HAL
open science

Short-Term Adaptations in Skeletal Muscle Mitochondrial Oxidative Capacity and Metabolic Pathways to Breaking up Sedentary Behaviors in Overweight or Obese Adults

Nathan de Jong, Michael Rudolph, Matthew Jackman, Rachel Sharp, Ken Jones, Julie Houck, Zhaoxing Pan, Jane Reusch, Paul Maclean, Daniel Bessesen, et al.

To cite this version:

Nathan de Jong, Michael Rudolph, Matthew Jackman, Rachel Sharp, Ken Jones, et al.. Short-Term Adaptations in Skeletal Muscle Mitochondrial Oxidative Capacity and Metabolic Pathways to Breaking up Sedentary Behaviors in Overweight or Obese Adults. Nutrients, 2022, 14 (3), pp.454. 10.3390/nu14030454 . hal-03538540

HAL Id: hal-03538540

https://hal.science/hal-03538540v1

Submitted on 31 Jan 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Article

Short-Term Adaptations in Skeletal Muscle Mitochondrial Oxidative Capacity and Metabolic Pathways to Breaking Up Sedentary Behaviors in Overweight or Obese Adults

Nathan P. De Jong[1], Michael C. Rudolph[2], Matthew Jackman[4], Rachel R. Sharp[2,3], Ken Jones[3], Julie Houck[4], Zhaoxing Pan[5], Jane Reusch[6,7,8], Paul MacLean[4], Daniel H. Bessesen[1,9] and Audrey Bergouignan[1,10]

[1]Division of Endocrinology, Metabolism, and Diabetes and Anschutz Health and Wellness Center, University of Colorado School of Medicine, Aurora, Colorado.
[2]Department of Physiology and Harold Hamm Diabetes Center, Oklahoma University Health Sciences Center, Oklahoma City, OK, USA.
[3]Laboratory of Molecular Biology and Cytometric Research, Oklahoma University Health Sciences Center, Oklahoma City, Oklahoma, USA.
[4]Division of Endocrinology, Metabolism, and Diabetes, Department of Medicine, Anschutz Medical Campus, University of Colorado, Aurora, Colorado, USA.
[5]Department of Biostatistics and Informatics, Anschutz Medical Campus, University of Colorado, Aurora, Colorado, USA.
[6]Center for Women's Health Research, University of Colorado School of Medicine, Aurora, Colorado, USA.
[8]Department of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, Colorado, USA.
[8]Rocky Mountain Regional Veterans Affairs Medical Center, Aurora, Colorado, USA.
[9]Denver Health Medical Center, Denver, Colorado, USA.
[10]Institut Pluridisciplinaire Hubert Curien, Université de Strasbourg, Centre National de la Recherche Scientifique (CNRS), UMR7178 Strasbourg, France.
* Correspondence author: Audrey.bergouignan@iphc.cnrs.fr

ABSTRACT (200/200 words): Breaking up sedentary behavior with short-frequent bouts of physical activity (PA) differentially influences metabolic health than the performance of a single-continuous bout of PA matched for total active time. The underlying mechanisms are however unknown. We compared skeletal muscle mitochondrial respiration (high-resolution respirometry) and molecular adaptations (RNA sequencing) to 4-day exposure to breaks vs energy-matched single-continuous PA bout in inactive adults with overweight/obesity. Participants (9M/10F, 32.2±6.4 years, 30.3±3.0 kg/m^2) completed three interventions of a randomized cross-over study, SED: sedentary control, MICRO: 5-min brisk walking each hour for 9hr/d, ONE: 45-min/d continuous brisk walking bout. Fasted muscle biopsies were collected on day 5. Mitochondrial coupling in the presence of lipid-associated substrates was higher after ONE (4.8±2.5) compared to MICRO (3.1±1.1, P=0.02) and SED (2.3±1.0, P=0.001). Respiratory rates did not differ across groups with carbohydrate-associated substrates. In pathways associated with muscle contraction-transcription signaling, ONE and MICRO similarly enhanced Oxidative Phosphorylation and Sirtuin Signaling expression (P<0.0001, for both). However, ONE (P<0.001, for all), but not MICRO, had greater pathway enrichment, including Ca++, mTOR, AMPK, and HIF1α signaling than SED. Although breaking up sedentary behavior triggers skeletal muscle molecular adaptations favoring oxidative capacity, it did not improve mitochondrial function over the short-term.

Keywords (6 max): Sedentary behavior; Mitochondria; Skeletal muscle molecular adaptations; mRNA sequencing; active breaks; obesity

1. INTRODUCTION

Prolonged sedentary behaviors (SB), defined as "any waking behavior characterized by an energy expenditure ≤ 1.5 metabolic equivalents while in a sitting, reclining, or lying posture" (46), are detrimental for health. It has been negatively associated with common risk factors for cardiometabolic disease such as waist

circumference, body mass index (BMI, kg/m^2), 2 hr postprandial glycemia, and fasting plasma glucose, triglycerides (TG) and C-reactive protein. Conversely, frequently breaking up SB with short bouts of physical activity (PA) confers metabolic health benefits independent of total time spent in moderate to vigorous physical activity (MVPA) and total daily sitting time (18, 19). It is now well established that, compared to sedentary control conditions, acute (≤ 12 hr) and short-term (≤ 4 days) exposures to active breaks reduce postprandial glucose and insulin concentrations compared to a sitting control in adults who are lean, with obesity and type 2 diabetes (3, 9, 10, 12, 22, 23, 28, 35, 47).

To investigate the independent effects of the active breaks, the metabolic response of short-frequent PA bouts performed throughout the day have been compared to those elicited by a time-matched single continuous bout (22, 23, 35). Those studies have highlighted that at same total active time and/or energy expenditure, differential metabolic effects are elicited by these different PA modalities. Short-frequent bouts of PA decreases postprandial plasma glucose and insulin concentration, while a single-continuous bout of PA reduces postprandial TG levels, both compared to prolonged sedentary periods (38). In a randomized cross-over trial, we further showed that breaking up SB with short-frequent bouts of moderate-intensity walking increases 24 hr carbohydrate (CHO) oxidation, while a single-continuous bout of moderate-intensity walking increases 24 hr fatty acid (FA) oxidation in physically inactive adults with overweight or obesity (8). The mechanisms underlying these differential metabolic effects are however poorly known (11).

So far, only two human clinical trials have investigated some of the underlying mechanisms at the skeletal muscle; skeletal muscle being a critical site for glucose disposal and nutrient metabolism both at rest and during PA (13). Acute light- and moderate-intensity walking breaks (2-min bouts every 20 min for 5 hr) have been associated with differentially expressed genes involved in carbohydrate (CHO) and lipid metabolism (pyruvate dehydrogenase kinase 4 [PDK4] and nicotinamide N-methyltransferase [NNMT], respectively) but also in cellular development, growth, and proliferation (myogenic factor 6 [MYF6], epithelial membrane protein 1 [EMP1], and dynein, light chain, LC8-type 1, transcript variant 1 [DYNLL1]) in adults with overweight or obesity (29). Performing these frequent active breaks for 3 days was shown to activate both non-oxidative and oxidative glucose uptake via insulin-independent and dependent pathways (2), which may optimize insulin action and both glucose oxidation and storage (42). It remains unclear whether these molecular adaptations align with or are different from those triggered by a time-matched single-continuous bout of PA, and whether they can explain the differential metabolic responses observed at the whole-body level.

In this ancillary study, we tested the hypothesis that 4-days of breaking up SB with short-frequent bouts of PA would enhance mitochondrial respiration in the presence of CHO-associated substrates and skeletal muscle would increase expression profiles favoring CHO metabolism. Because prolonged PA is known to be associated with increases in whole-body fat oxidation rates especially in post-absorptive conditions (15), we further hypothesized that 4-days following a daily single-continuous bout of PA matched for total active time would exhibit greater lipid-linked mitochondrial respiration and expression of genes associated with the regulation of lipid metabolism. Our findings provide muscle-specific evidence that molecular and functional adaptations depend on the terms of PA, i.e. frequency, duration and/or volume, and could contribute to whole-body energetics to improve metabolic health outcomes.

2. METHODS

2.1. Experimental Protocol

The detailed description of the study design and main outcomes have been published previously (8). Following the screening visit, three interventions were completed by each participant in a randomized balanced order. Each intervention period

lasted 4-days. The first 3-days in free-living conditions and the 4th day was in a whole-room calorimeter. All interventions were separated by a 28-day wash out period. Study procedures were performed during the same phase of the menstrual cycle in all women. All the visits were conducted at the Clinical and Translational Research Center of University of Colorado Hospital. This study was approved by the Colorado Multiple Institutional Review Board and listed at ClinicalTrials.gov: NCT02258438.

2.2. Participants

Study volunteers were recruited from October 2014 to October 2016. Inclusion criteria included: age 19 – 45 yr, BMI 27 – 33 kg/m^2, weight stable for ≥ 3 months, and being sedentary defined as sitting ≥ 6 h/day. Women were premenopausal but could use oral contraceptives. Exclusion criteria included clinically diagnosed diabetes, consumption of glucose- and/or lipid-lowering medication, dyslipidemia, smoking, or being physically active (> self-reported 150 min/wk MVPA).

2.3. Screening Visit

Written informed consent was obtained at the screening visit. Following verification of eligibility criteria, participants completed a treadmill walk test to determine a self-perceived moderate-intensity walking pace (mph). Participants were asked to walk at a similar moderate-intensity pace in free-living conditions and completed the same treadmill walking pace in the whole-room calorimeter.

2.4. Study Interventions

Participants were randomized to one of three possible trial-condition sequences using balanced blocks, separately prepared for male and female participants. The study statistician (Z.P.) prepared the computer generated randomization assignments and placed them in sealed envelopes which were opened the day before the first intervention to reveal the order of study interventions.

Sedentary behaviors (SED): During the 3-day free-living period, participants were asked to refrain from structured exercise and maintain habitual levels of daily PA and SB. On day 4, participants remained sedentary in the whole-room calorimeter.

Sedentary + single-continuous bout of walking (ONE): During the 3-day free-living period, participants were instructed to perform 45-min of moderate-intensity walking once per day and to maintain habitual levels of daily PA and SB the remainder of the day. On day 4, participants performed one 45-min bout of moderate intensity walking on a treadmill at 10:00 AM but otherwise remained sedentary in the whole-room calorimeter.

Sedentary + microbouts of walking (MICRO): During the 3-day free-living period, participants were instructed to perform a 5-min bout of moderate intensity walking each hour for 9 consecutive hours and to maintain habitual levels of daily PA and SB the remainder of the day. On day 4, participants performed a 5-min bout of moderate-intensity treadmill walking every hour for 9 consecutive hours from 10:00AM to 6:00PM and remained sedentary the rest of the day.

Compliance was verified with objectively measured time spent sitting and lying, stepping and daily steps with ActivPAL™ PA monitors (PAL Technologies Ltd., Glasgow, Scotland) and PA intensity was confirmed with ActiGraph GT3X tri-axial accelerometer (ActiGraph, Pensacola, FL, USA). Data on time spent sedentary and daily patterns of PA have been previously published (7).

2.5. Standardized Diet

Each participant consumed a 3-day standardized diet leading up to the whole-room calorimeter study visit. The macronutrient content of the control diet was 30% fat, 55% CHO, and 15% protein of total energy intake. On day 4, the macronutrient composition was 20% fat, 65% CHO and 15% protein. Daily energy needs were calculated based on resting metabolic rate and fat free mass measured by dual energy X-ray absorptiometry

(DXA, Hologic Delphi-W, Bedford, MA), as previously reported (8). By design, participants received the same amount of food across the three interventions and as a result were in similar state of negative energy balance in the two PA trials (8).

2.6. Skeletal Muscle Biopsy

Following an overnight fast, a *vastus lateralis* skeletal muscle biopsy was collected under local anesthesia (2% wt/vol lidocaine HCl) using the Bergstrom biopsy needle technique on the morning of day 5. From the total biopsy sample, ~10-20 mg was placed immediately in ice cold BIOPS solution (10mM Ca–EGTA buffer, 0.1M free calcium, 20mM imidazole, 20mM taurine, 50mM potassium 2-[N-morpholino]-ethanesulfonic acid, 0.5mM dithiothreitol, 6.56mM MgCl2, 5.77mM ATP, and 15mM phosphocreatine [PCr], pH 7.1) and ~40-60 mg was snap frozen in liquid nitrogen and stored at -80°C for future analysis.

2.7. Preparation of Permeabilized Muscle Fiber Bundles

Muscle fiber bundles were gently teased apart in a petri dish containing ice cold BIOPS solution with fine-nosed forceps and a dissecting microscope. Approximately 2-5 mg fiber bundles were then permeabilized in saponin (5 mg/ml) for 30 minutes at 4°C and then washed once for 10 minutes at 4°C with Mir05 respiration medium (0.5mM EGTA, 3mM MgCl2·6H2O, 60mM K-lactobionate, 20mM taurine, 10mM KH2PO4, 20mM HEPES, 110mM sucrose, and 1g/L BSA, pH 7.1), all on an orbital shaker (36). The permeabilized muscle fiber bundles were blotted, weighed, and placed in the high-resolution respirometry (HRR) chambers of an Oxygraph 2K (Oroboros Inc., Innsbruck, Austria) for analysis.

2.8. Skeletal Muscle Mitochondrial Respiration Protocols

HRR of permeabilized muscle fibers offers an integrative *ex vivo* measure of the dynamics of coupled metabolic pathways (14). Quantification of oxygen consumption in permeabilized fiber bundles was conducted at 37°C in the oxygen concentration above 200 nmol O_2/ml over a ~2 hr period. Substrate-uncoupler-inhibitor titration (SUIT) protocols for CHO and FA respiration was performed following stabilization of the O_2 trace. The addition of Cytochrome C was used to confirm the integrity of the outer mitochondrial membrane and the quality control threshold was set at ≤ 10% increase in respiration. Steady state O_2 flux for each respiratory state was determined and normalized to dry fiber bundle weight using Datlab 5 software (Oroboros Inc.).

In the CHO SUIT, LEAK state without adenylates respiration (i.e., substrate driven non-phosphorylating inner membrane proton leak) was determined by the addition of saturating concentrations of pyruvate (2 M) and malate (0.4 M). State 3 coupled respiration supported by electron flux through complex I was measured with the addition of ADP (0.5 M). Oxidative phosphorylation (OXPHOS) was achieved by adding saturating concentrations glutamate (2 M) and succinate (1 M). The addition of oligomycin (5 mM) inhibited ATP synthase and titrations of the uncoupler FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone 0.1 mM) were performed to assess maximal electron transport system (ETS c1+c2) respiration. Lastly, complex I inhibitor rotenone (1 mM) was then added to measure the rate of respiration through complex II alone (ETS c2). Finishing, malonic acid (2 M) and antimycin A (5 mM) were added to inhibit complex II and III, respectively. With total electron transport chain respiration inhibition, residual oxygen consumption was then quantified to be a correction factor for all mitochondrial respiratory rates.

In the FA SUIT, LEAK state without adenylates respiration was measured by the addition of octanoylcarnitine (0.1 M) and malate (0.4 M). This was followed by ADP (0.5 mM) for State 3 coupled respiration and then pyruvate (2 M), glutamate (2 M) and succinate (1 M) for OXPHOS. The remainder of the FA SUIT protocol was the same as the CHO SUIT.

For both substrate specific SUIT protocols, the respiratory control ratio (RCR) was calculated (State 3/LEAK) to determine the degree of coupled respiration and the flux control ratio (FCR, each respiratory state normalized to maximal respiratory rate, i.e. ETS c1+c2) to determine oxygen flux in different respiratory control states.

2.9. RNA sequencing

Samples were sequenced as previously described (21, 39). Briefly, frozen muscle samples were mechanically homogenized (~40-60 mg) in 1mL of TRIzol, and RNA was isolated according to manufacturer's instructions (Quick-DNA/RNA MiniPrep Plus Kit catalog# ZD7003, Research Products International, Mt. Prospect, IL, USA). RNA samples were submitted to the University of Colorado Denver's Genomics and Microarray Core where quality was assessed with the Agilent 2100 Bioanalyzer both before and after library preparation, which used Nugen's Universal Plus mRNA-seq kit according to manufacturer's protocols (NuGEN, Redwood City, CA). Sequencing was performed with the NovaSEQ6000 Ilumina sequencing platform as 150 nucleotide paired end reads, sequenced at 80 million paired reads per sample. Samples were run in two batches. Generated reads were trimmed, mapped to the human genome (GRCh38) using gSNAP, fragments per kilobase of transcript per million mapped reads (FPKM) were calculated using Cufflinks, and R for discovery of differential gene expression (45, 48). Genes were removed if their FPKM expression was less than 5 in either group of the statistical comparisons or if the difference in means was > 5. Raw mRNA sequencing data has been archived under the GEO accession number GSE186715.

2.10. Pathway Analysis

Canonical pathway and pathway comparison was conducted as previously described using IPA (Ingenuity Pathway Analysis, Qiagen) (31). Briefly, pathway analysis was performed using statistically significant differentially expressed genes with p-values of <0.05. Comparison analyses of the differentially expressed gene (DEG) lists between PA modes (ONE v SED, MICRO v SED, and MICRO v ONE) were used to calculate biological pathways and functions that were in common among DEG lists. The output lists were ordered by pathway enrichment significance (Benjamini-Hochberg false discovery rate [FDR]) and pathways were colored by predicted activation Z-score (red = positive, blue = negative, white = N/A).

2.11. Data and Statistical Analysis

The appropriateness of normality assumption for ANOVA and linear mixed effects model analysis was verified using Shapiro-Wilk test and visual examination of residual plots. One-way analysis of variance (ANOVA) was used to test for differences in participant characteristics between three subgroups: participants from the parent study (N=20) (8), participants included for Oroboros experiments to measure mitochondrial respiration in permeabilized muscle fibers (N=19) and participants for RNAseq analysis for molecular analysis (N=8). All initial respiratory states (LEAK, State 3, OXPHOS, ETS c1+c2 and ETS c2) that qualified for inclusion in data analysis (≤ 10% change in respiration after the addition of cytochrome C) were assessed with the ROUT method to identify and remove outliers from the data set (32). Linear mixed models tested for between-intervention differences in permeabilized muscle fiber respiratory states (LEAK, State 3, OXPHOS, ETS c1+c2 and ETS c2), and ratios (RCR and FCR) with intervention as repeated effect, sequence, period, and intervention as fixed effects and participants as random effects with variance components as covariance structure. A $P \leq 0.1$ was considered significant for model interactions. The least significant difference post-hoc test was used to examine between-intervention differences, a $P \leq 0.05$ was considered significant. Differentially expressed genes were analyzed with unpaired t-test between (SED vs. MICRO, SED vs. ONE, and ONE vs. MICRO) followed by FDR which was considered significant ≤ 0.05 by the Benjamini and Hochberg multiple testing correction. Spearman correlational analysis stratified by intervention was performed on

mitochondrial oxygen consumption dependent variables with previously published whole-body metabolic outcomes as independent variables (8). Linear mixed effect model and between-group comparison of correlations were fit to the dependent variables for the three interventions simultaneously while accounting for the correlation of repeated observations from the same participants. $P \leq 0.1$ was considered significant for model interactions and $P \leq 0.05$ was considered significant for between-group differences in correlation. Data are provided as mean ± SD, unless specified otherwise.

3. RESULTS

3.1. Participant Characteristics

As previously reported (8), twenty participants (n=10 males and 10 females) completed all study related procedures, mean age was 32.4 ± 6.3 yr and BMI 30.6 ± 2.9 kg/m^2. A subsample of participants had fasting muscle biopsies harvested for measurement of mitochondrial oxygen consumption in permeabilized muscle fibers (N=19, 9 males and 10 females) and a subsample for RNA sequencing and pathway analysis (N=8, 4 males and 4 females). No differences in the participant characteristics were noted between the two subsamples and the sample from the parent clinical trial (**Table 1**).

3.2. Mitochondrial capacity for fat oxidation is enhanced by a single continuous bout of PA, but not by multiple short bouts of PA

In the FA SUIT, no between-intervention differences in mitochondrial respiratory states were observed (**Figure 1A**), but RCR (i.e., degree of mitochondrial coupling) differed across interventions (intervention effect: P=0.008). In the presence of FA-associated substrates (Oct + M + ADP; **Figure 1E**), the RCR was higher after completing 4-days of ONE (4.8 ± 2.5) compared to both SED (2.3 ± 1.0, P=0.002) and MICRO (3.1 ± 1.1, P=0.024). The higher coupling in the presence of FA-associated substrates was likely driven by a decrease in LEAK respiration. LEAK FCR (i.e., contribution of mitochondrial respiratory state to overall flux; **Figure 1C**) differed across the three interventions (intervention effect: P = 0.020); it was lower in ONE (0.035 ± 0.008) compared to SED (0.075 ± 0.010, P=0.006), but not to MICRO (0.052 ± 0.005, P=0.21). In the CHO SUIT, there were no between-intervention differences observed for mitochondrial respiratory states (**Figure 1B**), FCR (**Figure 1D**), and RCR (**Figure 1F**).

3.3. Correlations between skeletal muscle mitochondrial oxidative capacity and whole-body metabolic outcomes

Mitochondrial coupling (i.e., RCR) in the presence of lipid-associated substrates was positively associated with fasting insulin concentrations in both PA interventions (slope ONE = 0.65, P = 0.0008 and slope MICRO = 0.24, P = 0.02; **Table 2**). The slopes of these associations were significantly different from SED (slope ONE vs. SED: P = 0.015 and slope MICRO vs SED: P = 0.05). Furthermore, ONE and MICRO differed (P = 0.03), the association was stronger in ONE than in MICRO. Similarly, mitochondrial coupling in the presence of lipid-associated substrates was positively associated with fasting insulin/glucose, an index of insulin sensitivity, in both PA interventions (slope ONE = 58.11, P = 0.001 and MICRO = 23.24, P = 0.012). The slope for ONE was significantly different from SED (slope ONE vs. SED: P = 0.002) and the slope for MICRO tended to differ from the one observed in SED (slope MICRO vs SED: P = 0.07). Sleeping CHO oxidation (i.e., proxy of fasting CHO oxidation) was negatively correlated with CHO-supported LEAK respiration (slope = -36.06, P = 0.03) but not with OXPHOS and ETS c1+c2 (P for interaction: OXPHOS P = 0.401 and ETS c1+c2 P = 0.194). Sleeping fat oxidation (i.e. proxy of fasting fat oxidation) was not correlated with fat-supported LEAK, OXPHOS, and ETS c1+c2 (P for interaction P = 0.919, P = 0.357, and P = 0.115, respectively).

3.4. Differential gene expression leads to similar pathway enrichment for MICRO and ONE with more biological pathways enriched in ONE

Differentially expressed genes were calculated (FDR ≤ 0.05) relative to the SED condition, and 1,126 DEG were identified in the ONE and 678 DEG were identified in the MICRO (**Figure 2A**). About 14% of DEG were shared between ONE and MICRO (**Figure 2B**). Ingenuity Pathway Analysis software was used to evaluate biological pathways and functions (**Figure 3**), providing a global view of the gene expression differences for muscle adaptations to PA mode. Significantly different gene lists were analyzed using the Comparison Analysis tool in IPA, and the pathway enrichment scores (sum of -log10(FDR)) were calculated as an indication of the proportion of DEGs that populate a given pathway. The fold changes of individual DEGs within a pathway were used to calculate whether a given pathway was predicted to be activated (red), inhibited (blue), or no prediction (white) (31). This pathway analysis revealed similar DEG enrichment and predicted activation states for ONE and MICRO compared to SED. The Oxidative Phosphorylation pathway was predicted to be activated (SED vs ONE: $P < 0.0001$; SED vs MICRO: $P < 0.0001$) and the Sirtuin Signaling pathway was predicted to be inhibited (SED vs ONE: $P < 0.0001$; SED vs MICRO: $P < 0.0001$). Critical cellular energy and metabolic sensor pathways were significantly enriched following ONE ($P < 0.01$, parenthetical values) but not MICRO ($P > 0.05$) when compared to SED. For instance, the mTOR, ERK/MAPK, AMPK, Nrf2-mediated oxidative stress response, insulin receptor, calcium signaling, necroptosis, and HIF1α pathways were enriched following ONE but not MICRO. Comparing both PA interventions (MICRO vs ONE) several pathways were enriched including Sirtuin Signaling, Oxidative Phosphorylation, mTOR, ERK/MAPK, AMPK, and NRF2-mediated oxidative stress response pathways ($P < 0.01$ for all, parenthetical values). Oxidative Phosphorylation pathway was predicted to be less activated following MICRO (MICRO vs ONE activation z-score: -3.357, $P < 0.001$). Additionally, while these pathways were not enriched, calcium signaling and necroptosis signaling pathways were upregulated for MICRO ($P < 0.05$). All pathways expressed by the interventions are listed in **Supplementary Tables 4 and 5**.

When comparing the significantly different genes modulated in the Oxidative Phosphorylation pathways for ONE or MICRO (relative to SED), more genes were similarly expressed in common between ONE and MICRO (N = 19/45) while some were unique only to MICRO (N=10/45) or ONE (N=16/45) (**Figure 4A**). Several genes regulated in common between both PA trials, included downregulation of the mitochondria trafficking inesin-binding protein 1 (TRAK1) and apoptosis activator Bcl2-associated agonist of cell death (BCL2), mitochondrial ATP synthase subunit epsilon 5E (ATP5E), cytochrome c oxidase genes (COX6, -7A2 and -7B), and NADH dehydrogenase subcomplex subunits (NDUFA2, -4, -8, 11 and -13, NDUFB5, -9, NDUFS3, and NDUFV2), the mitochondrial fission 1 protein (FIS1), and the mitochondrial peroxiredoxin-5 (PDX5).

The Sirtuin Signaling pathway, critical for sensing cellular energy status and metabolism (4), was also regulated following both ONE and MICRO. Significantly different genes that were regulated in common between ONE and MICRO included forkhead box protein O4 (FOXO4, decreased), mitochondrial import inner membrane translocase subunits (TIMM8, -23 and -50, and PAM16), and the mitochondrial cholesterol transport protein (TSPO) (**Figure 4B**). Significantly regulated genes in the ONE included 5'-AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1, decreased), superoxide dismutase 1 and 2 (increased SOD1 and decreased SOD2), lipogenic transcription factor sterol regulatory element-binding protein 1 (SREBF1, decreased), and inflammatory response signal transducer and activator of transcription 3 (STAT3, decreased). Furthermore, ONE upregulated unique mitochondrial import inner membrane translocase subunits (TIMM13 and -17A), as well as mitochondrial import receptor subunits (TOMM6, -7, and 22), and several MAP kinase signal transduction genes (MAPK1, -3, and -12). In contrast to ONE, MICRO had unique regulation of mitochondrial isocitrate dehydrogenase 2 (IDH2, decreased), skeletal muscle autophagy

regulators forkhead box protein O3 (FOXO3, decreased) and ubiquitin-like-conjugating enzyme (ATG3, increased), microtubule components (TUBA1A, TUBA1B, and TUB8), as well as complex V ATP synthase subunit gamma (ATP5C1). All significant DEGs organized by mode of PA are presented in supplemental data (**Supplemental Tables 1, 2, 3**).

4. DISCUSSION

In this randomized cross-over study, we compared the functional and molecular adaptations in skeletal muscle biopsies to short-frequent bouts of PA spread throughout the day to a time-matched single-continuous bout of PA, both compared to sedentary control conditions, in adults with overweight or obesity. We identified changes in the regulation of gene expression and mitochondrial functional capacity that are in common to both PA interventions and others that are unique to either ONE or MICRO. This indicates that biological pathways in skeletal muscle differentially respond to the terms of PA, i.e. volume, frequency and/or duration of the bouts.

Similar enrichment and activation of two key biological pathways known to regulate substrate oxidation, Oxidative Phosphorylation pathway (including electron transport chain components) and Sirtuin Signaling pathway, were observed after ONE and MICRO suggesting that adaptations within these pathways depend on total daily active time rather than bouts frequency and/or duration. These changes were observed 14 hr after the last microbout of PA and after 21 hr after the long continuous bout of PA, indicating a sustained response beyond the acute period. A potential regulator of muscle adaptation to acute forms of PA is FOXO4, that was commonly downregulated following both ONE and MICRO. FOXO4 and family members are transcription factors known to carry out the inhibitory effects of insulin and IGF-1 (30) potentially supporting improved insulin sensitivity in skeletal muscle following PA. This is in line with the improved insulin sensitivity noted at the whole-body level after both ONE and MICRO (8).

Contrary to our hypothesis, we observed no functional adaptations in mitochondrial oxidative capacity in the presence of CHO-associated substrates. This suggests that over the short-term short-frequent bouts of PA can trigger changes in the regulation of gene expression in pathways associated with the regulation of metabolism but these changes do not translate into higher mitochondrial function. This further suggests that mechanisms others than adaptations in mitochondrial oxidative capacity may contribute to the increase in 24 hr CHO oxidation we observed previously in MICRO (8). Because short-frequent bouts of brisk walking were completed throughout the day, participants were essentially in the postprandial state. The greater oxidative rates of CHO may therefore be the simple result of CHO availability to provide energy for body movements. Of note, substrate driven LEAK respiratory state in the presence of CHO-associated substrate was negatively correlated with sleeping CHO oxidation (an index of fasting CHO oxidation) and no associations were observed for OXPHOS and ETS mitochondrial respiratory states. A potential early mechanism for adaptations in mitochondrial function may be the decrease in substrate driven LEAK respiration through a more robust electron transport chain in which more protons are used for ATP production. The mitochondrial adaptations to MICRO may therefore take time. Longer-term studies will be needed to test if short-frequent bouts of PA can ultimately enhance overall skeletal muscle mitochondrial function.

We found that ONE, but not MICRO, resulted in a higher RCR when permeabilized muscle fibers were provided lipid-associated substrates, suggesting an improved coupling efficiency. The observed higher RCR in the presence of lipid-associated substrates may be due to a decrease in LEAK state FCR which indicates a reduced contribution of the LEAK respiratory state to overall electron flux and improved coupling efficiency because less protons are lost to non-coupled respiration. Besides one study testing the effect of an acute 60-min bout of PA in 21 healthy untrained male participants (42), the evidence on acute and short-term adaptions in mitochondrial

oxidative capacity are limited. Therefore, it is interesting to note that adaptations can be detected after 4-days of PA. In addition, this improved mitochondrial oxidative capacity for lipids along with the molecular adaptations favoring substrate oxidation may contribute to the higher 24 hr FA oxidation we observed previously in response to the single-continuous bout intervention (8).

The daily single-continuous bout intervention also had more biological pathways that were enriched with DEGs than the multiple short PA bouts intervention (**Figure 3**). These include the AMPK signaling pathway which is sensitive to changes in ATP turnover, calcium signaling pathway which is sensitive to oscillations in calcium concentrations from skeletal muscle contractile function, and HIF1α signaling pathway which is sensitive to the intracellular partial pressure of oxygen (13). Additionally, pathways associated with skeletal muscle nutrient uptake, growth, differentiation, survival, and the oxidative stress response were enriched after the daily single-continuous bout of PA intervention, including Insulin Receptor signaling, mTOR, ERK/MAPK signaling and NRF2-mediated Oxidative Stress Response. This constellation of enriched pathways after the single-continuous PA bout intervention is known to be associated with PGC-1α (5, 6, 33, 41), a master regulator of mitochondrial biogenesis (17), which may explain why we observed higher mitochondrial coupling. Of note, these results are confounded by an energy deficit as energy expended during PA was not replaced and future studies will need to confirm if similar results are observed in stable energy balance.

The enrichment of several PA associated transcriptional response pathways may be due to the sustained nature of the single-continuous PA bout intervention more than total active time. The longer bout length may create a greater challenge to skeletal muscle homeostasis therefore eliciting a greater adaptive stress response. This might be particularly true for the Oxidative Phosphorylation pathway because when comparing both PA interventions together this pathway was predicted to be less expressed in short-frequent bouts intervention. However, the Calcium Signaling pathway was predicted to be positively expressed following the exposure to frequent active breaks suggesting that the frequency rather than length of the PA bouts may impact this pathway. Sarcolemma depolarization during muscle contraction increases cytosolic Ca++ thereby activating the calcium signaling pathway. The frequent nature of the PA bouts may trigger waves of cytosolic Ca++ release that leads to increased expression of the Calcium Signaling pathway which has been shown to be associated with mitochondrial biogenesis (34).

A strength of the study was the randomized cross-over design of the interventions because each participant served as their own control and all females were studied in the same phase of their menstrual cycle. We were able to compare the respective effects of interrupting sedentary time from increased energy expenditure and/or PA by including a time-matched control. Compliance with each intervention period was verified with PA monitors. The results of this ancillary study should also be considered along with several limitations. Muscle biopsies were harvested at two different time intervals after the last bout of PA (21 hr post the single-continuous PA bout and 14 hr post the last bout of the short-frequent PA bouts intervention). The current experiments quantified mitochondrial respiratory capacity and not the actual respiration directly following completion of each 4-day PA trial. Additionally, the sample size for RNA sequencing was small and RNA sequencing results were not confirmed with RT-quantitative PCR due to limitations of muscle tissue availability.

5. CONCLUSION

Breaking up SB with short-frequent bouts of PA spread throughout the day over the short-term (4-days) is a sufficient stimulus to promote changes in the regulation of skeletal muscle gene expression in pathways associated with substrate oxidation. These changes were similar to those elicited by a time-matched daily single-continuous bout of PA, suggesting that total daily active time and/or energy expenditure is a primary trigger for those pathways. However, these molecular changes translated into an

improved capacity for mitochondrial fat oxidation and were associated with higher expression in muscle-contraction transcription signaling pathways only when PA was performed as a single-continuous bout. In contrast, the Calcium Signaling pathway was only activated when PA was completed as multiple short-frequent bouts. Therefore, this study showed for the first time that biological pathways in skeletal muscle are differentially regulated by the terms of PA, i.e. total active time, bout length and bout frequency. Future studies will need to determine whether these differential responses are sustained over time and translate into differential functional and whole-body changes.

AUTHOR CONTRIBUTIONS: M.R.J., D.H.B and A.B. conceived and designed the research; A.B. funding acquisition; N.P.D.J., M.C.R., J.H., D.H.B. and A.B. performed the experiments. N.P.D.J., M.C.R., M.R.J., R.S., K.J., Z.P., and A.B. analyzed data; N.P.D.J., M.C.R., M.J., K.J., J.R., P.S.M., D.H.B., and A.B. interpreted the results of the experiments; N.P.D.J. and A.B. prepared figures and drafted manuscript; N.P.D.J., M.C.R., M.J., R.S., K.J., J.H., Z.P., J.R., P.M., D.H.B., and A.B. reviewed and revised manuscript; all authors approved the final version of manuscript.

ACKNOWLEDGEMENTS: The authors thank study volunteers for their time and participation, as well as the medical, nutrition, and administrative staff of the University of Colorado Clinical and Translational Research Center.

FUNDING: This work was supported by the National Institutes of Health grant numbers F31DK125061 (N. De Jong), K99/R00DK100465 (A. Bergouignan), NORC P30DK048520 (A. Bergouignan, D.H. Bessesen, PS. MacLean), K01DK109079 and R03DK122189 (M. Rudolph). This publication was also supported by grant numbers TL1TR002533 (N. De Jong) and ULITR001082 (A. Bergouignan).

DISCLAIMER: Research reported in this publication was supported by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CONFLICTS OF INTEREST: There is nothing to disclose for any of the authors.

REFERENCES

1. **Benziane B, Burton TJ, Scanlan B, Galuska D, Canny BJ, Chibalin AV, Zierath JR, and Stepto NK**. Divergent cell signaling after short-term intensified endurance training in human skeletal muscle. *American journal of physiology Endocrinology and metabolism* 295: E1427-1438, 2008.
2. **Bergouignan A, Latouche C, Heywood S, Grace MS, Reddy-Luthmoodoo M, Natoli AK, Owen N, Dunstan DW, and Kingwell BA**. Frequent interruptions of sedentary time modulates contraction- and insulin-stimulated glucose uptake pathways in muscle: Ancillary analysis from randomized clinical trials. *Scientific reports* 6: 32044, 2016.
3. **Blankenship JM, Granados K, and Braun B**. Effects of subtracting sitting versus adding exercise on glycemic control and variability in sedentary office workers. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 39: 1286-1293, 2014.
4. **Chang HC, and Guarente L**. SIRT1 and other sirtuins in metabolism. *Trends in endocrinology and metabolism: TEM* 25: 138-145, 2014.
5. **Chau Long Y, Widegren U, and Zierath JR**. Exercise-induced mitogen-activated protein kinase signalling in skeletal muscle. *Proceedings of the Nutrition Society* 63: 227-232, 2004.
6. **Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, and Puigserver P**. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* 450: 736-740, 2007.
7. **De Jong NP, Debache I, Pan Z, Garnotel M, Lyden K, Sueur C, Simon C, Bessesen DH, and Bergouignan A**. Breaking up Sedentary Time in Overweight/Obese Adults on Work Days and Non-Work Days: Results from a Feasibility Study. *International journal of environmental research and public health* 15: 2018.
8. **De Jong NP, Rynders CA, Goldstrohm DA, Pan Z, Lange AH, Mendez C, Melanson EL, Bessesen DH, and Bergouignan A**. Effect of frequent interruptions of sedentary time on nutrient metabolism in sedentary overweight male and female adults. *Journal of applied physiology (Bethesda, Md : 1985)* 2019.

522 9. **Dempsey PC, Blankenship JM, Larsen RN, Sacre JW, Sethi P, Straznicky NE, Cohen ND, Cerin E, Lambert**
523 **GW, Owen N, Kingwell BA, and Dunstan DW**. Interrupting prolonged sitting in type 2 diabetes: nocturnal
524 persistence of improved glycaemic control. *Diabetologia* 60: 499-507, 2017.
525 10. **Dempsey PC, Larsen RN, Sethi P, Sacre JW, Straznicky NE, Cohen ND, Cerin E, Lambert GW, Owen N,**
526 **Kingwell BA, and Dunstan DW**. Benefits for Type 2 Diabetes of Interrupting Prolonged Sitting With Brief Bouts of
527 Light Walking or Simple Resistance Activities. *Diabetes Care* 39: 964-972, 2016.
528 11. **Dunstan DW, Barr EL, Healy GN, Salmon J, Shaw JE, Balkau B, Magliano DJ, Cameron AJ, Zimmet PZ, and**
529 **Owen N**. Television viewing time and mortality: the Australian Diabetes, Obesity and Lifestyle Study (AusDiab).
530 *Circulation* 121: 384-391, 2010.
531 12. **Dunstan DW, Kingwell BA, Larsen R, Healy GN, Cerin E, Hamilton MT, Shaw JE, Bertovic DA, Zimmet PZ,**
532 **Salmon J, and Owen N**. Breaking Up Prolonged Sitting Reduces Postprandial Glucose and Insulin Responses. *Diabetes*
533 *Care* 35: 976-983, 2012.
534 13. **Egan B, and Zierath JR**. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell*
535 *Metab* 17: 162-184, 2013.
536 14. **Gnaiger E**. Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis. 5th ed.
537 *Bioenerg Commun* 2020.
538 15. **Goodpaster BH, Katsiaras A, and Kelley DE**. Enhanced Fat Oxidation Through Physical Activity Is Associated
539 With Improvements in Insulin Sensitivity in Obesity. *Diabetes* 52: 2191-2197, 2003.
540 16. **Green HJ, Helyar R, Ball-Burnett M, Kowalchuk N, Symon S, and Farrance B**. Metabolic adaptations to
541 training precede changes in muscle mitochondrial capacity. *Journal of applied physiology (Bethesda, Md : 1985)* 72:
542 484-491, 1992.
543 17. **Halling JF, and Pilegaard H**. PGC-1α-mediated regulation of mitochondrial function and physiological
544 implications. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 45:
545 927-936, 2020.
546 18. **Healy GN, Dunstan DW, Salmon J, Cerin E, Shaw JE, Zimmet PZ, and Owen N**. Breaks in sedentary time:
547 beneficial associations with metabolic risk. *Diabetes Care* 31: 661-666, 2008.
548 19. **Healy GN, Matthews CE, Dunstan DW, Winkler EA, and Owen N**. Sedentary time and cardio-metabolic
549 biomarkers in US adults: NHANES 2003-06. *European heart journal* 32: 590-597, 2011.
550 20. **Helmerhorst HJF, Wijndaele K, Brage S, Wareham NJ, and Ekelund U**. Objectively measured sedentary time
551 may predict insulin resistance independent of moderate- and vigorous-intensity physical activity. *Diabetes* 58: 1776-
552 1779, 2009.
553 21. **Hohos NM, Elliott EM, Cho KJ, Lin IS, Rudolph MC, and Skaznik-Wikiel ME**. High-fat diet-induced
554 dysregulation of ovarian gene expression is restored with chronic omega-3 fatty acid supplementation. *Mol Cell*
555 *Endocrinol* 499: 110615, 2020.
556 22. **Holmstrup M, Fairchild T, Keslacy S, Weinstock R, and Kanaley J**. Multiple short bouts of exercise over 12-h
557 period reduce glucose excursions more than an energy-matched single bout of exercise. *Metabolism - Clinical and*
558 *Experimental* 63: 510-519, 2014.
559 23. **Homer AR, Fenemor SP, Perry TL, Rehrer NJ, Cameron CM, Skeaff CM, and Peddie MC**. Regular activity
560 breaks combined with physical activity improve postprandial plasma triglyceride, nonesterified fatty acid, and insulin
561 responses in healthy, normal weight adults: A randomized crossover trial. *Journal of clinical lipidology* 11: 1268-
562 1279.e1261, 2017.
563 24. **Hu FB, Leitzmann MF, Stampfer MJ, Colditz GA, Willett WC, and Rimm EB**. Physical activity and television
564 watching in relation to risk for type 2 diabetes mellitus in men. *Archives of internal medicine* 161: 1542-1548, 2001.
565 25. **Hu FB, Li TY, Colditz GA, Willett WC, and Manson JE**. Television watching and other sedentary behaviors in
566 relation to risk of obesity and type 2 diabetes mellitus in women. *Jama* 289: 1785-1791, 2003.
567 26. **Katzmarzyk PT, Church TS, Craig CL, and Bouchard C**. Sitting time and mortality from all causes,
568 cardiovascular disease, and cancer. *Med Sci Sports Exerc* 41: 998-1005, 2009.
569 27. **Koster A, Caserotti P, Patel KV, Matthews CE, Berrigan D, Van Domelen DR, Brychta RJ, Chen KY, and Harris**
570 **TB**. Association of sedentary time with mortality independent of moderate to vigorous physical activity. *PloS one* 7:
571 e37696, 2012.
572 28. **Larsen RN, Kingwell BA, Robinson C, Hammond L, Cerin E, Shaw JE, Healy GN, Hamilton MT, Owen N, and**
573 **Dunstan DW**. Breaking up of prolonged sitting over three days sustains, but does not enhance, lowering of

postprandial plasma glucose and insulin in overweight and obese adults. *Clinical science (London, England : 1979)* 129: 117-127, 2015.

29. **Latouche C, Jowett JB, Carey AL, Bertovic DA, Owen N, Dunstan DW, and Kingwell BA**. Effects of breaking up prolonged sitting on skeletal muscle gene expression. *Journal of applied physiology (Bethesda, Md : 1985)* 114: 453-460, 2013.

30. **Lee S, and Dong HH**. FoxO integration of insulin signaling with glucose and lipid metabolism. *J Endocrinol* 233: R67-r79, 2017.

31. **Martin Carli JF, Trahan GD, Jones KL, Hirsch N, Rolloff KP, Dunn EZ, Friedman JE, Barbour LA, Hernandez TL, MacLean PS, Monks J, McManaman JL, and Rudolph MC**. Single Cell RNA Sequencing of Human Milk-Derived Cells Reveals Sub-Populations of Mammary Epithelial Cells with Molecular Signatures of Progenitor and Mature States: a Novel, Non-invasive Framework for Investigating Human Lactation Physiology. *J Mammary Gland Biol Neoplasia* 25: 367-387, 2020.

32. **Motulsky HJ, and Brown RE**. Detecting outliers when fitting data with nonlinear regression – a new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics* 7: 123, 2006.

33. **Musci RV, Hamilton KL, and Linden MA**. Exercise-Induced Mitohormesis for the Maintenance of Skeletal Muscle and Healthspan Extension. *Sports* 7: 170, 2019.

34. **Ojuka EO**. Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle. *The Proceedings of the Nutrition Society* 63: 275-278, 2004.

35. **Peddie MC, Bone JL, Rehrer NJ, Skeaff CM, Gray AR, and Perry TL**. Breaking prolonged sitting reduces postprandial glycemia in healthy, normal-weight adults: a randomized crossover trial. *The American journal of clinical nutrition* 98: 358-366, 2013.

36. **Pesta D, and Gnaiger E**. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods in molecular biology (Clifton, NJ)* 810: 25-58, 2012.

37. **Pilegaard H, Saltin B, and Neufer PD**. Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* 546: 851-858, 2003.

38. **Plaisance EP, Mestek ML, Mahurin AJ, Taylor JK, Moncada-Jimenez J, and Grandjean PW**. Postprandial triglyceride responses to aerobic exercise and extended-release niacin. *The American journal of clinical nutrition* 88: 30-37, 2008.

39. **Presby DM, Checkley LA, Jackman MR, Higgins JA, Jones KL, Giles ED, Houck JA, Webb PG, Steig AJ, Johnson GC, Rudolph MC, and MacLean PS**. Regular exercise potentiates energetically expensive hepatic de novo lipogenesis during early weight regain. *American journal of physiology Regulatory, integrative and comparative physiology* 317: R684-R695, 2019.

40. **Remie CME, Janssens GE, Bilet L, van Weeghel M, Duvivier BMFM, de Wit VHW, Connell NJ, Jörgensen JA, Schomakers BV, Schrauwen-Hinderling VB, Hoeks J, Hesselink MKC, Phielix E, Houtkooper RH, and Schrauwen P**. Sitting less elicits metabolic responses similar to exercise and enhances insulin sensitivity in postmenopausal women. *Diabetologia* 2021.

41. **Schieke SM, Phillips D, McCoy JP, Jr., Aponte AM, Shen RF, Balaban RS, and Finkel T**. The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *The Journal of biological chemistry* 281: 27643-27652, 2006.

42. **Skovbro M, Boushel R, Hansen CN, Helge JW, and Dela F**. High-fat feeding inhibits exercise-induced increase in mitochondrial respiratory flux in skeletal muscle. *Journal of applied physiology (Bethesda, Md : 1985)* 110: 1607-1614, 2011.

43. **Spina RJ, Chi MM, Hopkins MG, Nemeth PM, Lowry OH, and Holloszy JO**. Mitochondrial enzymes increase in muscle in response to 7-10 days of cycle exercise. *Journal of applied physiology (Bethesda, Md : 1985)* 80: 2250-2254, 1996.

44. **Thyfault JP**. Setting the stage: possible mechanisms by which acute contraction restores insulin sensitivity in muscle. *American journal of physiology Regulatory, integrative and comparative physiology* 294: R1103-1110, 2008.

45. **Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, and Pachter L**. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 28: 511-515, 2010.

46. **Tremblay MS, Aubert S, Barnes JD, Saunders TJ, Carson V, Latimer-Cheung AE, Chastin SFM, Altenburg TM, Chinapaw MJM, Altenburg TM, Aminian S, Arundell L, Atkin AJ, Aubert S, Barnes J, Barone Gibbs B, Bassett-Gunter R, Belanger K, Biddle S, Biswas A, Carson V, Chaput J-P, Chastin S, Chau J, ChinAPaw M, Colley R, Coppinger T,**

Craven C, Cristi-Montero C, de Assis Teles Santos D, del Pozo Cruz B, del Pozo-Cruz J, Dempsey P, do Carmo Santos Gonçalves RF, Ekelund U, Ellingson L, Ezeugwu V, Fitzsimons C, Florez-Pregonero A, Friel CP, Fröberg A, Giangregorio L, Godin L, Gunnell K, Halloway S, Hinkley T, Hnatiuk J, Husu P, Kadir M, Karagounis LG, Koster A, Lakerveld J, Lamb M, Larouche R, Latimer-Cheung A, LeBlanc AG, Lee E-Y, Lee P, Lopes L, Manns T, Manyanga T, Martin Ginis K, McVeigh J, Meneguci J, Moreira C, Murtagh E, Patterson F, Rodrigues Pereira da Silva D, Pesola AJ, Peterson N, Pettitt C, Pilutti L, Pinto Pereira S, Poitras V, Prince S, Rathod A, Rivière F, Rosenkranz S, Routhier F, Santos R, Saunders T, Smith B, Theou O, Tomasone J, Tremblay M, Tucker P, Umstattd Meyer R, van der Ploeg H, Villalobos T, Viren T, Wallmann-Sperlich B, Wijndaele K, Wondergem R, and Participants oboSTCP. Sedentary Behavior Research Network (SBRN) – Terminology Consensus Project process and outcome. *International Journal of Behavioral Nutrition and Physical Activity* 14: 75, 2017.

47. **van Dijk JW, Venema M, van Mechelen W, Stehouwer CD, Hartgens F, and van Loon LJ**. Effect of moderate-intensity exercise versus activities of daily living on 24-hour blood glucose homeostasis in male patients with type 2 diabetes. *Diabetes Care* 36: 3448-3453, 2013.

48. **Wu TD, and Nacu S**. Fast and SNP-tolerant detection of complex variants and splicing in short reads. *Bioinformatics* 26: 873-881, 2010.

Tables

Table 1: Participant characteristics

	Muscle mitochondrial respiration (Oroboros)	**Muscle molecular adaptations (RNA seq)**
N (#)	19 (9m/10f)	8 (4m/4f)
Age (years)	32.2 ± 6.4	34.3 ± 8.4
BMI (kg/m²)	30.3 ± 3.0	29.9 ± 2.4
Body Mass (kg)	85.2 ± 10.9	84.8 ± 11.6
FFM (kg)	55.2 ± 10.6	55.9 ± 11.0
FM (kg)	30.0 ± 7.1	28.9 ± 8.3
Fat mass (%)	35.3 ± 7.7	34.2 ± 8.8
Fasting Glucose (mg/dL)	87.8 ± 4.6	86.6 ± 4.8
Fasting Triglyceride (mg/dL)	102.7 ± 52.8	115.9 ± 67.5
HOMA-IR (SED day fasting)	1.4 ± 0.7	1.2 ± 0.6
IPAQ self-reported sitting time (hrs/day)	10.6 ± 3.3	8.5 ± 3.0

Data are presented as mean ± SD. N, number of participants; BMI, body mass index; kg, kilograms; FFM, fat-free mass; FM, fat mass; HOMA-IR, homeostatic model assessment of insulin resistance was calculated from the blood draw collected on the morning of day 4 during the habitual sedentary behaviors (SED) intervention; IPAQ, international physical activity questionnaire.

Table 2: Relationship between mitochondrial oxidative capacity and whole-body metabolic measures

Dependent Variable	**Independent Variable**	**Intervention**	**Slope Estimate**	**P-value**	**P for interaction**
RCR fat	Fasting Insulin	SED	0.08	0.96	0.003**
		ONE	0.65	0.008##	
		MICRO	0.24	0.02#	
		SED - ONE	-0.64	0.015#	
		SED - BREAK	-0.24	0.05#	
		ONE - BREAK	0.40	0.03#	

RCR fat	Insulin/Glucose	SED	4.27	0.58	0.005**
		ONE	58.11	0.001##	
		MICRO	23.24	0.012#	
		SED - ONE	-53.84	0.002##	
		SED - BREAK	-18.97	0.07	
		ONE - BREAK	34.87	0.03#	
CHO LEAK	Sleeping CHO (g/min)	SED	-8.63	0.56	0.077*
		ONE	27.24	0.17	
		MICRO	-36.06	0.03#	
		SED - ONE	-35.87	0.11	
		SED - BREAK	27.45	0.31	
		ONE - BREAK	63.32	0.03#	
CHO OXPHOS	Sleeping CHO (g/min)	SED	42.22	0.76	0.401
		ONE	-97.52	0.55	
		MICRO	-320.65	0.15	
		SED - ONE	139.74	0.53	
		SED - BREAK	362.86	0.18	
		ONE - BREAK	223.13	0.42	
CHO ETS c1+c2	Sleeping CHO (g/min)	SED	-36.61	0.78	0.194
		ONE	229.91	0.15	
		MICRO	-200.32	0.33	
		SED - ONE	-266.53	0.18	
		SED - BREAK	163.71	0.49	
		ONE - BREAK	430.24	0.09	
FAT LEAK	Sleeping FAT (g/min)	SED	-2.47	0.94	0.919
		ONE	-8.59	0.81	
		MICRO	16.99	0.75	
		SED - ONE	6.11	0.89	
		SED - BREAK	-19.47	0.75	
		ONE - BREAK	-25.59	0.68	
FAT OXPHOS	Sleeping FAT (g/min)	SED	-105.42	0.71	0.357
		ONE	-460.21	0.15	
		MICRO	344.18	0.46	
		SED - ONE	354.79	0.41	
		SED - BREAK	-449.60	0.41	

		ONE - BREAK	-804.40	0.16	
FAT ETS c1+c2	Sleeping FAT (g/min)	SED	-61.82	0.85	0.115
		ONE	-170.15	0.57	
		MICRO	936.09	0.04	
		SED - ONE	108.33	0.80	
		SED - BREAK	-997.91	0.08	
		ONE - BREAK	-1106.24	0.04	

Spearman correlational analysis between fasting insulin concentration and mitochondrial coupling in the presence of lipid-associated substrates stratified by intervention. A linear mixed effect model was fit to the dependent variable for each trial simultaneously while accounting for the correlation of repeated observations from the same participant. Under the linear mixed effect model, slopes of the dependent variable vs independent variable were estimated and between-group comparison of the slopes were conducted. The between-group difference in slope assesses the difference between the two groups with respect to the correlation. SED – sedentary control, ONE – single-continuous bout of 45-min/day of moderate-intensity physical activity, MICRO – hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day. g/min: gram per minute substrate oxidation. Significant model interaction: * $P \leq 0.05$, ** $P \leq 0.01$. Significant between-intervention: $^{\#}$ $P \leq 0.05$, $^{\#\#}$ $P \leq 0.01$.

FIGURES

Figure 1

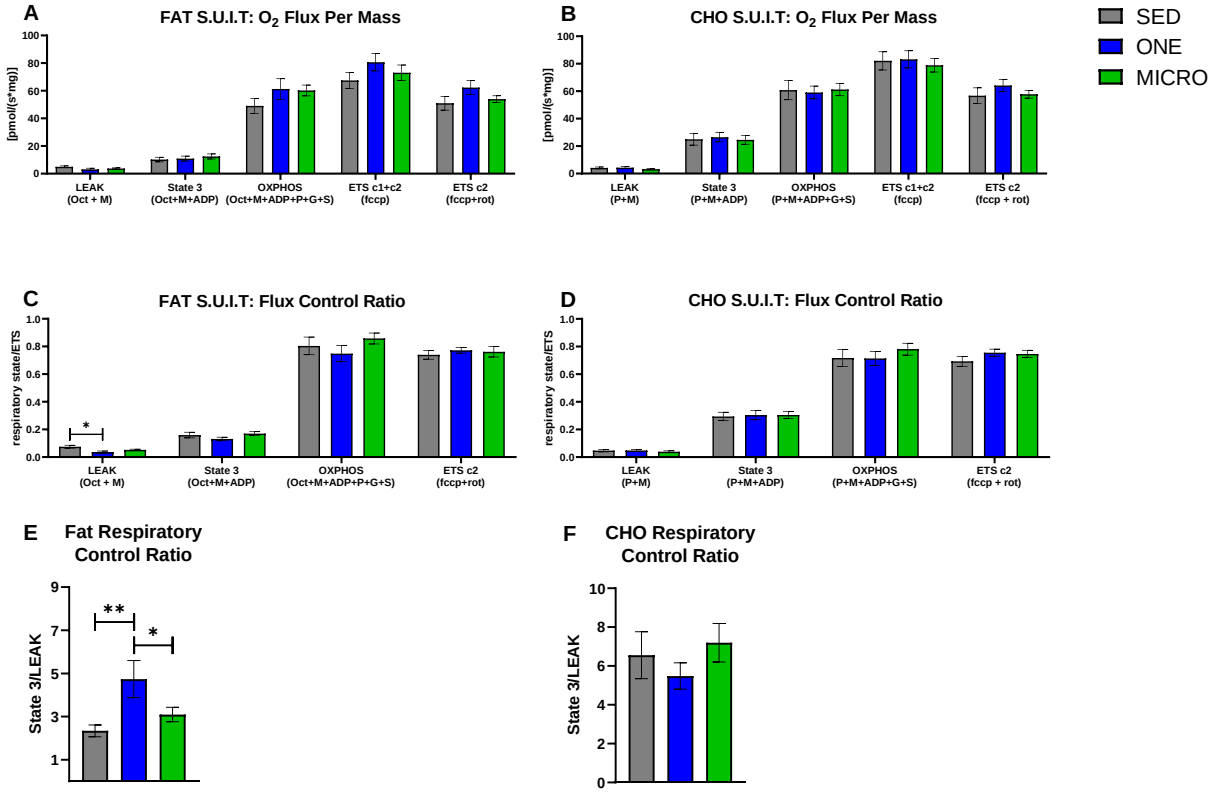


Figure 1: Carbohydrate- and fat-associated mitochondrial respiratory states, flux control and respiratory control in permeabilized skeletal muscles fibers. Substrate–uncoupler–inhibitor titration protocols associated with carbohydrate (CHO) and fat mitochondrial substrates were used for the evaluation of mitochondrial respiratory states, flux control ratio (FCR) and respiratory control ratio (RCR) in permeabilized skeletal muscle fibers collected in the fasting state from adults with overweight-to-obesity adults (n = 19, 9m/10f) after completing three 4-day study interventions, SED: sedentary control, ONE: single-continuous bout of 45-min/day of moderate-intensity physical activity, MICRO: hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day. **(A)** Fat-associated mitochondrial respiratory states: LEAK: octanol (Oct) + malate (M); State 3: Oct + M + adenosine diphosphate (ADP); Oxidative Phosphorylation (OXPHOS): Oct + M + ADP + Pyruvate (P) + Glutamate (G) + succinate (S); Electron transport system (ETS) through complex 1 and 2 (ETS c1+c2): fccp (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone); ETS through complex 2 (ETS c2): fccp + rotenone (rot). **(B)** CHO-associated mitochondrial respiratory states: LEAK: P + M; State 3: P + M + ADP; OXPHOS: P + M + ADP + G + S; ETS c1 & c2: fccp; ETS c2: fccp + rot. The FCR was calculated for each respiratory state by normalizing the respiratory state to ETS c1 &c2 which quantifies the contribution of each respiratory state to overall electron flux independent of mitochondrial content, mitochondrial preparations and experiment specific assay conditions. **(C)** Fat-associated FCR. **(D)** CHO-associated FCR. The RCR quantifies the degree of coupled respiration by State 3/LEAK. **(E)** Fat-associated RCR. **(F)** CHO-associated RCR. Data are mean ± SEM. Significant between-interventions * $P < 0.05$, ** $P < 0.01$.

FIGURE 2

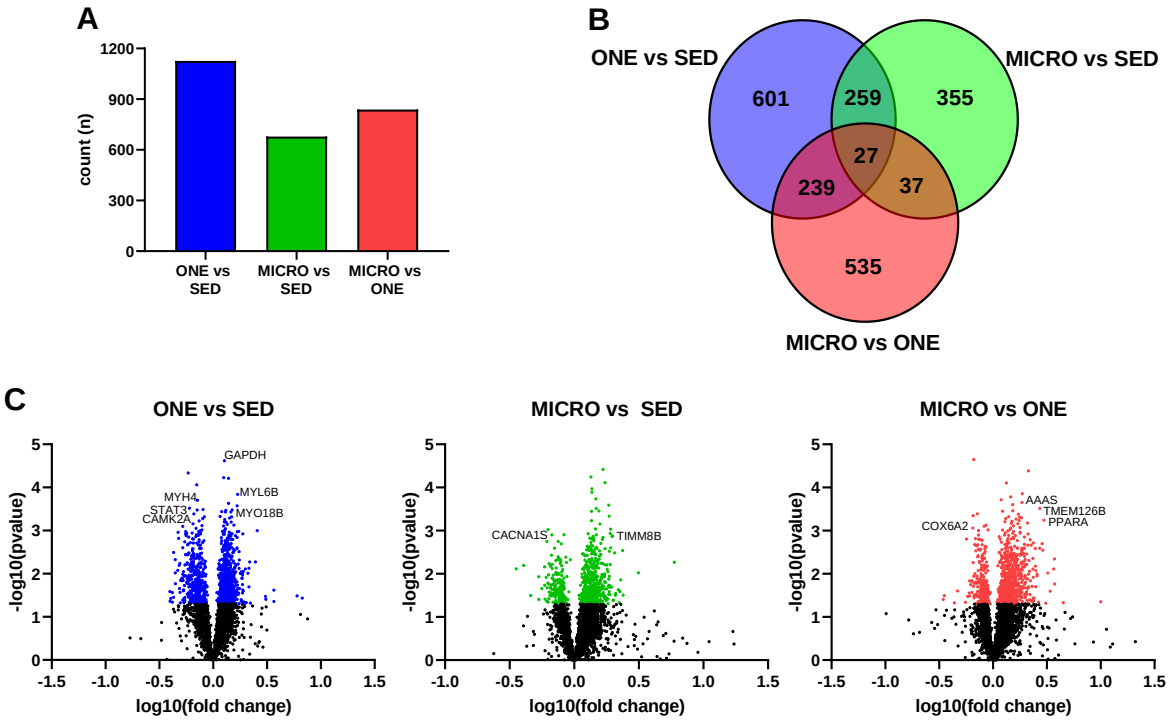


Figure 2: Comparison of muscle gene expression signatures in fasting skeletal muscle biopsies. (A) counts of differentially expressed genes by comparison, (B) Venn diagrams displaying the counts of differentially expressed genes for each comparison, (C) Volcano plots of differentially expressed genes. Genes related to metabolic outcomes selected from top 30 indicated on plot. Skeletal muscle biopsies were collected in the fasting state from adults with overweight-to-obesity adults (n = 8, 4m/4f) after completing three 4-day study interventions: SED: sedentary control, ONE: single-continuous bout of 45-min/day of moderate-intensity physical activity, MICRO: hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day.

FIGURE 3

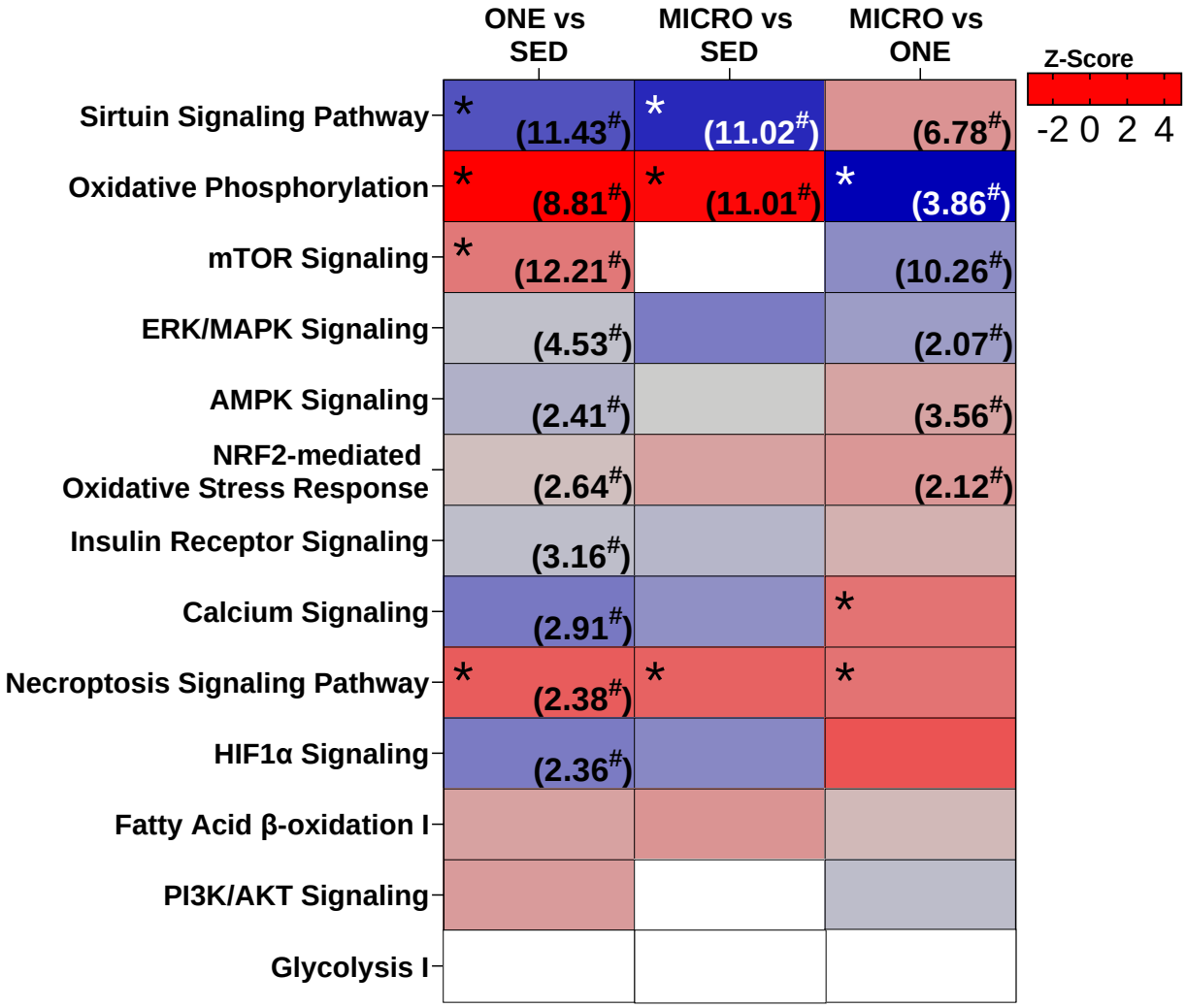
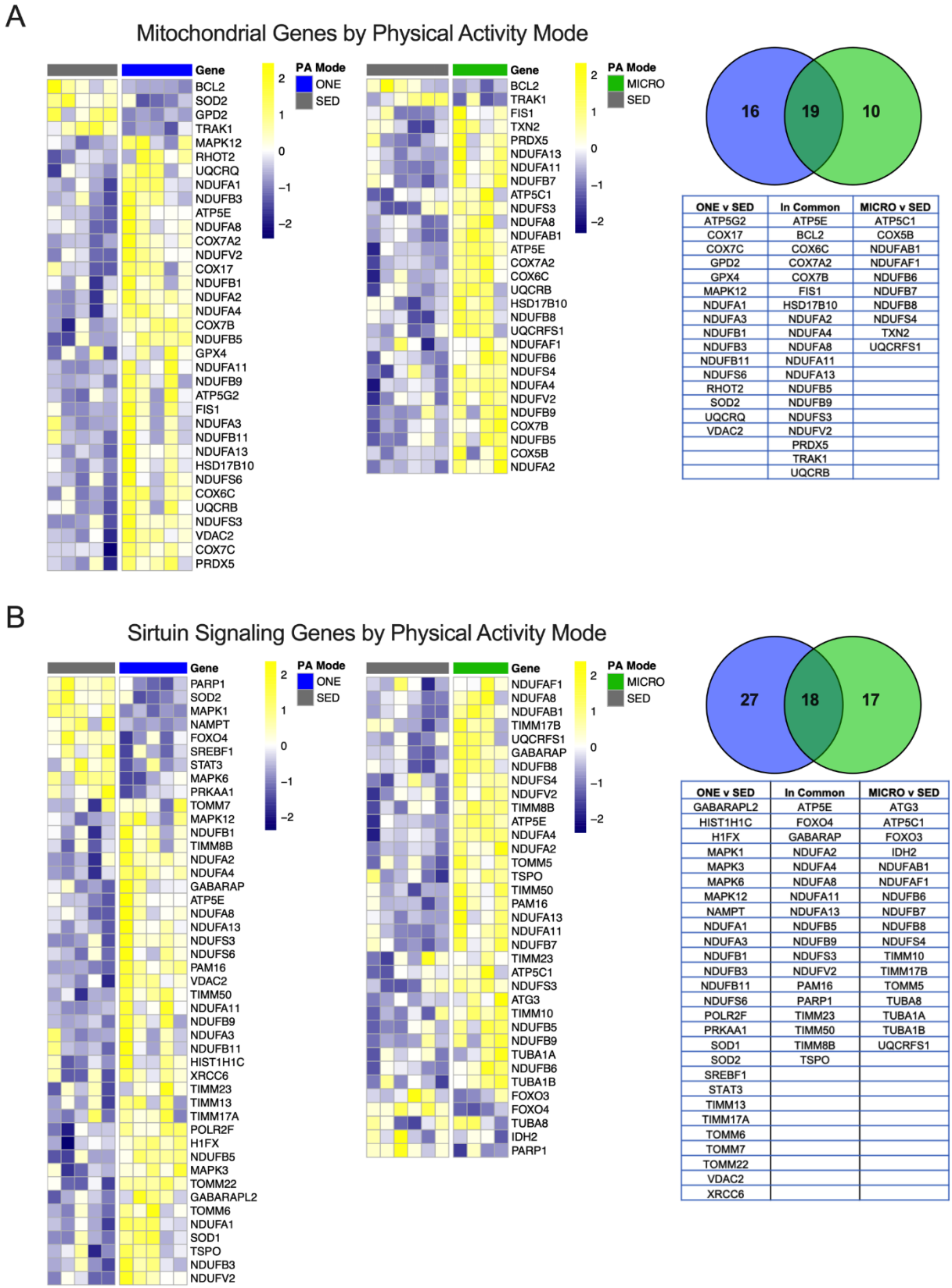


Figure 3: Comparison of metabolic and exercise-responsive pathways. Pathway enrichment was calculated -log10(FDR) for each comparison (value indicated in parentheses). For pathway ranking, total FDR score was calculated as the sum across the row for each FDR score. Pathway activation state colored by z-score (white = N/A, red = activated, blue = inhibited). A portion (n=8 – 4m/4f) of the skeletal muscle samples were analyzed by RNA sequencing to identify differentially expressed genes and pathway analysis was performed with Ingenuity Pathway Analysis (IPA, Qiagen Inc.). Skeletal muscle biopsies were collected in the fasting state from adults with overweight-to-obesity adults (n = 8, 4m/4f) after completing three 4-day study interventions: SED: sedentary control, ONE: single-continuous bout of 45-min/day of moderate-intensity physical activity, MICRO: hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day. * Significant z-score: $P < 0.05$. # Significant pathway enrichment: $P < 0.05$.

FIGURE 4



ONE v SED	In Common	MICRO v SED
ATP5G2	ATP5E	ATP5C1
COX17	BCL2	COX5B
COX7C	COX6C	NDUFAB1
GPD2	COX7A2	NDUFAF1
GPX4	COX7B	NDUFB6
MAPK12	FIS1	NDUFB7
NDUFA1	HSD17B10	NDUFB8
NDUFA3	NDUFA2	NDUFS4
NDUFB1	NDUFA4	TXN2
NDUFB3	NDUFA8	UQCRFS1
NDUFB11	NDUFA11	
NDUFS6	NDUFA13	
RHOT2	NDUFB5	
SOD2	NDUFB9	
UQCRQ	NDUFS3	
VDAC2	NDUFV2	
	PRDX5	
	TRAK1	
	UQCRB	

ONE v SED	In Common	MICRO v SED
GABARAPL2	ATP5E	ATG3
HIST1H1C	FOXO4	ATP5C1
H1FX	GABARAP	FOXO3
MAPK1	NDUFA2	IDH2
MAPK3	NDUFA4	NDUFAB1
MAPK6	NDUFA8	NDUFAF1
MAPK12	NDUFA11	NDUFB6
NAMPT	NDUFA13	NDUFB7
NDUFA1	NDUFB5	NDUFB8
NDUFA3	NDUFB9	NDUFS4
NDUFB1	NDUFS3	TIMM10
NDUFB3	NDUFV2	TIMM17B
NDUFB11	PAM16	TOMM5
NDUFS6	PARP1	TUBA8
POLR2F	TIMM23	TUBA1A
PRKAA1	TIMM50	TUBA1B
SOD1	TIMM8B	UQCRFS1
SOD2	TSPO	
SREBF1		
STAT3		
TIMM13		
TIMM17A		
TOMM6		
TOMM7		
TOMM22		
VDAC2		
XRCC6		

Figure 4. Mitochondrial and Sirtuin signaling genes unique to physical activity mode. Significantly different genes between either ONE vs SED or MICRO vs SED belonging to the mitochondria **(A)** or the Sirtuin Signaling pathway **(B)** were plotted by heatmap. In the mitochondria, 19 genes were in common, while 18 genes were in common between PA modes. Several NADH dehydrogenase subcomplex subunits (NDUF), cytochrome c oxidase (COX), and ATP synthase subunits were regulated specifically by a particular mode of PA. FOXO family member 4 was downregulated by both PA modes and may be an important regulatory transcription factor responding to acute PA in human skeletal muscle. All genes presented are

significant following multiple testing correction (FDR $P \leq 0.05$). PA: Physical activity, SED: sedentary control, ONE: single-continuous bout of 45-min/day of moderate-intensity physical activity, MICRO: hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day.