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2 Short-Term Adaptations in Skeletal Muscle Mitochondrial 3 Oxidative Capacity and Metabolic Pathways to Breaking Up 4 Sedentary Behaviors in Overweight or Obese Adults

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24 **ABSTRACT (200/200 words):** Breaking up sedentary behavior with short-frequent bouts of
25 physical activity (PA) differentially influences metabolic health than the performance of a single-
26 continuous bout of PA matched for total active time. The underlying mechanisms are however
27 unknown. We compared skeletal muscle mitochondrial respiration (high-resolution respirometry)
28 and molecular adaptations (RNA sequencing) to 4-day exposure to breaks vs energy-matched
29 single-continuous PA bout in inactive adults with overweight/obesity. Participants (9M/10F,
30 32.2±6.4 years, 30.3±3.0 kg/m²) completed three interventions of a randomized cross-over study,
31 SED: sedentary control, MICRO: 5-min brisk walking each hour for 9hr/d, ONE: 45-min/d
32 continuous brisk walking bout. Fasted muscle biopsies were collected on day 5. Mitochondrial
33 coupling in the presence of lipid-associated substrates was higher after ONE (4.8±2.5) compared to
34 MICRO (3.1±1.1, P=0.02) and SED (2.3±1.0, P=0.001). Respiratory rates did not differ across groups
35 with carbohydrate-associated substrates. In pathways associated with muscle contraction-
36 transcription signaling, ONE and MICRO similarly enhanced Oxidative Phosphorylation and
37 Sirtuin Signaling expression (P<0.0001, for both). However, ONE (P<0.001, for all), but not MICRO,
38 had greater pathway enrichment, including Ca⁺⁺, mTOR, AMPK, and HIF1α signaling than SED.
39 Although breaking up sedentary behavior triggers skeletal muscle molecular adaptations favoring
40 oxidative capacity, it did not improve mitochondrial function over the short-term.

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

44 1. INTRODUCTION

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

49 circumference, body mass index (BMI, kg/m²), 2 hr postprandial glycemia, and fasting
50 plasma glucose, triglycerides (TG) and C-reactive protein. Conversely, frequently
51 breaking up SB with short bouts of physical activity (PA) confers metabolic health
52 benefits independent of total time spent in moderate to vigorous physical activity
53 (MVPA) and total daily sitting time (18, 19). It is now well established that, compared to
54 sedentary control conditions, acute (≤ 12 hr) and short-term (≤ 4 days) exposures to
55 active breaks reduce postprandial glucose and insulin concentrations compared to a
56 sitting control in adults who are lean, with obesity and type 2 diabetes (3, 9, 10, 12, 22,
57 23, 28, 35, 47).

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

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

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

98 2. METHODS

99 2.1. *Experimental Protocol*

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

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

109 2.2. *Participants*

110 Study volunteers were recruited from October 2014 to October 2016. Inclusion
111 criteria included: age 19 – 45 yr, BMI 27 – 33 kg/m², weight stable for ≥ 3 months, and
112 being sedentary defined as sitting ≥ 6 h/day. Women were premenopausal but could use
113 oral contraceptives. Exclusion criteria included clinically diagnosed diabetes,
114 consumption of glucose- and/or lipid-lowering medication, dyslipidemia, smoking, or
115 being physically active (> self-reported 150 min/wk MVPA).

116 2.3. *Screening Visit*

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

122 2.4. *Study Interventions*

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

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

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

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

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

148 2.5. *Standardized Diet*

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

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

157 2.6. Skeletal Muscle Biopsy

158 Following an overnight fast, a *vastus lateralis* skeletal muscle biopsy was collected
159 under local anesthesia (2% wt/vol lidocaine HCl) using the Bergstrom biopsy needle
160 technique on the morning of day 5. From the total biopsy sample, ~10-20 mg was placed
161 immediately in ice cold BIOPS solution (10mM Ca-EGTA buffer, 0.1M free calcium,
162 20mM imidazole, 20mM taurine, 50mM potassium 2-[N-morpholino]-ethanesulfonic
163 acid, 0.5mM dithiothreitol, 6.56mM MgCl₂, 5.77mM ATP, and 15mM phosphocreatine
164 [PCr], pH 7.1) and ~40-60 mg was snap frozen in liquid nitrogen and stored at -80°C for
165 future analysis.

166 2.7. Preparation of Permeabilized Muscle Fiber Bundles

167 Muscle fiber bundles were gently teased apart in a petri dish containing ice cold
168 BIOPS solution with fine-nosed forceps and a dissecting microscope. Approximately 2-5
169 mg fiber bundles were then permeabilized in saponin (5 mg/ml) for 30 minutes at 4°C
170 and then washed once for 10 minutes at 4°C with Mir05 respiration medium (0.5mM
171 EGTA, 3mM MgCl₂·6H₂O, 60mM K-lactobionate, 20mM taurine, 10mM KH₂PO₄,
172 20mM HEPES, 110mM sucrose, and 1g/L BSA, pH 7.1), all on an orbital shaker (36). The
173 permeabilized muscle fiber bundles were blotted, weighed, and placed in the high-
174 resolution respirometry (HRR) chambers of an Oxygraph 2K (Oroboros Inc., Innsbruck,
175 Austria) for analysis.

176 2.8. Skeletal Muscle Mitochondrial Respiration Protocols

177 HRR of permeabilized muscle fibers offers an integrative *ex vivo* measure of the
178 dynamics of coupled metabolic pathways (14). Quantification of oxygen consumption in
179 permeabilized fiber bundles was conducted at 37°C in the oxygen concentration above
180 200 nmol O₂/ml over a ~2 hr period. Substrate-uncoupler-inhibitor titration (SUIT)
181 protocols for CHO and FA respiration was performed following stabilization of the O₂
182 trace. The addition of Cytochrome C was used to confirm the integrity of the outer
183 mitochondrial membrane and the quality control threshold was set at ≤ 10% increase in
184 respiration. Steady state O₂ flux for each respiratory state was determined and
185 normalized to dry fiber bundle weight using Datlab 5 software (Oroboros Inc.).

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

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

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

209 2.9. RNA sequencing

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

226 2.10. Pathway Analysis

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

236 2.11. Data and Statistical Analysis

237 The appropriateness of normality assumption for ANOVA and linear mixed effects
238 model analysis was verified using Shapiro-Wilk test and visual examination of residual
239 plots. One-way analysis of variance (ANOVA) was used to test for differences in
240 participant characteristics between three subgroups: participants from the parent study
241 (N=20) (8), participants included for Oroboros experiments to measure mitochondrial
242 respiration in permeabilized muscle fibers (N=19) and participants for RNAseq analysis
243 for molecular analysis (N=8). All initial respiratory states (LEAK, State 3, OXPHOS, ETS
244 c1+c2 and ETS c2) that qualified for inclusion in data analysis ($\leq 10\%$ change in
245 respiration after the addition of cytochrome C) were assessed with the ROUT method to
246 identify and remove outliers from the data set (32). Linear mixed models tested for
247 between-intervention differences in permeabilized muscle fiber respiratory states
248 (LEAK, State 3, OXPHOS, ETS c1+c2 and ETS c2), and ratios (RCR and FCR) with
249 intervention as repeated effect, sequence, period, and intervention as fixed effects and
250 participants as random effects with variance components as covariance structure. A $P \leq$
251 0.1 was considered significant for model interactions. The least significant difference
252 post-hoc test was used to examine between-intervention differences, a $P \leq 0.05$ was
253 considered significant. Differentially expressed genes were analyzed with unpaired t-
254 test between (SED vs. MICRO, SED vs. ONE, and ONE vs. MICRO) followed by FDR
255 which was considered significant ≤ 0.05 by the Benjamini and Hochberg multiple testing
256 correction. Spearman correlational analysis stratified by intervention was performed on

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

264 3. RESULTS

265 3.1. Participant Characteristics

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

274 3.2. Mitochondrial capacity for fat oxidation is enhanced by a single continuous bout of PA, but 275 not by multiple short bouts of PA

276 In the FA SUI, no between-intervention differences in mitochondrial respiratory
277 states were observed (Figure 1A), but RCR (i.e., degree of mitochondrial coupling)
278 differed across interventions (intervention effect: $P=0.008$). In the presence of FA-
279 associated substrates (Oct + M + ADP; Figure 1E), the RCR was higher after completing
280 4-days of ONE (4.8 ± 2.5) compared to both SED (2.3 ± 1.0 , $P=0.002$) and MICRO ($3.1 \pm$
281 1.1 , $P=0.024$). The higher coupling in the presence of FA-associated substrates was likely
282 driven by a decrease in LEAK respiration. LEAK FCR (i.e., contribution of mitochondrial
283 respiratory state to overall flux; Figure 1C) differed across the three interventions
284 (intervention effect: $P = 0.020$); it was lower in ONE (0.035 ± 0.008) compared to SED
285 (0.075 ± 0.010 , $P=0.006$), but not to MICRO (0.052 ± 0.005 , $P=0.21$). In the CHO SUI, there
286 were no between-intervention differences observed for mitochondrial respiratory states
287 (Figure 1B), FCR (Figure 1D), and RCR (Figure 1F).

288 3.3. Correlations between skeletal muscle mitochondrial oxidative capacity and whole-body 289 metabolic outcomes

290 Mitochondrial coupling (i.e., RCR) in the presence of lipid-associated substrates
291 was positively associated with fasting insulin concentrations in both PA interventions
292 (slope ONE = 0.65, $P = 0.0008$ and slope MICRO = 0.24, $P = 0.02$; Table 2). The slopes of
293 these associations were significantly different from SED (slope ONE vs. SED: $P = 0.015$
294 and slope MICRO vs SED: $P = 0.05$). Furthermore, ONE and MICRO differed ($P = 0.03$),
295 the association was stronger in ONE than in MICRO. Similarly, mitochondrial coupling
296 in the presence of lipid-associated substrates was positively associated with fasting
297 insulin/glucose, an index of insulin sensitivity, in both PA interventions (slope ONE =
298 58.11, $P = 0.001$ and MICRO = 23.24, $P = 0.012$). The slope for ONE was significantly
299 different from SED (slope ONE vs. SED: $P = 0.002$) and the slope for MICRO tended to
300 differ from the one observed in SED (slope MICRO vs SED: $P = 0.07$). Sleeping CHO
301 oxidation (i.e., proxy of fasting CHO oxidation) was negatively correlated with CHO-
302 supported LEAK respiration (slope = -36.06, $P = 0.03$) but not with OXPHOS and ETS
303 c1+c2 (P for interaction: OXPHOS $P = 0.401$ and ETS c1+c2 $P = 0.194$). Sleeping fat
304 oxidation (i.e. proxy of fasting fat oxidation) was not correlated with fat-supported
305 LEAK, OXPHOS, and ETS c1+c2 (P for interaction $P = 0.919$, $P = 0.357$, and $P = 0.115$,
306 respectively).

307 3.4. Differential gene expression leads to similar pathway enrichment for MICRO and ONE with
308 more biological pathways enriched in ONE

309 Differentially expressed genes were calculated ($FDR \leq 0.05$) relative to the SED
310 condition, and 1,126 DEG were identified in the ONE and 678 DEG were identified in
311 the MICRO (**Figure 2A**). About 14% of DEG were shared between ONE and MICRO
312 (**Figure 2B**). Ingenuity Pathway Analysis software was used to evaluate biological
313 pathways and functions (**Figure 3**), providing a global view of the gene expression
314 differences for muscle adaptations to PA mode. Significantly different gene lists were
315 analyzed using the Comparison Analysis tool in IPA, and the pathway enrichment
316 scores (sum of $-\log_{10}(FDR)$) were calculated as an indication of the proportion of DEGs
317 that populate a given pathway. The fold changes of individual DEGs within a pathway
318 were used to calculate whether a given pathway was predicted to be activated (red),
319 inhibited (blue), or no prediction (white) (31). This pathway analysis revealed similar
320 DEG enrichment and predicted activation states for ONE and MICRO compared to SED.
321 The Oxidative Phosphorylation pathway was predicted to be activated (SED vs ONE: P
322 < 0.0001 ; SED vs MICRO: $P < 0.0001$) and the Sirtuin Signaling pathway was predicted to
323 be inhibited (SED vs ONE: $P < 0.0001$; SED vs MICRO: $P < 0.0001$). Critical cellular
324 energy and metabolic sensor pathways were significantly enriched following ONE ($P <$
325 0.01 , parenthetical values) but not MICRO ($P > 0.05$) when compared to SED. For
326 instance, the mTOR, ERK/MAPK, AMPK, Nrf2-mediated oxidative stress response,
327 insulin receptor, calcium signaling, necroptosis, and HIF1 α pathways were enriched
328 following ONE but not MICRO. Comparing both PA interventions (MICRO vs ONE)
329 several pathways were enriched including Sirtuin Signaling, Oxidative Phosphorylation,
330 mTOR, ERK/MAPK, AMPK, and NRF2-mediated oxidative stress response pathways (P
331 < 0.01 for all, parenthetical values). Oxidative Phosphorylation pathway was predicted
332 to be less activated following MICRO (MICRO vs ONE activation z-score: -3.357 , $P <$
333 0.001). Additionally, while these pathways were not enriched, calcium signaling and
334 necroptosis signaling pathways were upregulated for MICRO ($P < 0.05$). All pathways
335 expressed by the interventions are listed in **Supplementary Tables 4 and 5**.

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

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

362 regulators forkhead box protein O3 (FOXO3, decreased) and ubiquitin-like-conjugating
363 enzyme (ATG3, increased), microtubule components (TUBA1A, TUBA1B, and TUB8), as
364 well as complex V ATP synthase subunit gamma (ATP5C1). All significant DEGs
365 organized by mode of PA are presented in supplemental data (**Supplemental Tables 1,**
366 **2, 3**).

367 4. DISCUSSION

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

376 Similar enrichment and activation of two key biological pathways known to
377 regulate substrate oxidation, Oxidative Phosphorylation pathway (including electron
378 transport chain components) and Sirtuin Signaling pathway, were observed after ONE
379 and MICRO suggesting that adaptations within these pathways depend on total daily
380 active time rather than bouts frequency and/or duration. These changes were observed
381 14 hr after the last microbout of PA and after 21 hr after the long continuous bout of PA,
382 indicating a sustained response beyond the acute period. A potential regulator of muscle
383 adaptation to acute forms of PA is FOXO4, that was commonly downregulated
384 following both ONE and MICRO. FOXO4 and family members are transcription factors
385 known to carry out the inhibitory effects of insulin and IGF-1 (30) potentially supporting
386 improved insulin sensitivity in skeletal muscle following PA. This is in line with the
387 improved insulin sensitivity noted at the whole-body level after both ONE and MICRO
388 (8).

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

408 We found that ONE, but not MICRO, resulted in a higher RCR when permeabilized
409 muscle fibers were provided lipid-associated substrates, suggesting an improved
410 coupling efficiency. The observed higher RCR in the presence of lipid-associated
411 substrates may be due to a decrease in LEAK state FCR which indicates a reduced
412 contribution of the LEAK respiratory state to overall electron flux and improved
413 coupling efficiency because less protons are lost to non-coupled respiration. Besides one
414 study testing the effect of an acute 60-min bout of PA in 21 healthy untrained male
415 participants (42), the evidence on acute and short-term adaptations 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 HIF1 α 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-1 α (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

470 improved capacity for mitochondrial fat oxidation and were associated with higher
471 expression in muscle-contraction transcription signaling pathways only when PA was
472 performed as a single-continuous bout. In contrast, the Calcium Signaling pathway was
473 only activated when PA was completed as multiple short-frequent bouts. Therefore, this
474 study showed for the first time that biological pathways in skeletal muscle are
475 differentially regulated by the terms of PA, i.e. total active time, bout length and bout
476 frequency. Future studies will need to determine whether these differential responses
477 are sustained over time and translate into differential functional and whole-body
478 changes.
479

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482 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.,
483 D.H.B., and A.B. interpreted the results of the experiments; N.P.D.J. and A.B. prepared figures and
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499 REFERENCES

- 500 1. **Benziene B, Burton TJ, Scanlan B, Galuska D, Canny BJ, Chibalin AV, Zierath JR, and Stepto NK.** Divergent
501 cell signaling after short-term intensified endurance training in human skeletal muscle. *American journal of*
502 *physiology Endocrinology and metabolism* 295: E1427-1438, 2008.
- 503 2. **Bergouignan A, Latouche C, Heywood S, Grace MS, Reddy-Luthmoodoo M, Natoli AK, Owen N, Dunstan**
504 **DW, and Kingwell BA.** Frequent interruptions of sedentary time modulates contraction- and insulin-stimulated
505 glucose uptake pathways in muscle: Ancillary analysis from randomized clinical trials. *Scientific reports* 6: 32044,
506 2016.
- 507 3. **Blankenship JM, Granados K, and Braun B.** Effects of subtracting sitting versus adding exercise on glycemic
508 control and variability in sedentary office workers. *Applied physiology, nutrition, and metabolism = Physiologie*
509 *appliquee, nutrition et metabolisme* 39: 1286-1293, 2014.
- 510 4. **Chang HC, and Guarente L.** SIRT1 and other sirtuins in metabolism. *Trends in endocrinology and metabolism:*
511 *TEM* 25: 138-145, 2014.
- 512 5. **Chau Long Y, Widegren U, and Zierath JR.** Exercise-induced mitogen-activated protein kinase signalling in
513 skeletal muscle. *Proceedings of the Nutrition Society* 63: 227-232, 2004.
- 514 6. **Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, and Puigserver P.** mTOR controls
515 mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* 450: 736-740, 2007.
- 516 7. **De Jong NP, Debache I, Pan Z, Garnotel M, Lyden K, Sueur C, Simon C, Bessesen DH, and Bergouignan A.**
517 Breaking up Sedentary Time in Overweight/Obese Adults on Work Days and Non-Work Days: Results from a
518 Feasibility Study. *International journal of environmental research and public health* 15: 2018.
- 519 8. **De Jong NP, Rynders CA, Goldstrohm DA, Pan Z, Lange AH, Mendez C, Melanson EL, Bessesen DH, and**
520 **Bergouignan A.** Effect of frequent interruptions of sedentary time on nutrient metabolism in sedentary overweight
521 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-1 α -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, Kesztyes 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, Rehner 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

- 574 postprandial plasma glucose and insulin in overweight and obese adults. *Clinical science (London, England : 1979)*
575 129: 117-127, 2015.
- 576 29. **Latouche C, Jowett JB, Carey AL, Bertovic DA, Owen N, Dunstan DW, and Kingwell BA.** Effects of breaking
577 up prolonged sitting on skeletal muscle gene expression. *Journal of applied physiology (Bethesda, Md : 1985)* 114:
578 453-460, 2013.
- 579 30. **Lee S, and Dong HH.** FoxO integration of insulin signaling with glucose and lipid metabolism. *J Endocrinol*
580 233: R67-r79, 2017.
- 581 31. **Martin Carli JF, Trahan GD, Jones KL, Hirsch N, Rolloff KP, Dunn EZ, Friedman JE, Barbour LA, Hernandez TL,**
582 **MacLean PS, Monks J, McManaman JL, and Rudolph MC.** Single Cell RNA Sequencing of Human Milk-Derived Cells
583 Reveals Sub-Populations of Mammary Epithelial Cells with Molecular Signatures of Progenitor and Mature States: a
584 Novel, Non-invasive Framework for Investigating Human Lactation Physiology. *J Mammary Gland Biol Neoplasia* 25:
585 367-387, 2020.
- 586 32. **Motulsky HJ, and Brown RE.** Detecting outliers when fitting data with nonlinear regression – a new method
587 based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics* 7: 123, 2006.
- 588 33. **Musci RV, Hamilton KL, and Linden MA.** Exercise-Induced Mitohormesis for the Maintenance of Skeletal
589 Muscle and Healthspan Extension. *Sports* 7: 170, 2019.
- 590 34. **Ojuka EO.** Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in
591 muscle. *The Proceedings of the Nutrition Society* 63: 275-278, 2004.
- 592 35. **Peddie MC, Bone JL, Rehrer NJ, Skeaff CM, Gray AR, and Perry TL.** Breaking prolonged sitting reduces
593 postprandial glycemia in healthy, normal-weight adults: a randomized crossover trial. *The American journal of clinical*
594 *nutrition* 98: 358-366, 2013.
- 595 36. **Pesta D, and Gnaiger E.** High-resolution respirometry: OXPHOS protocols for human cells and permeabilized
596 fibers from small biopsies of human muscle. *Methods in molecular biology (Clifton, NJ)* 810: 25-58, 2012.
- 597 37. **Pilegaard H, Saltin B, and Neufer PD.** Exercise induces transient transcriptional activation of the PGC-1alpha
598 gene in human skeletal muscle. *J Physiol* 546: 851-858, 2003.
- 599 38. **Plaisance EP, Mestek ML, Mahurin AJ, Taylor JK, Moncada-Jimenez J, and Grandjean PW.** Postprandial
600 triglyceride responses to aerobic exercise and extended-release niacin. *The American journal of clinical nutrition* 88:
601 30-37, 2008.
- 602 39. **Presby DM, Checkley LA, Jackman MR, Higgins JA, Jones KL, Giles ED, Houck JA, Webb PG, Steig AJ,**
603 **Johnson GC, Rudolph MC, and MacLean PS.** Regular exercise potentiates energetically expensive hepatic de novo
604 lipogenesis during early weight regain. *American journal of physiology Regulatory, integrative and comparative*
605 *physiology* 317: R684-R695, 2019.
- 606 40. **Remie CME, Janssens GE, Bilet L, van Weeghel M, Duvivier BMFM, de Wit VHW, Connell NJ, Jörgensen JA,**
607 **Schomakers BV, Schrauwen-Hinderling VB, Hoeks J, Hesselink MKC, Phielix E, Houtkooper RH, and Schrauwen P.**
608 Sitting less elicits metabolic responses similar to exercise and enhances insulin sensitivity in postmenopausal women.
609 *Diabetologia* 2021.
- 610 41. **Schieke SM, Phillips D, McCoy JP, Jr., Aponte AM, Shen RF, Balaban RS, and Finkel T.** The mammalian target
611 of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *The Journal of*
612 *biological chemistry* 281: 27643-27652, 2006.
- 613 42. **Skovbro M, Boushel R, Hansen CN, Helge JW, and Dela F.** High-fat feeding inhibits exercise-induced increase
614 in mitochondrial respiratory flux in skeletal muscle. *Journal of applied physiology (Bethesda, Md : 1985)* 110: 1607-
615 1614, 2011.
- 616 43. **Spina RJ, Chi MM, Hopkins MG, Nemeth PM, Lowry OH, and Holloszy JO.** Mitochondrial enzymes increase
617 in muscle in response to 7-10 days of cycle exercise. *Journal of applied physiology (Bethesda, Md : 1985)* 80: 2250-
618 2254, 1996.
- 619 44. **Thyfault JP.** Setting the stage: possible mechanisms by which acute contraction restores insulin sensitivity in
620 muscle. *American journal of physiology Regulatory, integrative and comparative physiology* 294: R1103-1110, 2008.
- 621 45. **Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, and Pachter L.**
622 Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell
623 differentiation. *Nat Biotechnol* 28: 511-515, 2010.
- 624 46. **Tremblay MS, Aubert S, Barnes JD, Saunders TJ, Carson V, Latimer-Cheung AE, Chastin SFM, Altenburg TM,**
625 **Chinapaw MJM, Altenburg TM, Aminian S, Arundell L, Atkin AJ, Aubert S, Barnes J, Barone Gibbs B, Bassett-Gunter**
626 **R, Belanger K, Biddle S, Biswas A, Carson V, Chaput J-P, Chastin S, Chau J, ChinAPaw M, Colley R, Coppinger T,**

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

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

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

642
643 Tables

644 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/m ²)	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

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

649
650 Table 2: Relationship between mitochondrial oxidative capacity and whole-body metabolic measures

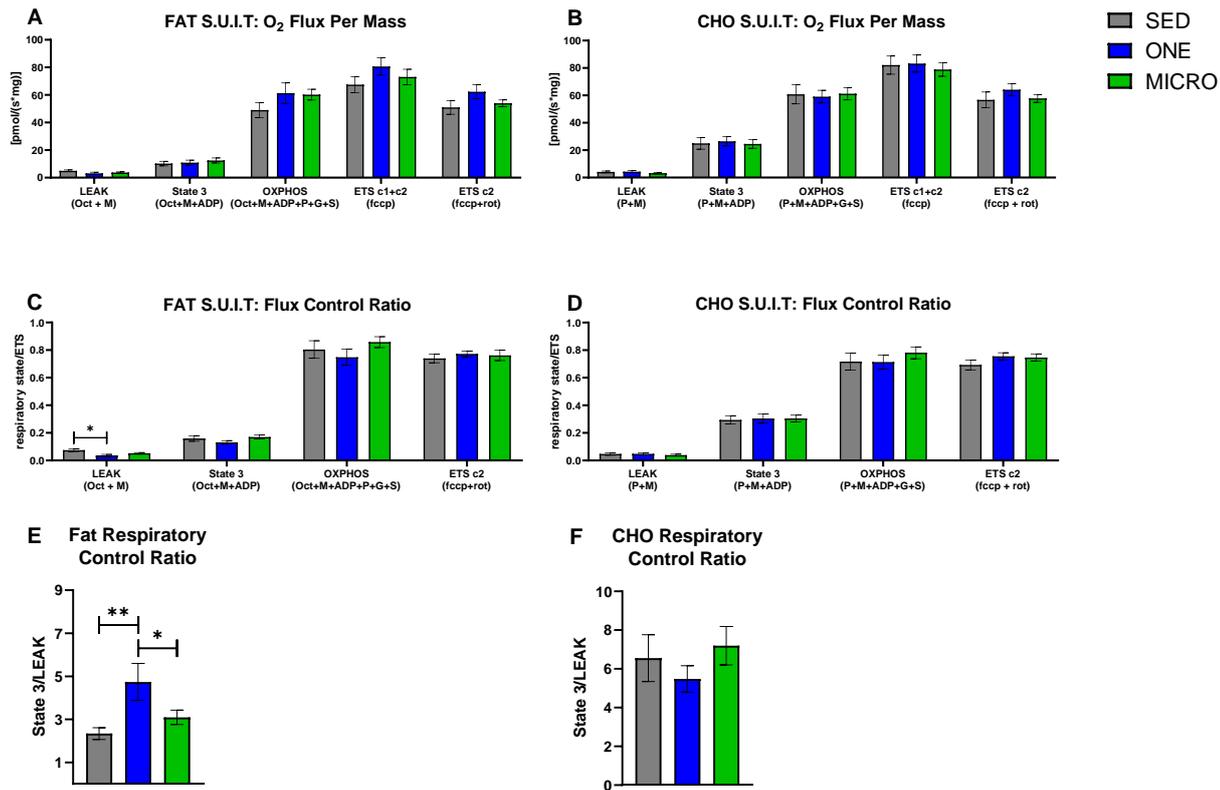
Dependent Variable	Independent Variable	Intervention	Slope Estimate	P-value	P for interaction
RCR fat	Fasting Insulin	SED	0.08	0.96	0.003**
		ONE	0.65	0.008##	
		MICRO	0.24	0.02#	
		SED - ONE	-0.64	0.015#	
		SED - BREAK	-0.24	0.05#	
		ONE - BREAK	0.40	0.03#	

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	

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

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

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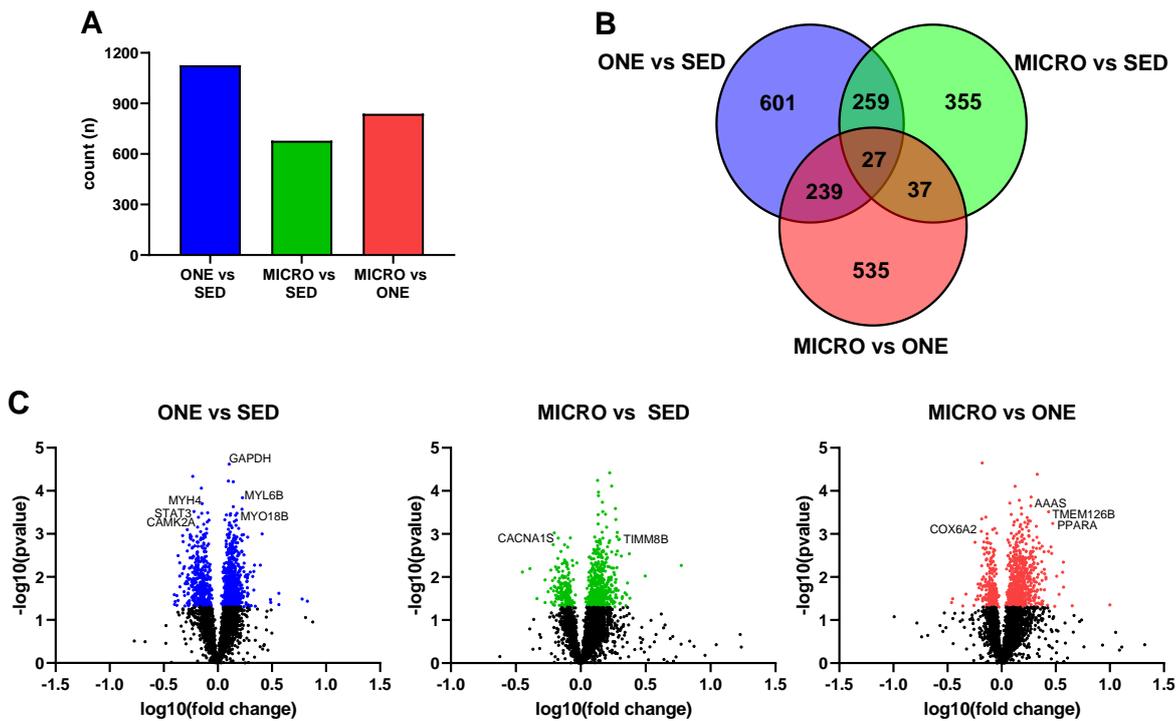
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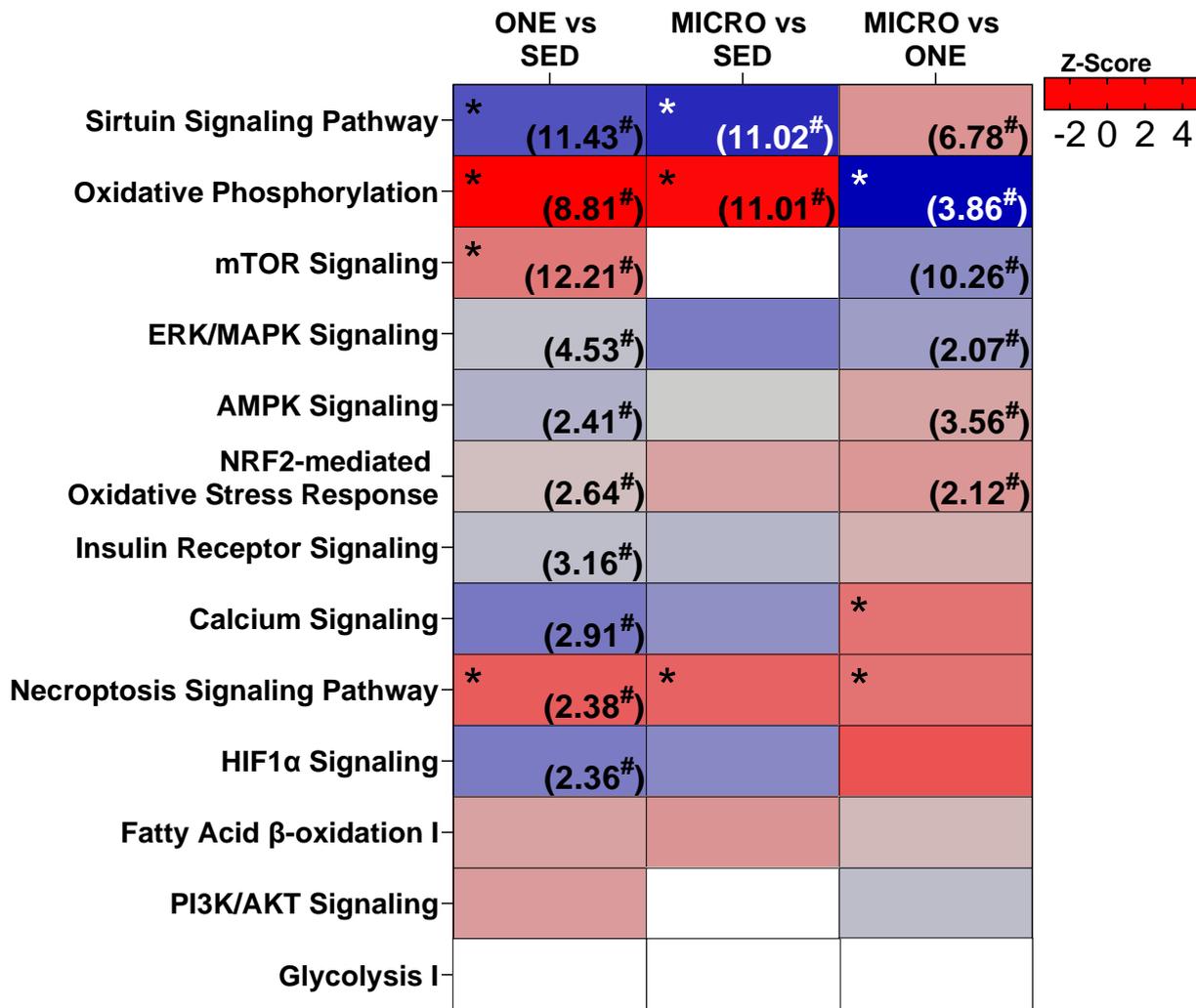
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702 **Figure 2: Comparison of muscle gene expression signatures in fasting skeletal muscle biopsies.** (A) counts of differentially
703 expressed genes by comparison, (B) Venn diagrams displaying the counts of differentially expressed genes for each
704 comparison, (C) Volcano plots of differentially expressed genes. Genes related to metabolic outcomes selected from top 30
705 indicated on plot. Skeletal muscle biopsies were collected in the fasting state from adults with overweight-to-obesity adults (n
706 = 8, 4m/4f) after completing three 4-day study interventions: SED: sedentary control, ONE: single-continuous bout of 45-
707 min/day of moderate-intensity physical activity, MICRO: hourly 5-min bouts of moderate intensity physical activity
708 performed for 9 consecutive hours per day.

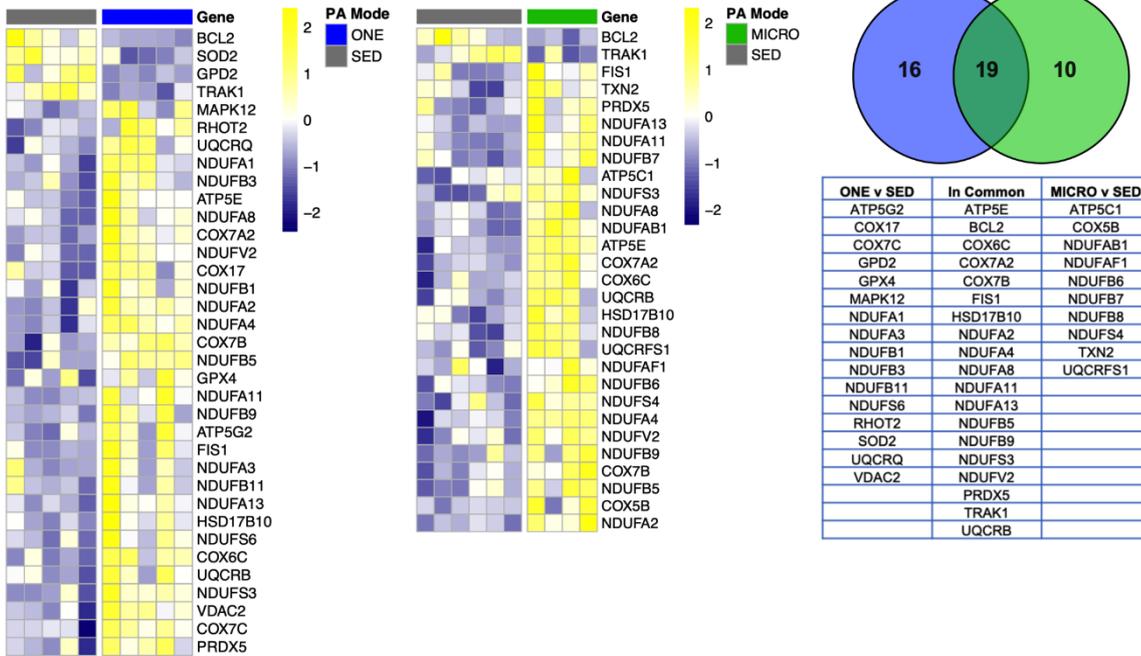
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739 **Figure 3: Comparison of metabolic and exercise-responsive pathways.** Pathway enrichment was calculated $-\log_{10}(\text{FDR})$ for each
740 comparison (value indicated in parentheses). For pathway ranking, total FDR score was calculated as the sum across the row
741 for each FDR score. Pathway activation state colored by z-score (white = N/A, red = activated, blue = inhibited). A portion ($n=8$
742 – 4m/4f) of the skeletal muscle samples were analyzed by RNA sequencing to identify differentially expressed genes and
743 pathway analysis was performed with Ingenuity Pathway Analysis (IPA, Qiagen Inc.). Skeletal muscle biopsies were collected
744 in the fasting state from adults with overweight-to-obesity adults ($n = 8, 4m/4f$) after completing three 4-day study
745 interventions: SED: sedentary control, ONE: single-continuous bout of 45-min/day of moderate-intensity physical activity,
746 MICRO: hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day. * Significant z-
747 score: $P < 0.05$. # Significant pathway enrichment: $P < 0.05$.
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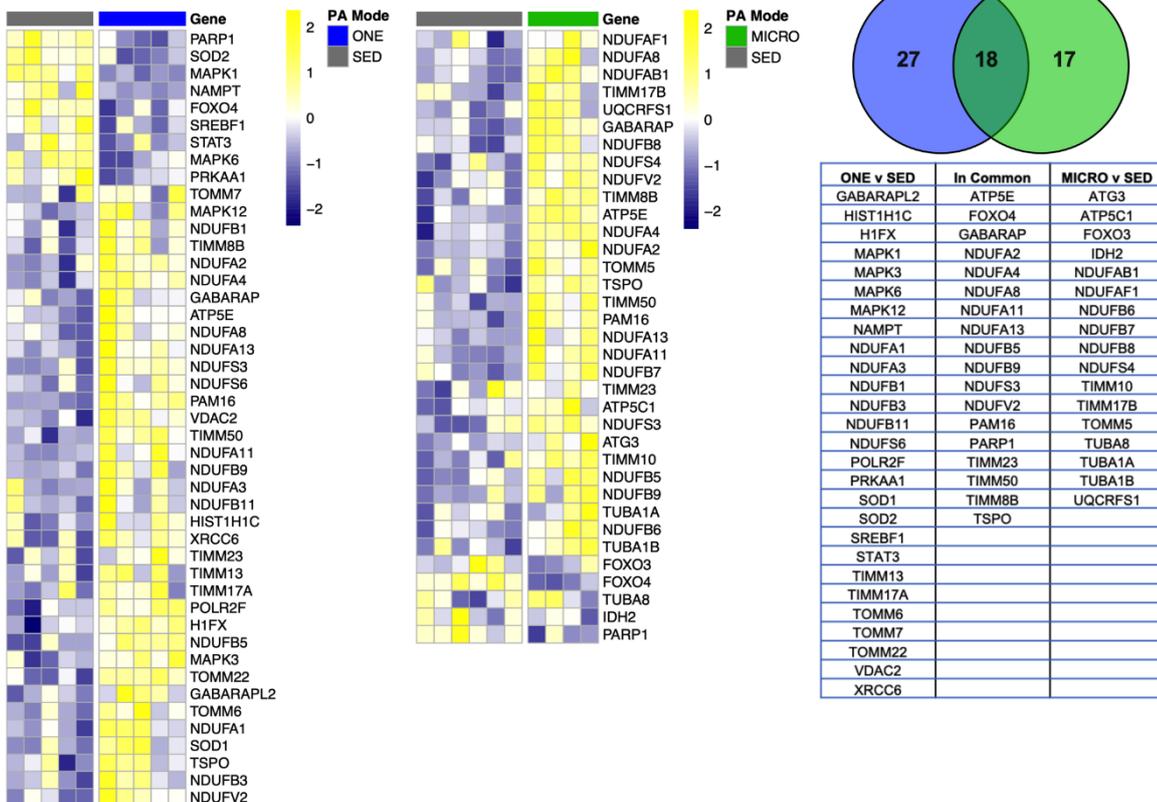
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Mitochondrial Genes by Physical Activity Mode



B

Sirtuin Signaling Genes by Physical Activity Mode



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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

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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.