulouse,

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France) and was provided as a single pill per meal (3 pills day^{-1}). This ω-3 fatty acid pill comprised 1.1 g of eicosapentaenoic acid and 1 g of docosahexaenoic acid. Vitamin E and selenium were given as a single daily pill providing 168 mg of vitamin E in combination with 80 μg of selenium (Solgar, Marne la Vallée, France). The control group did not receive any supplementation or placebo.

RMR and respiratory quotient

RMR was obtained for all subjects following an overnight fast and prior to breakfast on BDC −14 and −2 and on days 4, 25, 39 and 58 during HDT. Subjects lay in bed and the RQ and RMR were calculated using canopy dilution respirometry (Quark, Cosmed, Rome, Italy) with the classical equation of indirect calorimetry corrected for urinary nitrogen excretion (Ferrannini, 1988; Frayn, 1983).

Skeletal muscle biopsies

Prior to and just before the end of the 60 day bed rest period (BDC-6 and HDT56) skeletal muscle specimens were obtained by muscle biopsy from the vastus lateralis using the standardized Bergstrom needle technique when subjects were in the fasted state. Muscle biopsy samples were either frozen in liquid nitrogen for nucleic acid and protein analysis, and subsequently stored at −80°C, or placed into a preservation medium, BIOPS solution (in mM: 2.77 CaK_2 EGTA, 7.23 K_2 EGTA, 20 immidazole, 20 taurine, 6.65 $MgCl_2$, 5.77 ATP, 3.95 phosphocreatine, 0.5 dithiothreitol, 50 K-Mes, pH 7.1 at 0˚C) and prepared

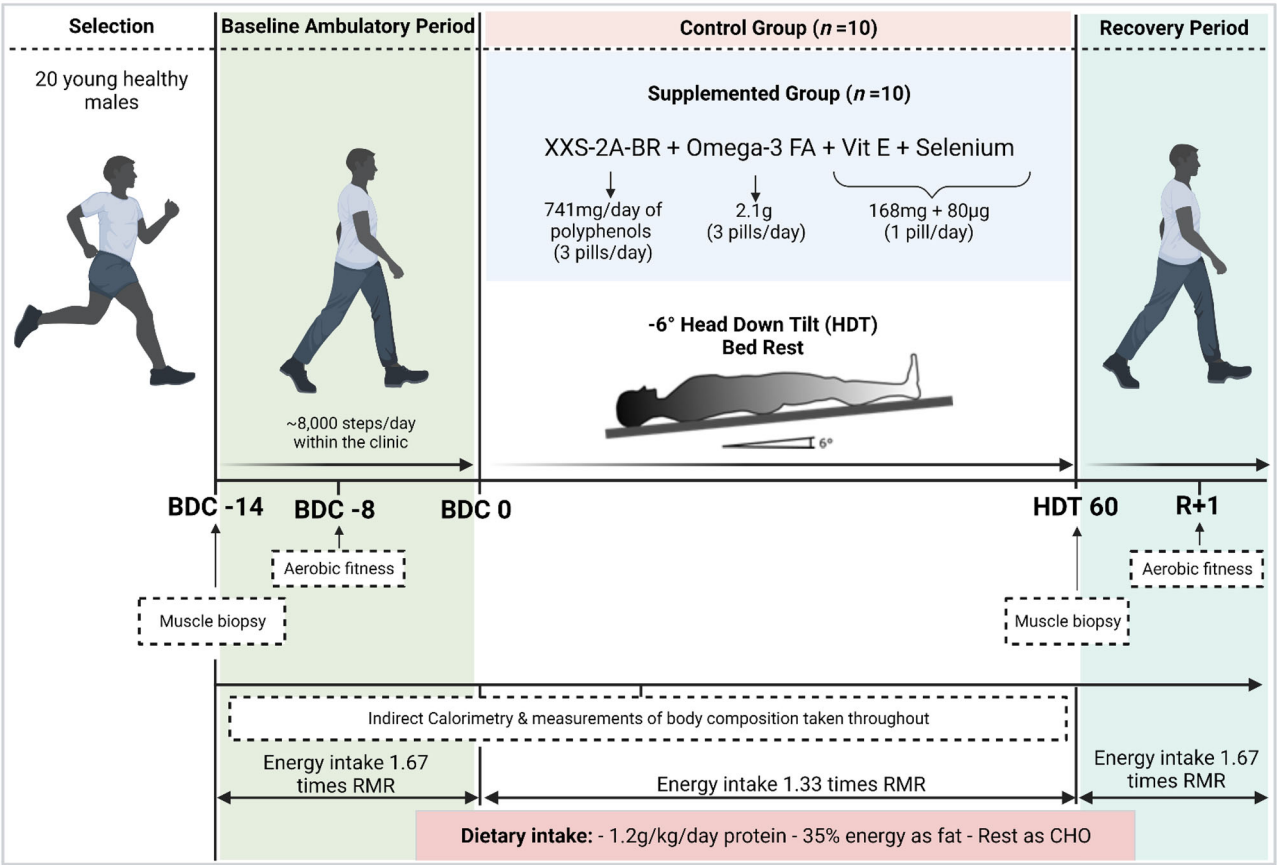

Figure 1. 60-day −6° bed rest study design
Twenty young healthy males were selected to participate in the present study. All 20 subjects were taken to the study clinic and required to adjust their energy intake to 1.67 times their RMR and their daily step count to ∼8000 steps during the BDC for 14 days. Aerobic fitness was measured on BDC-8 and again at Recovery +1 (R+1), whereas a skeletal muscle biopsy from the vastus lateralis was taken on BDC-14 and HDT 60. Indirect calorimetry was used throughout the testing period to calculate subject's substrate oxidation during BDC and during −6° HDT. Subjects were required to lay at −6° HDT for 60 days with their energy intake adjusted to be 1.33 times their RMR. Subject's dietary intake consisted of 1.2 g kg^{-1} day^{-1} protein, 35% of energy was fat and the rest consisted of carbohydrate (CHO). Subjects within the supplemented group (n = 10) were required to take 741 mg day^{-1} polyphenols (XXS-2A-BR2) (three pills per day), 2.1 g of omega-3 fatty acids (three pills per day), 168 mg of vitamin E and 80 μg selenium (one pill per day). [Colour figure can be viewed at wileyonlinelibrary.com]

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immediately for analysis of mitochondrial respiration using the High-Resolution Respirometery, O2k Oroboros (Oroboros Instruments GmbH, Innsbruck, Austria).

High-resolution respirometry

Respiration was measured at 37°C in each chamber of an Oroboros Oxygraph-O2k containing 2 mL of MiR05 (EGTA 0.5 mM, $MgCl_2 \cdot 6H_2O$ 3 mM, taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, bovine serum albumin 1 g L^{-1}, potassium-lactobionate 60 mM, mannitol 110 mM, dithiothreitol 0.3 mM, pH 7.1, adjusted with 5 M KOH). The oxygen concentration in each chamber was set at 200 nmol O_2 mL^{-1}. DatLab (Oroboros Instruments GmbH, Innsbruck, Austria) was used for data acquisition at 2 s time intervals. The carbohydrate substrate-uncoupler-inhibitor-titration (i.e. SUIT) protocol for high resolution respirometry was performed in saponin-permeabilized skeletal muscle fibres. LEAK (state 4) respiration was measured by the addition of pyruvate (5 mM per chamber) and malate (5 mM per chamber). Oxidative phosphorylation (OXPHOS) (state 3) was subsequently measured with the addition of ADP (2.5 mM per chamber). Glutamate (10 mM per chamber) was then added as an additional substrate to generate NADH.

Uncoupler titrations of carbonyl cyanide *p*-trifluoro-methoxyphenyl hydrazine (0.001 mM per chamber) were performed in the ADP activated state, to achieve complex I linked electron transport system (ETS) capacity (non-coupled). Maximal ETS capacity with convergent electrons flow from complex I and II was reached after addition of succinate (10 mM per chamber) (complex I and II linked ETS capacity). Complex I was inhibited by the injection of rotenone (0.005 mM per chamber) to measure respiration through complex II only. Malonic acid (Mna) (5 mM per chamber) and antimycin A (Ama) (0.0025 mM per chamber) were then added to inhibit complex II and III, respectively (residual oxygen consumption, ROX). Lastly, ascorbate (2 mM per chamber) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (0.5 mM per chamber) were added to calculate complex IV activity. All mitochondrial respiratory states were corrected for ROX, which accounts for non-mitochondrial cellular oxygen consuming processes. Data were subsequently represented as O_2 flux per mass [pmol(s*mg)] and O_2 flux per mass relative to mtDNA:nDNA [(pmol(s*mg)/mtDNA:nDNA].

Skeletal muscle lysate preparation and analysis

Muscle that had been stored at −80°C was prepared for protein and nucleic acid extraction. Samples were initially lyophilized and dissected prior to homogenization.

Western blotting. Protein was extracted and quantified using the Bradford technique. Samples were prepared as 2 mg mL^{-1} in 5× Laemmli buffer and homogenization buffer (200 μM Okadiac buffer, 50× protease inhibitor (P8340; Sigma-Aldrich, St Louis, MO, USA), 100 mM dithiothreitol, 100 mM Na_3VO_4, 100 mM phenylmethylsulfonyl fluoride). Samples were separated on 4% to 12% precast gradient gels and transferred onto nitrocellulose membranes respectively (iBlot Device; Thermo Fisher Scientific Inc., Waltham, MA, USA). Membranes were blocked in Tris-buffered saline (TBS) Odyssey Blocking Buffer (927-50000; Li-Cor, Lincoln, NE, USA) for 2 h before incubation in primary antibody of target proteins (see Supporting information, Table S2 S1), which was prepared in the Odyssey Blocking Buffer and antibody dilution buffer (99.8% TBS, 0.1% sodium azide, 0.1% bovine serum albumin), overnight at 4°C. Following primary incubation overnight, the membrane was washed six times for 10 min in TBS-Tween prior to incubation with Li-Cor secondary antibody (donkey anti-rabbit 800CW (925-32213; Li-Cor)/donkey anti-mouse 800CW (925-32212; Li-Cor) or donkey anti-rabbit 680 RD (925-68073; Li-Cor)/donkey anti-mouse 680RD (925-68072; Li-Cor) prepared within Odyssey Blocking Buffer only). Following secondary incubation, blots were washed six times as before using TBS-Tween. Proteins were subsequently visualized at 700 nm/800 nm (Odyssey Infrared Imaging System). Protein loading was normalized to GAPDH or α-tubulin and a standardized sample was used to control gel-to-gel variation. Positive controls were generated to ensure the correct densitometry target was captured. Relative protein abundance was quantified using the Quant densitometry, version 1.0 (TotalLab, Gosforth, UK).

Reverse transcriptase-PCR (RT-PCR). RNA was isolated from lyophilized samples using the Tri reagent guanidinium thiocyanate-phenol-chloroform (T9424; Sigma-Aldrich) extraction method (Chomczynski & Sacchi, 1987). RNA was subsequently quantified, using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) and reverse transcribed into cDNA in equal amounts of High-Capacity cDNA Reverse Transcription Kit 200 Rxn in accordance with the manufacturer's instructions (4368814; Thermo Fisher Scientific Inc.) within a PCR thermocycler (Bio-Rad, Hercules, CA, USA). All cDNA samples were made to 200 ηg μL^{-1}. A quantitative RT-PCR (RT-qPCR) was subsequently performed on all extracted cDNA samples. All RT-qPCR reactions were conducted on cDNA samples prepared with a mastermix consisting of SYBR Green (FastStart Essential DNA Green Master; 06402712001; Roche, Basel, Switzerland), nuclease-free water and the forward (10 μM) and reverse (10 μM) primer of the target of interest in

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accordance with the manufacturer's instructions. A LightCycler 96 instrument (Roche) was used for all RT-qPCR reactions with quantification cycle (Cq) values analyzed using the standard delta-delta Cq method (ΔΔCq). GAPDH was used as a loading control to normalize all probed targets. All primers were custom designed in-house and synthesized at 0.025 μM in dry format by Merck (Darmstadt, Germany). Primers were subsequently made to 100 μM using nuclease-free water in a Rnase free environment. Quality control was performed on all primers to ensure that consistency between experiments was managed by maintaining Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline standards with primer efficiency accepted between 90% and 110% (Bustin et al., 2009). All samples were measured within RNAse free conditions. A list of the primers and their sequences used within the present study is provided in the Supporting information (Table S3 S2).

mtDNA:nDNA ratio. The mitochondrial DNA to nuclear DNA ratio (mtDNA:nDNA) was used to normalize all skeletal muscle respirometry data to mitochondrial content. Following reverse transcription, both mitochondrial and nuclear DNA targets were analyzed using RT-qPCR. Delta Cq (ΔCq) was calculated by normalizing the Cq gene of interest to the Cq value of the control gene (GAPDH). Delta Delta Cq (ΔΔCq) was subsequently calculated by subtracting mitochondrial DNA ΔCq from the nuclear DNA ΔCq. The fold change for each was subsequently calculated as $2^{(-\Delta\Delta\,Cq)}$. Finally, skeletal muscle respirometry data was normalized for mtDNA:nDNA (mitochondrial content) by dividing the muscle wet weight normalized by ROX data by the previously calculated mtDNA:nDNA ratio [(pmol/(s*mg)/mtDNA:nDNA]. mtDNA:nDNA ratio was taken as NDUFA4:POLG expression.

Statistical analysis

The Shapiro–Wilk and Kolmogorov–Smirnov tests of normal distribution were used to determine whether the data were normally distributed and accepted with $P > 0.05$ allowing for parametric analysis. A two-way (treatment × time) mixed model analysis of variance (ANOVA) accounting for repeated measures among participants with a compound symmetry covariance structure was used to identify differences in the response to time, treatment and the interaction effect (treatment × time) for all clinical outcomes. As such, the main trial effect is represented by the interaction effect (i.e. the modulation of the bed rest as a consequence of micronutrient supplementation). As a result of no significant change in clinical parameters, pre (BDC) to post (HDT) bed rest comparisons in all non-clinical measures (i.e. protein, mRNA expression and mitochondrial respiration) were assessed in combination using paired samples *t* tests. The statistical analysis was performed using Prism (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ (two-sided) was considered statistically significant and data are reported as the mean ± SD.

Results

Body composition

The body composition changes during bed rest are presented in Table 1. Body mass (kg) progressively decreased during bed rest with a significant effect of time ($P < 0.001$) but not trial ($P = 0.674$). The change in body mass could be explained by a decrease in whole body FFM ($P < 0.001$) and a small increase in fat mass ($P < 0.001$) with time but not trial. The loss of lean body mass was predominately in the legs and not the arms. Although procedures aimed to maintain subjects in energy balance, the increase in fat mass indicates a small positive energy balance during bed rest.

Resting metabolic rate

The RMR adjusted for FFM is presented in Fig. 2*A*. There was a modest decrease in RMR [(kJ·min^{-1})/kgFFM^{-1}] from BDC-14 (0.019 ± 0.003 and 0.021 ± 0.004 [(kJ min^{-1})/kg FFM^{-1}] for the control and intervention trial respectively) to HDT58 (0.019 ± 0.003 and 0.021 ± 0.002 [(kJmin^{-1})/kg FFM^{-1}] for the control and intervention trial respectively), with a significant effect of time ($P < 0.001$) but not trial. The fasting RQ progressively increased as an effect of time ($P < 0.001$) but not trial (Fig. 2*B*), indicating an increased reliance on carbohydrate oxidation at rest that was not mitigated by the micronutrient intervention.

Aerobic fitness

There was a significant effect of time ($P < 0.001$), but not trial ($P = 0.862$), on peak oxygen consumption relative to body weight (mL kg^{-1} min^{-1}) following bed rest (Fig. 2*C*). Peak power was reduced ($P < 0.001$) and peak heart rate increased ($P = 0.021$) following bed rest (Fig. 2*D* and *E*) with a significant effect of time but not trial.

Mitochondrial respiration

Given the insignificant effect of the micronutrient cocktail in mitigating against changes in clinical measures of body

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Table 1. Control and intervention anthropometric data, before, during and following 60 days of bed rest

	Control (n = 10)			*Intervention (n = 10)*					
	Pre(BDC-2)	HDT 25	Post(HDT 58)	Pre(BDC −2)	HDT 25	Post(HDT 58)	Significant effect of time	Significant effect of trial	Interaction effect
Weight (kg)	73.69 ± 8.48	72.35 ± 8.34	71.60 ± 8.08	72.09 ± 5.71	71.07 ± 5.62	70.75 ± 5.68	$P < 0.001$	$P = 0.674$	$P = 0.503$
Body Fat (%)	25.37 ± 3.24	26.53 ± 3.23	27.48 ± 3.53	27.06 ± 4.08	28.23 ± 3.85	29.64 ± 4.05	$P < 0.001$	$P = 0.298$	$P = 0.362$
WB FFM (kg)	54.89 ± 5.78	53.06 ± 5.65	51.81 ± 5.32	52.49 ± 3.89	50.91 ± 3.59	49.6 ± 3.31	$P < 0.001$	$P = 0.305$	$P = 0.839$
WB FM (kg)	18.80 ± 3.88	19.30 ± 3.88	19.79 ± 4.08	19.60 ± 3.95	20.16 ± 3.87	21.09 ± 4.19	$P < 0.001$	$P = 0.618$	$P = 0.055$
Arm fat (kg)	2.45 ± 0.54	2.42 ± 0.51	2.51 ± 0.53	2.45 ± 0.51	2.48 ± 0.62	2.55 ± 0.61	$P < 0.001$	$P = 0.890$	$P = 0.789$
Arm lean (kg)	7.58 ± 1.04	7.36 ± 1.06	7.48 ± 0.97	6.70 ± 0.81	6.54 ± 0.71	6.45 ± 0.65	$P = 0.106$	$P = 0.020$	$P = 0.583$
Leg fat (kg)	6.55 ± 1.64	6.73 ± 1.58	7.04 ± 1.80	6.62 ± 1.60	6.79 ± 1.54	7.06 ± 1.77	$P < 0.001$	$P = 0.774$	$P = 0.972$
Leg lean (kg)	17.72 ± 1.88	16.87 ± 2.02	15.96 ± 1.64	17.16 ± 1.85	16.11 ± 1.82	15.18 ± 1.69	$P < 0.001$	$P = 0.454$	$P = 0.436$

Data are presented as the mean ± SD. WB FFM, whole body fat-free mass; WB FM – whole body fat mass (CONT, $n = 10$; INT, $n = 10$). The only significant effect of the trial was observed with respect to arm lean (kg) ($P = 0.020$). No other significant differences were observed between control and intervention, but there were significant differences between BDC and HDT as an effect of time for weight (kg)($P < 0.001$), body fat (%) ($P < 0.001$), whole body free-fat mass (kg) ($P < 0.001$), whole body fat mass (kg) ($P < 0.001$), leg lean mass (kg) ($P < 0.001$) and leg fat mass (kg) ($P < 0.001$).

composition, metabolic rate and aerobic fitness, as per other studies within the same clinical trial (Hoffmann et al., 2022; Trim et al., 2022, 2023), a combined analysis of all non-clinical measures (i.e. protein, mRNA expression and mitochondrial respiration) was performed pre (BDC) and post (HDT) bed rest.

The skeletal muscle mitochondrial respiration data, expressed relative to muscle wet weight [pmol/(s* mg)], is presented in Fig. 3. There was significant decrease in maximal ETS cellular respiration following bed rest. These differences were evident for complex I alone ($P = 0.004$) and complex I and II following the addition of succinate ($P = 0.031$). mtDNA:nDNA ratio significantly decreased in both groups following bed rest ($P < 0.001$) (BDC: 0.67 ± 0.19 HDT: 0.46 ± 0.19 relative mRNA expression). There were no significant differences in ETS or other measures when the data were further normalized to mitochondrial content, using the mtDNA:nDNA ratio (Fig. 3*B*), indicating that the differences observed when expressed relative to muscle wet weight were the result fo a decrease in mitochondrial content during bed rest.

Mitochondrial fusion and fission protein expression

A series of mitochondrial fusion and fission proteins were measured using western blotting with the data presented in Fig. 4. The total protein content of OPA1 significantly decreased following 60 days of −6° HDT bed rest ($P = 0.032$). This is in line with the observed decreases in OPA1 mRNA expression ($P = 0.028$) (see Supporting information, Figure S1 B). These decreases may be driven the observed significant decrease in the OPA1 short isoform (S-OPA1) ($P = 0.022$) following bed rest. We noted no significant differences in the OPA1 long isoform (L-OPA1), Mfn1, Mfn2 or the OPA1 regulatory proteins, OMA1 or YME1L, following bed rest.

The protein expression of Drp1 decreased following bed rest with a significant effect of time ($P = 0.003$). Furthermore, we observed a significant increase in $pDrp1^{s616}$ following bed rest ($P = 0.016$), suggestive of an increase in mitochondrial fission following bed rest. There was no effect of bed rest on the protein expression of Fis1 or the $pDrp1^{s637}$.

We observed no significant change with respect to markers of mitochondrial breakdown/mitophagy that we assessed, suggesting that bed rest had no effect on mitochondrial maintenance (Fig. 5).

Discussion

The present study suggests that the micronutrient cocktail has no effect in mitigating against changes in whole-body metabolism following 60 days of bed rest. However, interestingly, prolonged bed rest appears to significantly

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alter the regulation of key markers of mitochondrial dynamics within the skeletal muscle in combination with significantly reducing mitochondrial content. This decrease in mitochondrial content helps to explain the observed reduction in mitochondrial respiration following 60 days of bed rest.

The whole-body physiological changes following 60 days of bed rest in the present study are in agreement with previous studies of shorter duration (Kenny et al., 2017, 2020; Rudwill et al., 2018). Bed rest resulted in a decrease in FFM and body weight and an increase in percentage body fat. Absolute and relative aerobic capacity ($\dot{V}_{O_2peak}$) decreased following bed rest in both groups, as did peak power output and peak heart rate. RMR adjusted for FFM also significantly decreased as an effect of time in both groups with an increase in RQ indicating a greater reliance upon carbohydrate as fuel in fasting state.

Our previous research, using a very similar micronutrient cocktail, indicated positive metabolic effects following 20 days of reduced ambulatory activity (Damiot et al., 2019). In that study, healthy male adults reduced activity to a maximum of 2500 to 4000 steps day^{-1} and were randomly assigned to micronutrient supplementation or a control group. There was a significant increase in percent body fat and no change in lean body mass, but the intervention maintained type II muscle fibre cross-sectional area and lipid oxidation. In the present study, we did not observe mitigation of whole-body metabolic parameters and this may be related to the increased duration of the study or the more extreme nature of the bed rest model.

Bed rest is associated with metabolic changes in skeletal muscle and previous studies have shown a decrease in mitochondrial content and metabolic flexibility (Goodpaster & Sparks, 2017; Rudwill et al., 2018). Previously published literature from the same clinical trial provided insight into the futile effect of this micronutrient cocktail on measures of adipose tissue immunometabolism (Trim et al., 2022), glucose disposal (Trim et al., 2023), cardiac (Hoffmann et al., 2022) and bone deconditioning (Austermann et al., 2021). Evidence from (Arc-Chagnaud et al., 2020) exposed a similar light with

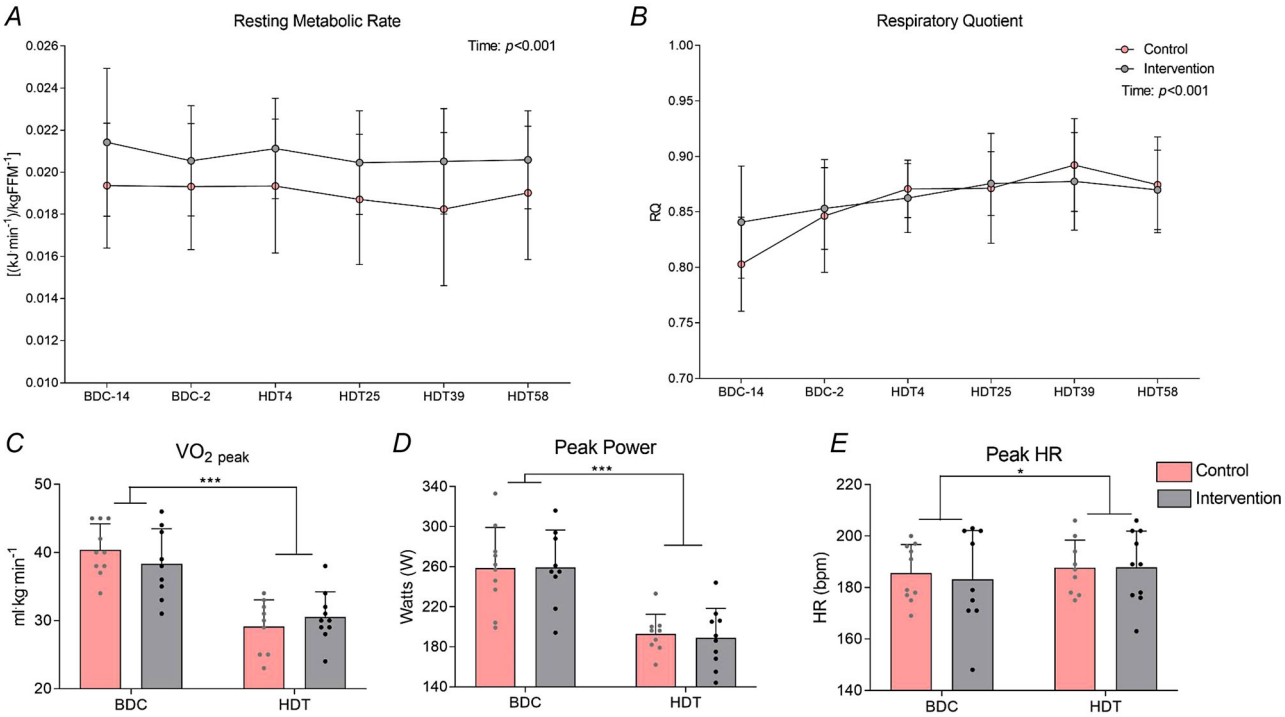

Figure 2. RMR, RQ and aerobic fitness measurements before and after 60 days of −6° HDT bed rest
RMR (*A*) adjusted to whole-body fat-free mass (kg FFM) [(kJmin^{-1})/kg FFM^{-1}] and RQ (*B*) calculated throughout BDC and −6° HDT for both the control (red) ($n = 10$) and intervention (light grey) ($n = 10$) groups. Peak aerobic capacity ($\dot{V}_{O_2peak}$) (*C*) presented relative to subject weight (CONT, $n = 10$; INT, $n = 9$), peak power (CONT, $n = 10$; INT, $n = 9$) (*D*) and peak heart rate (HR) (CONT, $n = 10$; INT, $n = 10$) (*E*) calculated following aerobic fitness testing on BDC −8 and Recovery+1 (R+1) for both the control (red) and intervention group (light grey). Individual response represented by dots; control (grey), intervention (black). Data are presented as the mean ± SD. There was a significant effect of time noted at $P < 0.001$ for RMR [(kJmin^{-1})/kg FFM^{-1}] (***) and $P < 0.001$ for RQ (***). $\dot{V}_{O_2peak}$ peak (mL^{-1} kgmin^{-1}) significantly decreased in both groups as an effect of time (***$P < 0.001$). Peak power (Watts) significantly decreased in both groups as an effect of time (***$P < 0.001$). Peak HR significantly increased in both groups as an effect of time (*$P = 0.021$). [Colour figure can be viewed at wileyonlinelibrary.com]

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respect to skeletal muscle, underling a greater decrease in muscle fibre cross-sectional area within the intervention (−22.5%) compared to the control group (−11.7%). In light of these and our own findings with respect to a lack of a whole-body metabolic response to the intervention, and in accordance with other studies within the same clinical trial (Hoffmann et al., 2022; Trim et al., 2022, 2023), measures of mitochondrial respiration, content, dynamics and mitophagy were assessed in combination.

Many studies have reported significant downregulation in pathways predominantly associated with mitochondrial biogenesis with bed rest (Alibegovic et al., 2010; Buso et al., 2019), potentially explaining the noted reduction in mitochondrial content. However, the impact that prolonged periods of bed rest poses on measures of mitochondrial function and dynamics has not been explored in great detail. Recent work from (Marshall et al., 2022) emphasized how short-term bed rest (5 days) presented no significant change in measures of skeletal muscle mitochondrial dynamics, mitophagy-related signalling or mitochondrial content in healthy, older males (71 ± 4 years). Given the observed reduction in quadricep cross-sectional area (Smeuninx et al., 2021), such results infer that these alterations in mitochondrial metabolism may be secondary to changes in skeletal muscle mass. These findings support similarly designed studies into short-term bed rest deducing no change in regulators of mitochondrial dynamics following 10 days of bed rest in older adults (67 ± 2 years) despite a significant, increase in H_2O_2 emissions combined with a reduction in mitochondrial respiration, content and leg lean mass (Standley et al., 2017, 2020). Nevertheless, animal models of disuse have helped to indicate significant adaptations in the expression of proteins involved in the regulation of mitochondrial dynamics. These adaptations have been denoted by decreases in regulators of fusion (Cannavino et al., 2015; Iqbal et al., 2013; Kang et al., 2016; Memme et al., 2021), aligned with increases in regulators of fission contributing to skeletal muscle atrophy and remodelling (Dulac et al., 2020; O'Leary et al., 2012; Romanello et al., 2010; Touvier et al., 2015). Figure 6 illustrates the key changes with respect to mitochondrial morphology, mitophagy, content and respiration following 60 days of -6° HDT bed rest.

In the present study, we observed a decrease in skeletal muscle ETS (complex I) and ETS (complex I + II) respiration following 60 days of bed rest when normalized to tissue wet weight. However, when we express our data relative to mitochondrial content, these changes are no longer evident, supported by other studies of shorter duration bed rest (Kenny et al., 2017; Standley et al., 2020). Therefore, the adaptations in metabolic regulation are synonymous with changes in mitochondrial content. Reductions in mitochondrial content have been shown to be initiated by changes in the regulators of mitochondrial morphology/dynamics, resulting in fewer and smaller mitochondria (Kelley et al., 2002). Despite the potential role that they may have in this respect, the only evidence to date exists from bed rest studies of much shorter duration (Marshall et al., 2022; Standley et al., 2017).

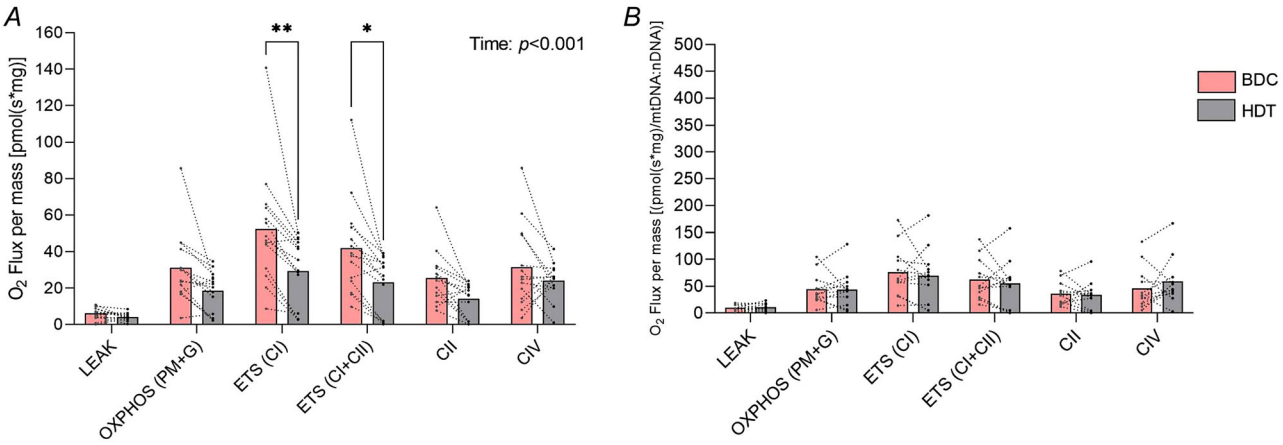

Figure 3. Mitochondrial respiration calculated before and following 60 days of −6° HDT bed rest
xA carbohydrate substrate uncoupler inhibitor titration (i.e. SUIT) protocol was performed using the Oroboros Oxygraph O2-k. Data expressed as (*A*) oxygen flux per mass normalized to wet weight [pmol/(s*mg)] (BDC, n = 14; HDT, n = 14) and (*B*) normalized to wet weight and mitochondrial DNA to nuclear DNA (mtDNA:nDNA) ratio [(pmol/(s*mg)/mtDNA: nDNA)] (BDC, n = 12; HDT, n = 12). Red bars, BDC; grey bars, HDT. Individual response represented by dots; BDC (grey), HDT (black); ETS(CI), maximal respiration through complex I; ETS(CI+CII), maximal respiration through complex I and II; CII, respiratory capacity through complex II only when complex I is inhibited; CIV, complex IV. Data are expressed as the mean ± SD. Maximal respiration through CI [pmol/(s*mg)] significantly decreased in both groups (P = 0.004). Maximal respiration through CI + CII [pmol/(s*mg)] significantly decreased in both groups (P = 0.031). [Colour figure can be viewed at wileyonlinelibrary.com]

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With respect to this point, we found that the expression of the inner mitochondrial membrane (IMM) fusion protein, OPA1, significantly decreased following this prolonged period of bed rest. This change was further supported by a decrease in the expression of the short isoform of OPA1 (S-OPA1). As such, there is potential that, similar to the coarse models of disuse used within animal studies, 60 days of bed rest alters the expression of regulators of mitochondrial dynamics. These changes were coupled with the maintenance in expression of the metalloendopeptidase OMA1 and i-AAA peptidase YME1L. OMA1 cleaves the L-OPA1 to S-OPA1 isoform

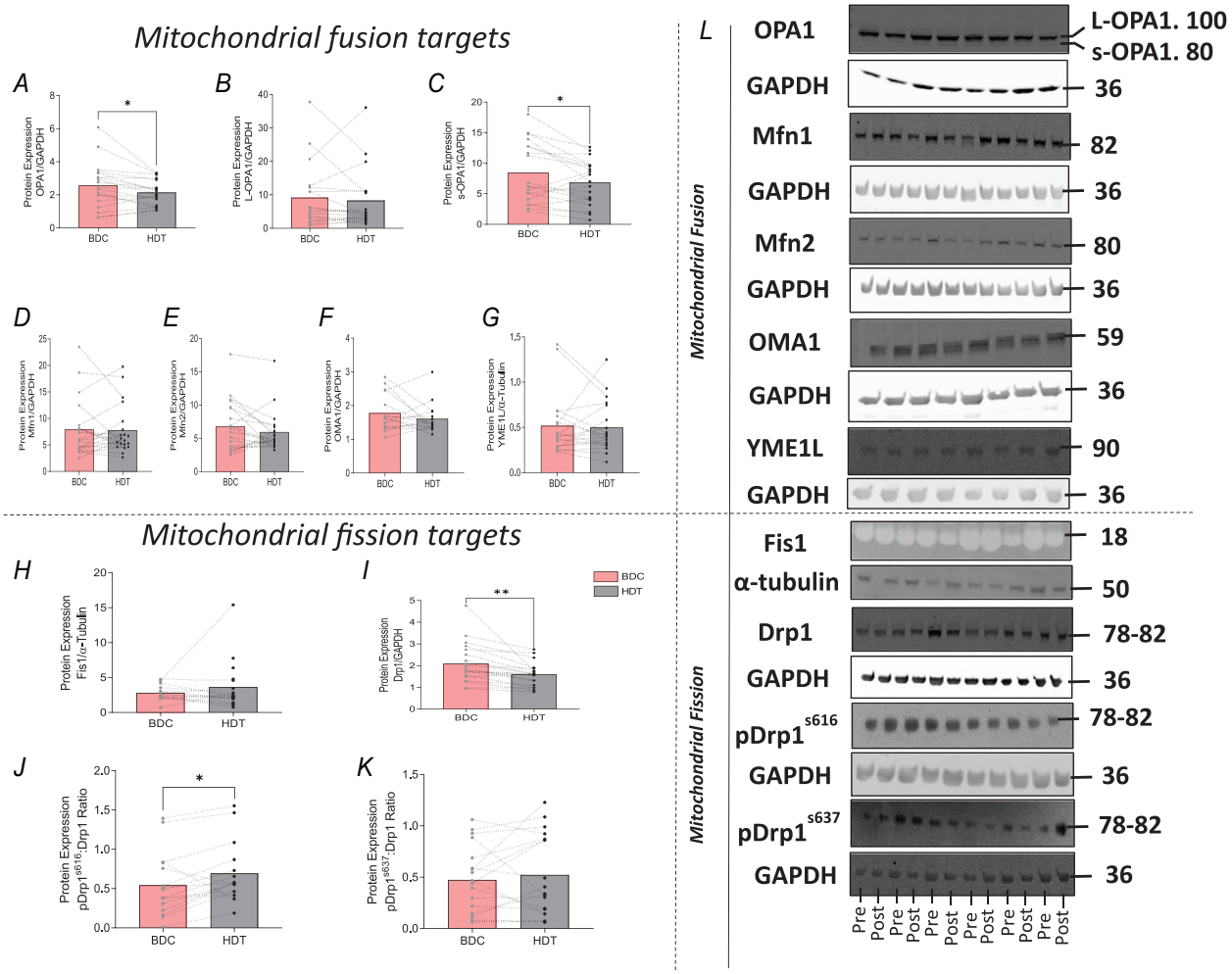

Figure 4. Protein expression of mitochondrial fusion and fission targets before (BDC) and following (HDT) 60 days of −6° HDT bed rest

Mitochondrial fusion proteins (*A*) optic atrophy 1 (OPA1) (BDC, n = 19; HDT, n = 19), (*B*) the long isoform OPA1 (L-OPA1) (BDC, n = 19; HDT, n = 19), (*C*) the short isoform OPA1 (s-OPA1) (BDC, n = 19; HDT, n = 19), (*D*) mitofusin-1 (Mfn1) (BDC, n = 20; HDT, n = 20), (*E*) mitofusin-2 (Mfn2) (BDC, n = 20; HDT, n = 20), (*F*) metalloendopeptidase OMA1 (OMA1) (BDC, n = 16; HDT, n = 16) and (*G*) ATP-dependent metalloprotease YME1L1 (YME1L) (BDC, n = 19; HDT, n = 19) were measured for protein expression before and following 60 days of −6° HDT bed rest. Mitochondrial fission targets, (*H*) fission protein 1 (Fis1) (BDC, n = 14; HDT, n = 20), (*I*) dynamin related protein 1 (Drp1) (BDC, n = 20; HDT, n = 20), (*J*) phospho-dynamin-related protein 1 serine 616 (pDrp1^{s616}) (BDC, n = 16; HDT, n = 16) and (*K*) phospho-dynamin related protein 1 serine 637 (pDrp1^{s637}) (BDC, n = 18; HDT, n = 18) were measured for protein expression before and following 60 days of −6° HDT bed rest. Protein expression expressed as raw data controlled against internal control and loading control GAPDH or α-tublulin. A positive control was used on all blots for the identification of the correct protein band. Data are expressed as the mean ± SD, Red bars, BDC; light grey bars, HDT. Individual response represented by dots; BDC (grey), HDT (black). OPA1 protein expression significantly decreased as an effect of time following bed rest (*P = 0.032). S-OPA1 protein expression significantly decreased as an effect of time following bed rest (*P = 0.022). Drp1 protein expression significantly decreased as an effect of time following bed rest (**P = 0.003). pDrp1^{s616} protein expression significantly increased as an effect of time following bed rest (*P = 0.016). All protein expression was quantified using western blot with densitometry represented in (*L*). [Colour figure can be viewed at wileyonlinelibrary.com]

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in response to changes in the mitochondrial membrane potential, particularly during times of mitochondrial dysfunction/stress (Cipolat et al., 2006; Fessler et al., 2020; Ishihara et al., 2006; Madreiter-Sokolowski et al., 2019; Satoh et al., 2003; Song et al., 2007; Zick et al., 2009; Zorzano et al., 2010). By contrast, YME1L acts to cleave IMM bound L-OPA1 to soluble S-OPA1 in response to changes in cellular ATP, given its function as an ATP-dependent proteolytic complex of the IMM (Ohba et al., 2020; Wai & Langer, 2016). Additionally, we observed no changes in the OMM fusion proteins Mfn1 or Mfn2, suggesting that adaption of IMM fusion proteins (OPA1) may be particularly sensitive to the effects of prolonged bed rest. This adaptation suggests that the reduction in mitochondrial content may be related to a reduction in OPA1-mediated mitochondrial fusion, namely stress-induced fission (SiF), in a futile attempt to mitigate against the loss in mitochondrial content.

In addition to the reduction in OPA1, Drp1 and mitochondrial content, we observed no change in the regulators of mitochondrial breakdown and removal (mitophagy). These data are suggestive that, although mitochondrial content decreases, being aligned with an increase in mitochondrial fission following bed rest, mitochondrial turnover does not change. Without further measures of mitochondrial activity in respect to mitophagy, we can only theorize that this may be an adaptive response to preserve a declining mitochondrial number, combined with a reduction in mitochondrial biogenesis (PGC-1α transcriptional expression; see Supporting information, Figure S1 C).

As with mitochondrial fusion, we observed specific differences in the expression of mitochondrial fission regulators before and after bed rest. Although we found a significant decrease in the expression of Drp1, interestingly, its phosphorylation at serine 616 (pDrp1^{s616}) was increased, further supporting the notion of mitochondrial stress-induced fission following bed rest. Based on these observational changes to mitochondrial respiration, protein and mRNA expression, we speculate that 60 days of bed rest resulted in fewer,

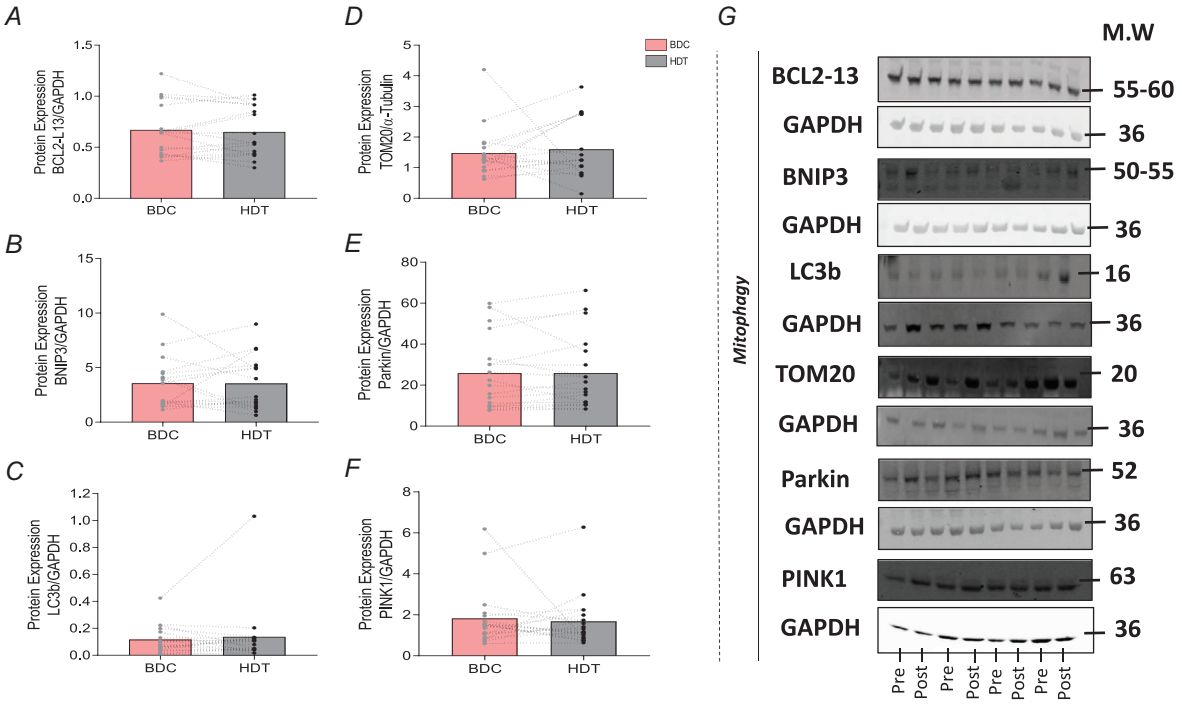

Figure 5. Protein expression of mitophagy targets before (BDC) and following (HDT) 60 days of −6° HDT bed rest as quantified by western blotting

Mitophagy proteins (*A*) B-cell lymphoma 2-like protein 13 (BCL2-L13) (BDC, n = 17; HDT, n = 17), (*B*) BCL-2/adenovirus E1B interacting protein 3 (BNIP3) (BDC, n = 17; HDT, n = 17), (*C*) light chain 3b (LC3b) (BDC, n = 18; HDT, n = 18), (*D*) translocase of the outer membrane protein 20 (TOM20) (BDC, n = 17; HDT, n = 17), (*E*) Parkin (BDC, n = 18; HDT, n = 18) and (*F*) phosphatase and tensin homolog-induced kinase 1 (PINK1) (BDC, n = 19; HDT, n = 19) were measured for protein expression before and following 60 days of −6° HDT bed rest. Protein expression expressed as raw data controlled against internal control and loading control GAPDH or α-tublulin. A positive control was used on all blots for the identification of the correct protein band. Red bars, BDC; light grey bars, HDT. Individual response represented by dots; BDC (grey), HDT (black). Data are expressed as the mean ± SD. All protein expression was quantified using western blot with densitometry represented in (*L*). [Colour figure can be viewed at wileyonlinelibrary.com]

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more fragmented mitochondria, which explains the reduction in mitochondrial function.

Aerobic exercise has been observed to initially increase Mfn2-mediated OMM fusion, leading to an increase in OPA1-mediated IMM fusion (Arribat et al., 2018; Noone et al., 2022). We speculate that prolonged bed rest may pose an alternative change to these proteins, initiated with a reduction in OPA1-mediated IMM fusion. Loss of OPA1 has been shown to widen the cristae lumen and junction, reduce IMM potential, increase the basal mitochondrial calcium level, and induce apoptosis. Furthermore, a reduction in mitochondrial surface area has been shown to diminish ETS/OXPHOS activity by increasing the diffusion distances of substrates and electron transfer (Madreiter-Sokolowski et al., 2019; Trewin et al., 2018). Our results point to an integrated response between fusion–fission dynamics and mitochondrial function, with OPA1 being the principal protagonist in response to prolonged bed rest.

Subject measurements during the baseline period (baseline data collection, BDC) were obtained when subjects were ambulatory. It could be argued that this

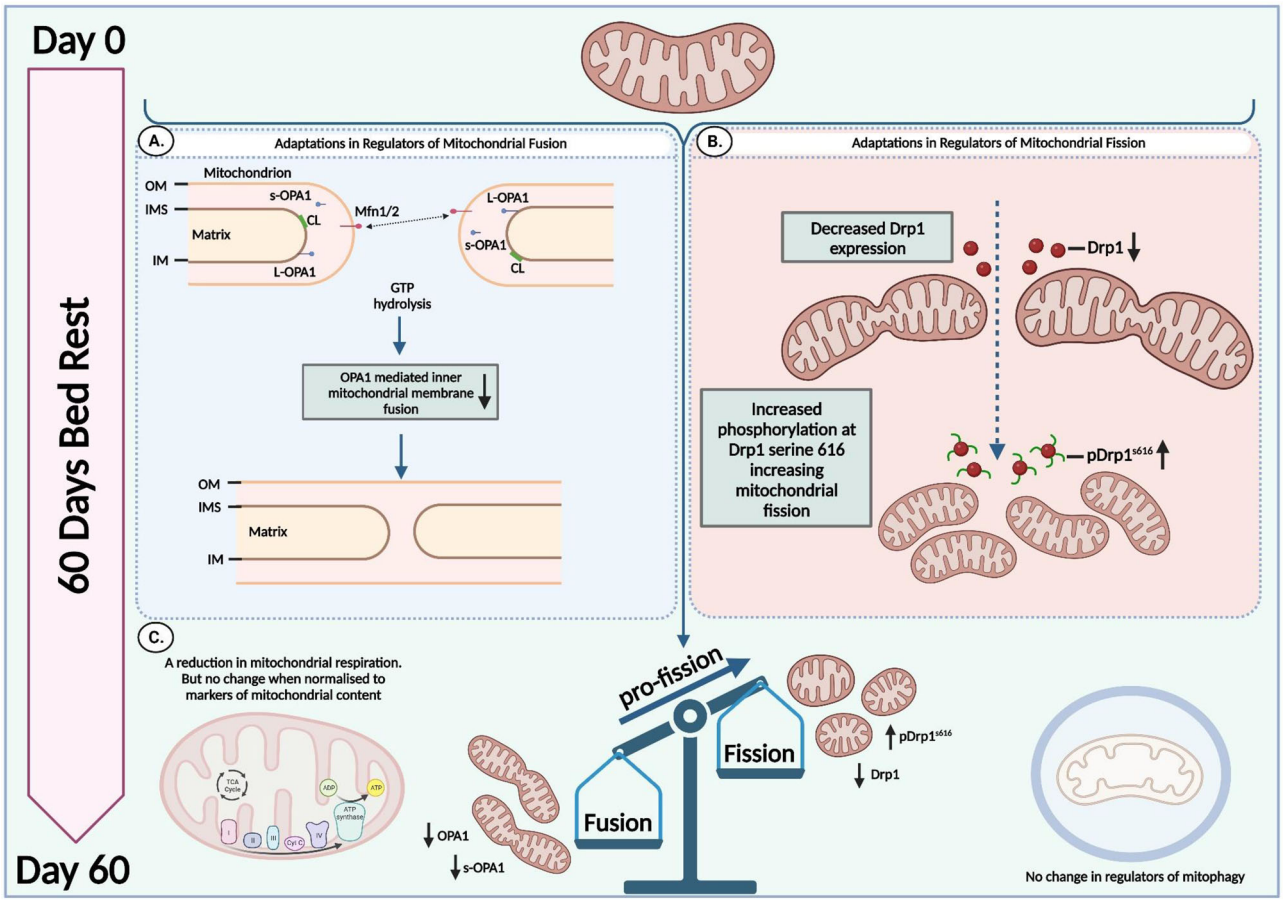

Figure 6. The relationship between mitochondrial fusion, fission, content and mitophagy following 60 days of -6° head down tilt bed rest

Schematic interpretation of the effect of 60 days −6° HDT bed rest on (*A*) regulators of mitochondrial fission, (*B*) regulators of mitochondrial fission and (*C*) their balance with mitochondrial function and regulators of mitophagy. OM, outer mitochondrial membrane; IMS, intermembrane space; IM, inner mitochondrial membrane; s-OPA1, short isoform OPA1; L-OPA1, long isoform OPA1; Mfn, mitofusin; CL, cardiolipin; ω-3, omega-3 fatty acid. *A*, we observed no change in protein expression of outer mitochondrial membrane fission proteins, Mfn1 and 2, following 60 days of bed rest following 60 days of bed rest suggestive of a maintenance in outer mitochondrial membrane (OM) fission through the homotypic interaction between mitofusin proteins. The significant decrease in OPA1 protein expression, along with its short isoform (S-OPA1), following 60 days of bed rest is suggestive of a decrease in inner mitochondrial membrane (IM) fission. *B*, we observed a decrease in the expression of mitochondrial fission protein Drp1, with an increase in its phosphorylation at serine 616 (pDrp1^{s616}) indicative of an increase in mitochondrial fission. *C*, this decrease in OPA1, combined with an increase in pDrp1^{s616}, a reduction in mitochondrial content and maintenance of regulators of mitophagy, suggests an increase in mitochondrial fission following 60 days of −6° HDT bed rest. [Colour figure can be viewed at wileyonlinelibrary.com]

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is reflective of habitual free-living conditions; however, given that subjects are confined to the clinic throughout these measurements, this conditional effect may be somewhat reduced. On a related note, it is possible that the healthy, young subjects recruited for the present study were more metabolically adaptable to the physiological stress posed by 2 months of bed rest than an age-matched, sedentary cohort. However, considering the demands such a lengthy period in bed poses on the body, this hypothesis is difficult to test ethically in an unhealthy population. In addition, the present study is reflective of the changes posed by an acute bout of inactivity and, as such, the changes do not represent those posed by decades of sedentary behaviour. Given the regulation of mitochondrial dynamics is a perpetual cycle of fusion and fission, measurement of changes in the mitochondrial phenotype using techniques such as transmission electron microscopy at multiple timepoints throughout the best rest period may help to further the observations found, but its obsoletism here is a limitation for this work. Further experimentation focused on this process should consider employing this technique and design. Lastly, protein expression was normalized using GAPDH and α-tubulin loading controls. Bed rest resulted in a decrease in lean muscle mass and has been shown to change glycolytic enzyme expression (Brocca et al., 2012), meaning that any changes observed may be consistent with these observations. However, given the similar changes in whole-body FFM (kg) (HDT58 CON: 51.809 $\pm$ 5.319, INT: 49.656 $\pm$ 3.314, no significant difference between groups following trial, $P = 0.299$) and RQ (HDT58 CON: 0.874 $\pm$ 0.043, INT: 0.869 $\pm$ 0.036, no significant difference between groups following trial, $P = 0.630$) within both groups of this between-patient designed study, these changes are probably not indicative of shifts in glycolysis and body composition, but are symptomatic of bed rest.

In conclusion, the findings of the present study suggest that a micronutrient cocktail has no significant effect in mitigating against changes in whole-body physiological changes induced by 60 days of bed rest. Although the demands of such a prolonged period of bed rest may be too high to counteract with a micronutrient cocktail alone, a combined approach with exercise and/or drug therapies may prove beneficial in preventing the adverse effects of prolonged bed rest.

Furthermore, as observed with shorter termed bed rest models, mitochondria appear to efficiently adapt to the 'new' energy state (i.e. less energy requirement as a result of physical inactivity) imposed by 60 days of bed rest as reflected by changes in mitochondrial number and function. This adaptation is further supported by an increase in OPA1 and Drp1-mediated mitochondrial fission, potentially acting to counteract the decrease in mitochondrial content to meet the limited energy demands posed by prolonged bed rest. As opposed to the OMM fusion regulators, such as Mfn1 and 2, IMM fusion as regulated by OPA1 appears to be particularly sensitive. Given the importance the integrity of the IMM has in hosting the complexes associated with the electron transport chain along its cristae, a reduction in OPA1 may be linked to the reduction in mitochondrial functional capacity observed following bed rest, similar to observations shown with cell and animal models (Cogliati et al., 2013; Hu et al., 2020; Patten et al., 2014). This could be particularly integral in respect to bed rest as a result of strong evidence linking the mitochondrial phenotype to substrate oxidation, with more fragmented mitochondria, driven by an decrease in regulators of mitochondrial fusion, preferring carbohydrate over fatty acid oxidation as a source of fuel, comprising observations that we noted with respect to a whole-body perspective from our indirect calorimetry data (Dai & Jiang, 2019; Parra et al., 2014; Pich et al., 2005; Yao et al., 2019; Zanna et al., 2008).

Given these preliminary findings, further studies are required to extrapolate the connection between mitochondrial dynamics and function in human skeletal muscle *in vivo* in the context of prolonged bed rest, as well as to fully elucidate the mechanism of action we are observing with 60 days of bed rest.

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Additional information

Data availability statement

The original data generated and analyzed during the present study are included in the published article or in the data repositories listed in the References.

Competing interests

H.K. is currently employed by Bayer Ltd. The work described in the present study does not have any direct or commercial connection work carried out at Bayer Ltd and the authors declare that they have no competing interests.

Author contributions

J.N., A.D., S.N., F.C., I.D.G., M.P.B., C.S., A.B., S.B. and D.O'G. were responsible for the study conception and design. J.N., A.D., I.C., A.Z., S.N., F.C., I.D.G., C.L., M.P.B., C.S., A.B., S.B. and D.O'G. were responsible for data collection. J.N., A.D., H.K., I.C, A.Z., S.N., F.C., I.D.G., K.D.R., C.L., M.P.B., C.S., A.B., S.B. and D.O'G. were responsible for analysis and interpretation of results. J.N., A.D., H.K., I.C., A.Z., S.N., F.C., I.D.G., K.D.R., C.L., M.P.B., C.S., A.B., S.B. and D.O'G. were responsible for draft manuscript preparation. All authors have reviewed the results and approved the final version of the manuscript submitted for publication.

Funding

This was a European Space Agency (ESA) sponsored study organized at the Institute of Space Medicine and Physiology (MEDES-IMPS) in Toulouse, France. The study was funded by the European Space Agency PRODEX programme and Enterprise Ireland, the French Space Agency (CNES) and by the Federal Ministry of Economics and Energy (BMWi) through the German Aerospace Centre (DLRe.V.) (Grants 50WB1231 and 50WB1421 to DB).

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Acknowledgements

We are grateful for help and support provided by the MEDES-IMPS staff with respect to study co-ordination. We also thank all of the volunteers who enabled us to carry out this study.

Open access funding provided by IReL.

Keywords

bed rest, energy expenditure, metabolism, mitochondrial dynamics, mitochondrial function, OPA1, skeletal muscle

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