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## **A nutrient cocktail prevents lipid metabolism alterations induced by 20 days of daily steps reduction and fructose overfeeding: Result from a randomized study**

Anthony Damiot, Rémi Demangel, John Noone, Isabelle Chery, Alexandre Zahariev, Sylvain Normand, Thomas Brioché, François Crampes, Isabelle de Glisezinski, Etienne Lefai, et al.

### ► To cite this version:

Anthony Damiot, Rémi Demangel, John Noone, Isabelle Chery, Alexandre Zahariev, et al.. A nutrient cocktail prevents lipid metabolism alterations induced by 20 days of daily steps reduction and fructose overfeeding: Result from a randomized study. *Journal of Applied Physiology*, 2019, 126 (1), pp.88-101. 10.1152/jappphysiol.00018.2018 . hal-01918097

**HAL Id: hal-01918097**

**<https://hal.science/hal-01918097>**

Submitted on 26 May 2020

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1 **A nutrient cocktail prevents lipid metabolism alterations induced by 20 days of daily steps**  
2 **reduction and fructose overfeeding: Result from a randomized study**

3  
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36

37 **Running Head:** Nutrient cocktail protects against physical inactivity

38

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47

48

49 **ABSTRACT**

50 **Context:** Physical inactivity and sedentary behaviours are independent risks factors for numerous  
51 diseases.

52 **Objective:** We examined the capacity of a nutrient cocktail composed of polyphenols, omega-3 fatty  
53 acids, vitamin E and selenium to prevent the expected metabolic alterations induced physical activity  
54 and sedentary behaviors.

55 **Design/Setting/Participants:** Twenty healthy trained men (averaging~14,000 steps/d and engaged  
56 in sports) were randomly divided into a control group (no supplementation) and a cocktail group for  
57 a 20-day free-living intervention during which they stopped exercise and decreased their daily steps  
58 to averaging~3000. During the last 10 days metabolic changes were further triggered by fructose  
59 overfeeding. On days 0, 10 and 20 body composition (DXA), blood chemistry, glucose tolerance  
60 (OGTT) and substrate oxidation (indirect calorimetry) were measured. Glucose tolerance included  
61 1% fructose labelled with (U-<sup>13</sup>C) fructose to assess liver *de novo* lipogenesis. Histological changes  
62 and related cellular markers were assessed from muscle biopsies collected on days 0 and 20.

63 **Results:** While the cocktail did not prevent the decrease in insulin sensitivity and its muscular  
64 correlates induced by the intervention, it fully prevented the hypertriglyceridemia, the drop in fasting  
65 HDL and total fat oxidation, and the increase in *de novo* lipogenesis. The cocktail further prevented  
66 the decrease in type-IIa muscle fiber cross-sectional area and was associated with lower protein  
67 ubiquitination content. The circulating anti-oxidant capacity was improved by the cocktail following  
68 the OGTT.

69 **Conclusion:** A cocktail of nutrient compounds from dietary origin protects against the alterations in  
70 lipid metabolism induced by physical inactivity and fructose overfeeding.

71

72 **New & Noteworthy:**

73 This is the first study to test the efficacy of a novel dietary nutrient cocktail on the metabolic and  
74 physiological changes occurring during 20 days of physical inactivity with fructose overfeeding.

75 The main findings of this study are that (i) reduction in daily steps lead to decreased insulin  
76 sensitivity and total fat oxidation, resulting in hyperlipemia and increased de novo lipogenesis; and  
77 (ii) a cocktail supplement prevents the alterations on lipid metabolism.

78

79 **Keywords:** nutrition; physical inactivity; sedentary behaviors; countermeasure; polyphenols; omega-  
80 3 fatty acids; selenium; vitamins; OGTT

81

82 **Abbreviations:**

83 Akt: Protein kinase B

84 AUC: Area under the curve

85 BSA: Bovine serum albumine

86 CSA: Cross sectional area

87 CPT1: Carnitine palmitoyl transferase 1

88 DHA: Docosahexaenoic acid

89 DRI: Dietary references intake

90 DXA: Dual energy X-ray

91 EPA: Eicosapentaenoic acid

92 FABPpm: Fatty acid binding protein plasma membrane

93 FAT/CD 36: Fatty acid transport CD36

94 FATP1: Fatty acid transport protein 1

95 FM: Fat mass

96 GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

- 97 GSH: Glutathione
- 98 GSK3: Glycogen synthase kinase 3
- 99 GSSG: Glutathione disulfide
- 100 HDL: High density lipoprotein
- 101 HMW: High molecular weight
- 102 HPLC: High performance liquid chromatography
- 103 IRMS: Isotope ratio mass spectrometry
- 104 LBM: Lean body mass
- 105 LCcoA: Long chain acyl co-A
- 106 LPL: Lipoprotein lipase
- 107 MDA: Malondialdehyde
- 108 MET: Metabolic equivalent
- 109 MPE: Molar percent enrichment
- 110 mtGPAT: mitochondrial glycerol-3phosphate acyltransferase
- 111 NEFA: Non-esterified fatty acids
- 112 NFkB: Nuclear factor kappa beta
- 113 NPRQ: non-protein respiratory quotient
- 114 OGTT: Oral glucose tolerance test
- 115 PBS: Phosphate buffered saline
- 116 PGC1 $\alpha$ : Peroxisome proliferator-activated receptor gamma co-activator 1 alpha
- 117 ROS: Reactive oxygen species
- 118 TG: Triglycerides
- 119 UL: Upper limit
- 120 VL: Vastus lateralis
- 121 VLDL: Very low-density lipoprotein



## 123 INTRODUCTION

124

125 Over the past decade, physical inactivity has emerged as an important risk factor for a number of  
126 chronic metabolic and cardiovascular diseases. In addition, sedentary behaviors have been associated  
127 with adverse health effects (35) even in subjects who meet the levels of current recommendations on  
128 physical exercise (14). This *sedentary death syndrome* (41) is responsible for an equivalent number  
129 of deaths to smoking (19) and has prompted numerous research studies to understand the role of  
130 physical inactivity and sedentary behaviors in diseases aetiology and develop efficient preventive  
131 strategies.

132

133 Research on the physiology of physical inactivity in humans has been pioneered by researchers  
134 investigating physiological adaptations of astronauts to space environment. To do so, they used a  
135 ground-based model analogue to microgravity, the bed-rest model (28). Indeed, the hypokinesia and  
136 hypodynamia induced during prolonged bed-rest are largely responsible for the described adaptations  
137 to space. Over the past 60 years, bed-rest studies ranging from as little as 3 days to 120 days have  
138 demonstrated that physical inactivity leads to muscle atrophy and a shift from slow oxidative fibers  
139 towards fast glycolytic fibers (59). These structural adaptations are strongly associated with the  
140 development of metabolic inflexibility, low-grade inflammation and oxidative stress (56, 66, 70).  
141 There is an increase in fasting and post-prandial glucose oxidation concomitant to an increased  
142 spillover of dietary lipids along with a reduced capacity to burn fat (see (9) for review). The resultant  
143 hypertriglyceridemia leads to ectopic fat storage in the liver, muscle and bone marrow (81) and  
144 contributes to the development of insulin resistance at the muscle level (9). The role of physical  
145 inactivity and sedentary behaviors in the onset and progression of metabolic diseases have attracted  
146 an increasing attention from the scientific biomedical community over the past decade. Some  
147 investigators have proposed to reduce the number of daily steps of physically active individuals to



148 study the direct metabolic effects of physical inactivity (38, 39, 62). While these studies confirmed  
149 the findings observed during the bed rest studies at levels of physical inactivity closer to what is  
150 observed in the general population, they did not examine the underlying mechanisms. The  
151 physiology of physical inactivity therefore needs to be further delineated in order to develop  
152 strategies to prevent its deleterious effects.

153

154 Recent studies conducted to develop preventive strategies suggest that bioactive nutrients such as  
155 polyphenols, vitamins and essential fatty acids may mitigate some metabolic features of physical  
156 inactivity. However so far, most studies have been tested in rodent analogs of microgravity to induce  
157 muscle disuse atrophy and metabolic disorders. A large body of data exists in human, rodents and  
158 primates demonstrating the effect of polyphenols such as quercetin, resveratrol, cinnamon, grape or  
159 green tea extracts on insulin sensitivity, lipid metabolism, inflammation and oxidative stress (see (2,  
160 24, 33, 44, 55, 77, 78, 85) for recent reviews). In addition, polyphenols supplemented as pure  
161 molecules such as 8-phenylnaringenin (flavanones, (53)), quercetin, (flavonols, (54)), resveratrol  
162 (oligostilbens, (51)), epigallocatechin 3 (catechins, (46)) or as extracts from dietary sources (green  
163 tea, apple extracts or grape seed extracts) prevent several aspects of rodent muscle atrophy and  
164 metabolic disorders induced by disuse while some promote a fatigue resistant muscle fiber  
165 phenotype (3, 40, 46, 50, 63).

166

167 Vitamin E is also known to have anti-oxidant capabilities, by acting as a reactive oxygen species  
168 (ROS) scavenger, and anti-inflammatory properties through inhibition of the NF-kB pathway which  
169 is well described in disuse atrophy models to activate proteolytic pathways (32, 37). Vitamin E  
170 supplementation mitigates disuse atrophy in rats (71), reduces adipose tissue fibrosis, inflammation,  
171 oxidative stress and blood lipids in obese humans (1). Vitamin E intake is also related to muscle  
172 strength in the elderly (12, 16). Selenium is often co-supplemented with vitamin E as it scavenges

173 ROS and boosts the intracellular effects of vitamin E. The combination of these two micronutrients  
174 was also shown to be beneficial on specific dystrophy (65). Furthermore, selenium has independent  
175 effects on insulin sensitivity and reduces insulin secretion (79). Although these effects are still  
176 debated there are clear anti-oxidant and anti-inflammatory effects of selenium (22), but not on lipid  
177 metabolism (79).

178

179 The role of omega 3 fatty acids ( $\omega$ -3) has been studied for decades. Studies in healthy and unhealthy  
180 humans and animals showed that they improve hepatic insulin sensitivity, lower very low density  
181 lipoproteins (VLDL) production and *de novo* lipogenesis by the liver, and reduce inflammation and  
182 oxidative stress (15, 29, 60). All of these are metabolic features observed in the inactive/sedentary  
183 states (9). These observations were also reported in presence of fructose overfeeding (21).  
184 Surprisingly, besides the protective effects of  $\omega$ -3 supplementation during bed-rest and spaceflight  
185 on bone markers (88), no studies have investigated their impact on muscle function during disuse.  
186 However, some studies suggest they could have beneficial effects to prevent against muscle atrophy.  
187 In rodent cancer-induced cachexia,  $\omega$ -3 fatty acids supplementation prevented muscle atrophy (74)  
188 and in the elderly, they increased activation of the protein synthesis mTOR pathway in response to  
189 insulin stimulation in the elderly (72, 73).

190

191 While the available literature suggests that some bio-active molecules, taken individually or as food  
192 extracts, improve several aspects of muscle and whole-body metabolic control, it is unlikely that any  
193 single micronutrient will be sufficiently powerful to reverse the wide range of deleterious effects  
194 induced by physical inactivity. Recently the notion of nutrient cocktails, to trigger additive and/or  
195 synergistic effects between bio-active compounds, has been proposed (6). Several studies have  
196 shown that co-supplementing with  $\omega$ -3 & green tea extracts (49),  $\omega$ -3 & vitamin E (20, 83), vitamin  
197 E & selenium (25), fish oil & selenium (84), epigallocatechin & resveratrol (52) improve, to some

198 extent, insulin sensitivity, body composition, fat oxidation, inflammation and oxidative stress in  
199 aging and diseases associated with muscle atrophy including obesity and type 2 diabetes.

200

201 While these cocktails have the potential to reduce the risk of metabolic disease there are no studies in  
202 the literature examining their impact on physical inactivity-induced metabolic alterations in humans.

203 The purpose of this study was to determine the preventive effect of a nutrient cocktail composed of  
204 polyphenols,  $\omega$ -3, vitamin E and selenium on the metabolic and physiological changes that occur  
205 during 20 days of reduced daily steps and exercise in trained men. To further trigger metabolic  
206 deterioration, dietary fructose supplementation was provided during the last 10 days of the trial. We  
207 hypothesized that the cocktail supplementation would reduce and/or prevent the deterioration in  
208 glucose and lipid metabolism, insulin sensitivity and muscle atrophy that are associated with reduced  
209 physical activity and fructose overfeeding.

210

## 211 **METHODS**

212

### 213 **Subjects**

214 Twenty healthy trained young men were recruited from the local community. Characteristics are  
215 presented in **Table 1**. Habitual physical activity was assessed using hip-worn triaxial accelerometry  
216 (Actigraph GT3x+<sup>TM</sup>, Actigraph USA) for 7 days. Subjects were included if they walk >10,000  
217 steps/day, participated in at least two leisure sport sessions per week and were free of any known  
218 diseases. Subjects were excluded if not able to drastically reduce professional, leisure and transport-  
219 related physical activities. This study was approved by the local ethical committee and all subjects  
220 provided written informed consent (NCT03313869, ID-RCB number: 2015-A00665-14).

221

### 222 **Experimental design**

223 The experiment outflow is presented in **Figure 1**. Participants were randomly assigned to control  
224 (n=10) or daily nutrient cocktail supplemented (n=10) groups. Random tables were generated by the  
225 study statistician. The participants, nurses and physicians were not blinded as no placebo pills were  
226 given to the control group; however, the statistician was blinded. During the 20-day study in free-  
227 living conditions, volunteers from both groups were asked to stop exercising and drastically reduce  
228 their daily physical activity. Subjects were instructed to walk a maximum of 2,500-4,000 steps/day.  
229 This was controlled on a daily basis by the subject and the investigators through a wrist-worn Fitbit  
230 pedometer (Fitbit, USA). Accurate data were assessed using an Actigraph GT3x+™ hip-worn  
231 throughout the twenty days of the experiment. Metabolic stress induced by physical inactivity was  
232 boosted during the last ten days of the protocol by ingesting 3g/kg/d of fructose (Vivis® fructose)  
233 mixed with 0.5g/kg/d glucose in water to alleviate intestinal problems (61, 80), on top of their  
234 regular diet. Fasting blood collection, glucose tolerance, substrate oxidation and *de novo* lipogenesis  
235 were measured at baseline and after 10 and 20 days of reduced activity. Muscle biopsies and body  
236 composition measurement were completed at baseline and after 20 days (**Figure 1**). Diet was not  
237 controlled during the intervention; subjects were instructed to eat according to their appetite.

238

### 239 **Cocktail composition and doses**

240 The supplemented group received a polyphenol nutrient cocktail derived from food sources that  
241 consist of Liliaceae, Vernenaceae, Lamiaceae, Vitaceae, Rubiaceae, Theaceae and Rutaceae Genres  
242 consisting of *Allium cepa*, *Lippia citriodora*, *Ajuga reptans*, *Vitis vinifera*, *Coffea robusta*, *Camellia*  
243 *sinensis*, and *Citrus aurantium*. The cocktail is referred to as XXS-2A and was designed by Spiral  
244 Company (Dijon, France). The daily dose was achieved by the ingestion of 3 pills (one at breakfast,  
245 lunch and dinner) to reach a total dose of 529.5 mg/d of polyphenols that was composed of 120 mg/d  
246 flavonols (including quercetin 50 mg), 75 mg/d oligostilbens (including resveratrol 20 mg), 91.5  
247 mg/d hydroxycinnamic acids (including chlorogenic acid 40 mg), 135 mg/d flavanols (including

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248 epigallocatechin gallate 60 mg) and 108 mg/d flavanones (including naringin 30 mg). As there is no  
249 Dietary References Intake (DRI) available for polyphenols, the ~500 mg/d dose was based on several  
250 reviews on the bioavailability and bioefficacy of polyphenols in humans and others studies that  
251 tested the effects of polyphenols on exercise performance and oxidative stress (43, 57, 75). The 3 g  
252 daily dose of  $\omega$ -3 (Omacor, Pierre Fabre Laboratories, Toulouse France) was based on French  
253 pharmacopeia recommendations for hypolipemic effects (2-4 g/day) and was provided as 1 pill per  
254 meal which is within the daily dose used in most clinical studies (29, 60). This daily dose thus  
255 corresponded to 1.1 g of eicosapentaenoic acid (EPA) and 1 g of docosahexaenoic acid (DHA).  
256 Vitamin E and selenium were given as a single daily pill providing 168 mg of vitamin E associated  
257 with 80  $\mu$ g of selenium (Solgar, Marne la Vallée, France). The DRI for Vitamin E is set at 15 mg/d.  
258 The tolerable upper limit (UL) for intake is set at 1 g in adults, therefore the dose from the  
259 commercially available pill was 6 times lower than the UL but 11 times higher than DRI. To  
260 capitalize on the cocktail effect, the dose of vitamin E was two-fold lower the doses that have  
261 provided positive effects on metabolism and muscle (25, 65). Regarding selenium, the intake  
262 reported in most countries presents a large variability. DRI and UL are respectively set at 55  $\mu$ g and  
263 400  $\mu$ g per day. The selected dose provided a daily supplement that set the daily intake at 135  $\mu$ g/d  
264 which is 3 times lower than the UL and lower than the dose reported in previous studies (25, 65) in  
265 order to capitalise on cocktail effects. The control group did not receive any supplementation or  
266 placebo.

267

## 268 **Body composition**

269 Fat mass (FM) and lean body mass (LBM) were assessed by using a dual-energy X-ray  
270 absorptiometer (DXA, HOLOGIC QDR 4500W, USA) at baseline and on day 20 of the experiment.

271

## 272 **Physical Activity Patterns**

273

274 Time spent in sitting and active was determined using a tri-axial accelerometer (ActiGraph GT3X+;  
275 ActiGraph, Pensacola, Fla., USA). Participants were instructed to wear the accelerometer at their  
276 right hip at all times except for bathing during one week before the inclusion and during the all  
277 experimental protocol. At each visit, both raw accelerometry and activity-counts per min were  
278 downloaded using manufacturer software (Actilife 6.13, Pensacola, USA). An automatic activity-  
279 recognition algorithm (7), that identifies sitting time, and an activity-specific energy expenditure  
280 model (23), both developed by our group, were used to determine time spent in a sitting position and  
281 in different activity intensities; cut-points of 1.5-3 METs and >3METs were used for light intensity  
282 activity and moderate-to-very vigorous activity, respectively.

283

#### 284 **Glucose tolerance and substrate use**

285 Subjects reported to the clinic the evening prior to the test. A standard dinner was given containing  
286 47.7% carbohydrates, 32.5% lipids and 17.8% protein for a total of 918 Kcal intake. An oral glucose  
287 tolerance test (OGTT) was performed after an overnight fast using a mix of 1g/kg of glucose and  
288 0.5g/kg of fructose diluted in 300mL of water; 1% of fructose was labelled with U-<sup>13</sup>C-fructose  
289 (Eurisotop, Paris). Following baseline collection and glucose/fructose ingestion, blood samples were  
290 collected every 15-min for the first 3 hours and every 30-min for the 4<sup>th</sup> hour. Carbohydrate and fat  
291 oxidation rates were determined every hour using canopy dilution respirometry (Quark, Cosmed,  
292 Italy) and the classical equations of indirect calorimetry corrected for urinary nitrogen excretion.  
293 Insulin sensitivity was estimated using the Matsuda Index (45). Metabolic flexibility, defined as the  
294 ability to adjust nutrient oxidation to nutrient availability and demand, was assessed during the  
295 OGTT by examining the relationship between the variance of plasma insulin and the variance of  
296 non-protein respiratory quotient (NPRQ). As explained previously (10) the variance-derived indexes  
297 assume a metabolically flexible state when the variance in insulin is low and the variance in NPRQ is

298 high; in other words, when the body has a high capacity to switch from fat to carbohydrate oxidation  
299 in association with small changes in insulin concentration in response to the OGTT. A transition  
300 towards a metabolically inflexible state is assumed when the variance in insulin increases and/or the  
301 variance in NPRQ decreases.

302

### 303 **Exogenous fructose oxidation**

304 Exhaled breath samples were collected at the same time as blood samples. Breath  $^{13}\text{C}/^{12}\text{C}$  isotopic  
305 ratio was measured in triplicate on a GasBench system (Thermo Scientific, Germany) connected to a  
306 continuous-flow isotope ratio mass spectrometer (IRMS, DELTA V, Thermo Scientific, Germany).  
307  $\text{U-}^{13}\text{C}$ -fructose oxidation was calculated as the cumulated percentage dose recovery of  $^{13}\text{C}$  in expired  
308  $\text{CO}_2$  per hour over the OGTT, as previously described (42).

309

### 310 **Liver *de novo* lipogenesis**

311 *De novo* lipogenesis was measured as the apparition of  $1\text{-}^{13}\text{C}$ -palmitate from  $\text{U-}^{13}\text{C}$ -fructose in very  
312 low-density lipoprotein (VLDL). In brief, as previously described (42) VLDL were separated by  
313 sequential ultracentrifugation and total lipids were extracted from VLDL by a modified Folch  
314 technique. TGs were further separated by solid phase extraction and derivatized into methyl esters.  
315 The absolute concentration of both unlabelled and labelled palmitate was measured by gas  
316 chromatography/mass spectrometry (Agilent 5975, Inert XL) through a dual acquisition program in  
317 single ion monitoring  $m/z$  ratios of 270 and 271, and calculated by reference to internal standards  
318 added to the plasma. The concentration of  $1\text{-}^{13}\text{C}$ -palmitate was calculated by multiplying its molar  
319 percent enrichment (MPE) by the concentration of total palmitate.

320

### 321 **Blood sample analyses**



322 Plasma insulin was assessed by radio-immuno assay, and glucose, NEFA and TG were measured by  
323 colorimetric assays and enzymatic methods, as previously described (42). Reduced and oxidized  
324 glutathione (GSH and GSSG, respectively) concentrations were simultaneously measured by  
325 reverse-phase high performance liquid chromatography (HPLC) as previously described (87). Total  
326 plasma malondialdehyde (MDA) was determined by reverse-phase HPLC (82). The antioxidant  
327 defenses were examined using a test based on *in vitro* free-radical-induced blood hemolysis KRL test  
328 (Kirial International/Spiral, Couternon, France) as previously described (68).

329

### 330 **Muscle biopsies and parameters**

331 Muscle biopsies were performed on the mid *Vastus Lateralis* (VL) under aseptic conditions and after  
332 anaesthesia (2% w/v lidocaine HCl) using Bergström skeletal muscle biopsy needle (Lauran  
333 Médical®, KBM 5/10). One piece was mounted in tragacanthum gum (OCT Compound) for  
334 histological analysis, cooled to the temperature of liquid nitrogen in isopentane, and the remainder  
335 was snap frozen in liquid nitrogen and stored at -80°C until further analysis.

336

### 337 ***Immunohistochemical classification of muscle fibers and cross-sectional area measurements:***

338 Transverse serial cross sections (10 µm thick) of vastus lateralis muscle samples were obtained using  
339 a cryostat at -25°C (HM-560, Microm H), and mounted onto glass microscope slides. Before  
340 labeling, sections were dried and fixed for 10 min in acetone. Sections were then washed in  
341 phosphate buffered saline (PBS), blocked and permeabilized with 0.1% Triton-X100 and 20% horse  
342 serum. For muscle fiber typing and cross sectional areas (CSA) determination, sections were  
343 incubated with anti-MyHC primary antibodies (anti-slow (I) MyHC, BA-D5, Developmental Studies  
344 Hybridoma Bank, 1:10; anti-fast (II) MyHC, M4276, Sigma-Aldrich, 1:200), and anti-fast (IIA)  
345 MyHC, SC-71, Developmental Studies Hybridoma Bank, 1:10) for 1 h at 37°C, followed by washes  
346 in PBS and incubation with the secondary antibodies (ALEXA 488, A11029, Invitrogen, 1:800;



347 ALEXA 568, A11031, Invitrogen, 1:800) for 1 h. Fiber typing was manually determined, and the  
348 fiber sizes were analyzed with Image J software (1.46r version). A total of approximately 300  
349 myofibers per sample were thus measured in double-immunostained pre- and post-DI VL  
350 cryosections.

351

352 **Protein expression:** Muscle samples were homogenized in 10 volumes of lysis buffer (50 mM Tris–  
353 HCl [pH 7.5], 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 100 mM NaF, 5 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton  
354 X-100, 1% SDS, 40 mM β-glycerophosphate and protease inhibitor mixture [P8340; Sigma-  
355 Aldrich]) and centrifuged at 10,000 g for 10 min (4°C). Sixty micrograms of protein extract were  
356 loaded into Stain-Free 4-20% precast gels (4568095; Bio-Rad) before electrophoretic transfer onto  
357 nitrocellulose membranes (Bio-Rad; Trans-Blot Turbo Blotting System). After transfer, the  
358 membranes were blocked with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 (TBS-  
359 T) containing 5% skimmed milk or bovine serum albumin (BSA) and incubated overnight at 4°C  
360 with primary antibodies. The membranes were then incubated for 1 h with a peroxidase-conjugated  
361 secondary antibody. The immunoblots were revealed using a Pierce ECL kit (32106; Thermo  
362 Scientific), and proteins were visualized by enhanced chemiluminescence using the ChemiDoc  
363 Touch Imaging System and quantified with Image Lab™ Touch Software (version 5.2.1). Stain-Free  
364 technology or red Ponceau or GAPDH were used as loading control.

365

366 **Citrate Synthase Activity:** Citrate synthase activity of muscle samples was measured using a  
367 commercially available kit, as reported before (36).

368

### 369 **Data and statistical analysis**

370 The effects of the intervention and the cocktail supplementation on the outcomes were assessed by  
371 using mixed linear models with group, intervention, group-by-intervention interaction and baseline

372 values as fixed effects and subjects as random effect. Additional adjustment for changes in FM and  
373 LBM was performed for substrate oxidation. Between-group and within-group differences were  
374 assessed by using post-hoc tests. Data obtained during OGTT are presented as area under the curve  
375 (AUC). Significance was set up at 0.05 for main effects and 0.10 for interaction effects. Values are  
376 mean  $\pm$  SEM, unless otherwise stated. Statistical analysis were performed with SAS version 9.4  
377 (SAS Institute, Cary, USA).

378

## 379 **RESULTS**

380

### 381 **Subjects' characteristics and changes in time spent physically active and sitting**

382 Subjects' characteristics and changes in activities are presented in **Table 1**. Participants' compliance  
383 was very good; over the 20-day intervention daily steps went down from  $14,952 \pm 1,720$  steps to  
384  $3,009 \pm 298$  steps in the supplemented group and from  $13,032 \pm 875$  steps to  $2,645 \pm 331$  steps in the  
385 control group. We observed in both groups a reduction of both light-intensity activity and moderate-  
386 to-very vigorous intensity activity, along with a significant increase in time spent sitting during  
387 waking hours. Although the intervention did not modify body and lean mass, we observed a  
388 significant increase in FM, of less than 1kg, independent of the cocktail supplementation.

389

### 390 **Glucose tolerance and insulin sensitivity**

391 Fasting plasma glucose and insulin were neither affected by the intervention nor by the cocktail  
392 supplementation (**Table 2**). Concentrations of glucose, TG and insulin during the OGTT are  
393 presented in **Figures 2A, 2B, 2C** respectively. To increase readability of the results, data are also  
394 presented as AUC in **Figure 3**. During the OGTT, glucose concentration did not change between  
395 control and supplemented groups (**Figure 3B**); insulin increased after 10 days of reduction in daily  
396 steps (**Figure 3A**) but did not further change after 10 more days of inactivity combined with fructose

397 overfeeding. Reduction in daily steps decreased insulin sensitivity in both groups as indicated by the  
398 decrease in Matsuda index (**Figure 3C**). Fasting high molecular weight (HMW) adiponectin  
399 increased during the intervention in both groups, but the increase was greater in the supplemented  
400 group compared to the control group after 20 days of intervention (**Table 2**). Total carbohydrate  
401 oxidation (**Figure 3D**) and exogenous fructose oxidation (**Figure 3E**) during the OGTT increased  
402 similarly in both groups after both 10 and 20 days of intervention. While a group-by-intervention  
403 interaction almost reached significance for an increase in insulin variance, significant decrease in  
404 NPRQ variance was observed during the OGTT in the control group after both 10 and 20 days of  
405 intervention, that was fully prevented by the supplementation (**Figure 3F**). This indicates a  
406 development of metabolic inflexibility in the control group but not in the supplemented group, that  
407 was likely due to decreased oxidative capacities in association with whole body metabolic  
408 adaptations. Of note, most metabolic changes were observed after 10 days of inactivity only; the  
409 addition of 10 more days of reduced daily steps combined with fructose overfeeding did not lead to  
410 further modifications in metabolic flexibility.

## 412 **Lipid metabolism**

413 Fasting TG and HDL respectively increased and decreased in the control group after both 10 and 20  
414 days of intervention (**Table 2**). Similar responses were observed during the OGTT. TG (**Figure 4A**),  
415 VLDL-TG (**Figure 4B**), palmitate VLDL-TG (**Figure 4C**) and *de novo* lipogenesis from fructose  
416 (**Figure 4D**) gradually and significantly increased after 10 and 20 days of intervention in the control  
417 group. The intervention decreased total lipid oxidation in association with an increase in net lipid  
418 synthesis both in the fasting states and following the OGTT (**Table 4 & Figure 4E**), as indicated by  
419 the negative values of lipid oxidation, thus confirming the increase in *de novo* lipogenesis as  
420 measured by stable isotopes. The cocktail supplementation fully prevented the increase in lipemia  
421 (**Table 2**) in both fasting state and during the OGTT (**Figure 4A**), the decrease in total lipid

422 oxidation (**Table 2 and Figure 4E**) and the increase in *de novo* lipogenesis measured both by tracer  
423 technique (**Figure 4D**) and indirect calorimetry (**Table 2**) induced by the daily step reduction with or  
424 without fructose overfeeding.

425

### 426 **Anti-oxidant capacity**

427 Fasting oxidative markers remained unaffected by either the intervention or the supplementation  
428 (**Table 3**). However, blood anti-oxidant capacity following the OGTT decreased in the control group  
429 when the fructose overfeeding was added to the daily step reduction while the supplemented group  
430 remained at significantly higher values of whole blood anti-oxidant capacity (**Figure 3F**).

431

### 432 **Skeletal muscle analysis**

433 Muscle parameters are presented in **Table 5**. While type I fibers CSA was lower in both groups at  
434 the end of the intervention, the decrease in type IIa CSA observed in the control group, was  
435 prevented by the cocktail supplementation. In line with this observation, ubiquitination content, a  
436 protein marker of proteolysis processes, increased only in the control group. At the mitochondrial  
437 level both Mitofusin-2 and PGC1 $\alpha$  contents dropped in response to the intervention. We failed to see  
438 a protective effect of the cocktail. FATP1, involved in fatty acid transportation at the mitochondrial  
439 level, tended to rise in the cocktail group only ( $p=0.07$ ), supporting the maintenance of lipid  
440 oxidation during the intervention in the supplemented but not in the control group. No changes in  
441 citrate synthase activity, skeletal muscle intracellular signaling protein contents and oxidative stress  
442 markers were observed.

443

### 444 **DISCUSSION**

445

446 The main findings of this randomized interventional study in lean healthy trained young male are  
447 that (i) a reduction in daily light intensity activity and moderate-to-vigorous activity along with an  
448 increase in time spent sedentary leads to decreased insulin sensitivity, total fat oxidation,  
449 hyperlipemia and increased de novo lipogenesis; and (ii) a nutrient cocktail supplement prevents the  
450 alterations on lipid metabolism but not insulin sensitivity. This is the first study to test the efficacy of  
451 a nutrient cocktail to counteract the negative effects of physical inactivity along with fructose  
452 overfeeding.

453  
454 During the intervention, subjects decreased on average their activity to <3,000 steps per day. Body  
455 weight and LBM remained stable during the study but FM and the percentage of body fat increased.  
456 The effect on body weight is not surprising. It is likely to be influenced by 1) the known delayed  
457 compensatory adjustment in energy intake, 2) the duration of the intervention, given some (27, 38),  
458 but not all studies (5, 26) with 7-14 days decreased activity reported weight gain, and 3) the fructose  
459 overfeeding between 10-20 days, as others have also reported an increase in FM with overfeeding  
460 (38). The increase in FM is thus in line with the design of the study. Overall, LBM was preserved  
461 during the study though there was a decrease in type II muscle fibers CSA in the control group,  
462 suggesting that the cocktail supplementation may delay muscle disuse atrophy.

463  
464 There was a decrease in insulin sensitivity during the 20-day intervention, in agreement with other  
465 studies when activity was reduced for 3-14 days (5, 27, 31, 38, 48, 67). This was mainly due to  
466 increased insulin AUC during the OGTT while we did not show a change in glucose, in agreement  
467 with some (2,9) but not all studies (5, 31). The additional fructose provided after day 10 did not  
468 affect insulin sensitivity but there was a significant increase in carbohydrate oxidation and  
469 exogenous fructose oxidation. Knudsen et al. (38) reported similar glucose and insulin responses to  
470 an OGTT following 14 days of reduced activity and a 50% increase in energy intake. While the

471 Matsuda index, in that study, was significantly lower at day 7 but not day 14, clamp-derived insulin  
472 sensitivity had decreased by ~44%. In agreement with other studies of decreased activity (38) or bed-  
473 rest (34), we reported an increase in fasting plasma adiponectin after 20 days of decreased activity.  
474 This is in contrast with studies where decreased adiponectin is associated with insulin resistance. It is  
475 possible that, in these healthy individuals, an increase in adiponectin may be a short-term  
476 compensatory mechanism to preserve insulin sensitivity. In support of this we did not find a change  
477 in Akt and GSK3 proteins following the intervention. This would need to be further investigated but  
478 given the preservation of metabolic flexibility, the effects appear to be more pronounced on lipid  
479 metabolism.

480

481 By using bed-rest ranging from 7 to 90 days in men and women we have shown that a hallmark of  
482 physical inactivity physiology is a decrease in exogenous lipid clearance due to both a reduced  
483 uptake and oxidative capacity at the muscle level (see (10) for review). We further showed in the  
484 general population through training/detraining studies that activity energy expenditure is a major  
485 determinant of lipid trafficking (plasma pools, chylomicrons, VLDL) and partitioning through uptake  
486 (LPL, FAT/CD36, FABPpm) between oxidation (LCCoA synthase, CPT1) and storage (mtGPAT)  
487 within the skeletal muscle (8). In the present study, we observed that physical inactivity by voluntary  
488 step reduction and physical inactivity plus fructose overfeeding gradually decreased fasting and  
489 postprandial fat oxidation to an extent where only net lipid synthesis was observed during the OGTT;  
490 similar to our observation during an OGTT following 7-days bed-rest in men and women (11). One  
491 of the striking results of the present study is the capacity of the cocktail to fully prevent the effects on  
492 lipid oxidation, thus likely preventing the hypertriglyceridemia of the control group.

493

494 Although no similar data exist, numerous recent studies investigating the impact of various bio-  
495 active compounds from the diet, taken as cocktails, in various physio-pathological states and models

496 support our results (4, 13, 30, 52). However, mechanistic evidence primarily comes from rodent  
497 models. Vitamin E plus  $\omega$ -3 fatty acids supplementation was hepato-protective in naturally aging  
498 rats, with major anti-oxidant properties, especially in the brain (58). It was argued that vitamin E  
499 reduces the peroxidation of  $\omega$ -3 fatty acids thus allowing synergistic effects. In support of that, fish  
500 oils associated with grape polyphenols in rats fed a high sucrose diet improved numerous blood  
501 biochemical parameters such as HDL and TG, as in our study, by significantly enhancing fatty acid  
502 beta oxidation (47). Interestingly, the same study showed a major suppression of lipogenic enzymes  
503 by their cocktail, in agreement with our human data. A recent review (69) reported evidence that  
504 polyphenols play key controlling roles in suppressing *de novo* lipogenesis in various models of non-  
505 alcoholic fatty liver diseases. Selenium is often co-supplemented with vitamin E but is likely to have  
506 had limited impact in this study. Supplementation with selenium has independent effects on insulin  
507 sensitivity and insulin secretion but no effect on lipid profiles (79). In this study the main changes  
508 were in lipid metabolism with no changes in glucose/insulin parameters.

509  
510 The beneficial effects of fish oils  $\omega$ -3 fatty acids on lipemia by lowering TG and rising HDL have  
511 been well known for decades (86). In overweight/obese subjects supplemented with  $\omega$ -3 fatty acids  
512 and polyphenols there is a substantial improvement in fasting plasma HDL and their sub-classes (13)  
513 as well as post-prandial TG and triglyceride-rich lipoproteins (4). Annuzzi et al. (4) showed reduced  
514 oxidative damage through a reduced excretion of 8-isoprostane. This data supports the improved  
515 global anti-oxidant capacity we observed in our study during the OGTT. The impact on oxidative  
516 stress could have been augmented by the co-supplementation of vitamin E and selenium, both  
517 recognized as ROS scavengers (32, 37, 65). Of note, the impact of our cocktail on various markers of  
518 anti-oxidant capacity or oxidative markers was quite modest. One may thus argue that, in our study,  
519 the principal effects were due to the  $\omega$ -3 fats, known to boost the whole muscle machinery of lipid  
520 metabolism (18), and to a lesser extent to the polyphenols and vitamin E/selenium. However, a



521 recent study of 12-week supplementation with epigallocatechin-3-gallate and resveratrol showed  
522 major impact on mitochondrial capacity and fat oxidation in overweight/obese men and women (52).  
523 In agreement with our data, they did not observe an improvement in insulin sensitivity. In addition,  
524 Annuzzi et al. (4) found that the polyphenols reduced circulating TG and VLDL while  $\omega$ -3 reduced  
525 postprandial cholesterol and VLDL apolipoprotein B-48. While the cocktail may benefit physically  
526 inactive and unhealthy individuals, it could also optimize the effects of training on lipid metabolism.  
527 In this line, Ota et al. showed supplementation in green tea extract beverage rich in catechins for two  
528 months, concomitant to aerobic exercise training increased fat oxidation rates during exercise (64).  
529 Altogether these data support the positive effect on lipid metabolism associated with the cocktail  
530 supplementation in our study.

531

532 While there is strong evidence which support our results on lipid metabolism following polyphenols  
533 and  $\omega$ -3 supplementation, we also observed interesting effects on the muscle phenotype itself.  
534 Indeed, the muscle is known to be plastic in response to mechanical loading and unloading and it was  
535 expected that reduction in physical activity, by ~10000 steps/d, would have a detraining and  
536 deconditioning effect in healthy trained subjects. There was a global decrease in muscle fiber CSA  
537 with significant reductions in type 1 and type 2 fibers. Such plasticity in response to exercise or  
538 chronic hypoactivity is well known (17). Interestingly, these adverse effects on type 2 muscle fibers  
539 were prevented by the cocktail and may be associated with the decrease in ubiquitination content,  
540 factors known to be associated to muscle deconditioning and fiber atrophy. We have previously  
541 shown that resveratrol prevented disuse muscle atrophy of hind-limb suspended rats (51) and a recent  
542 study reported that  $\omega$ -3 fatty acids delays muscle degradation in mice (76). It is possible that these  
543 components of the nutrient cocktail are responsible for preserving type 2 fiber CSA in this study as  
544 there is no evidence for a role of vitamin E or selenium. However, there are no mechanistic studies  
545 examining the effects of nutrient cocktails and further experiments are required.



546

547 Limitations must be acknowledged. The control group did not take a placebo supplement due to  
548 difficulties to find a neutral oil to encapsulate. This being said the magnitude of the effects we  
549 observed suggest this would have a minor impact on the conclusion of the study. The molecular  
550 parameters we selected did not allow us to fully unravel the mechanisms at play and further  
551 mechanistic studies are required. Finally, we acknowledge that subjects taking >14,000 step/d does  
552 not represent the general population and the interpretation of the results are delimited to young active  
553 men. While recommending physical activity is a central component of health promotion policies, not  
554 enough people achieve the recommendations and therefore nutritional strategies to offset some of the  
555 deleterious effects of physical inactivity are important. Therefore, further studies are needed on less  
556 active populations and in at-risk populations for developing metabolic diseases.

557

558 In conclusion, we have demonstrated that the negative effects of physical inactivity on lipid  
559 metabolism can be mitigated by micronutrient supplementation. We have established that the early  
560 decrements in lipid oxidation contribute to increased *de novo* lipogenesis but that a nutrient cocktail  
561 containing polyphenols, ω-3 fatty acids and other compounds can effectively prevent these changes  
562 during 20-days of decreased physical activity. These data highlight the importance of regular  
563 physical activity to maintain carbohydrate and lipid metabolism. It is possible that this  
564 supplementation could be an effective prevention strategy for chronic diseases such as cardiovascular  
565 disease and type 2 diabetes but long-term studies, especially in high risk groups, are now required.

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815 **Acknowledgements**

816 We are thankful to the participants and the medical, nutrition and administrative staff of the  
817 MEDES – Institut de Médecine et Physiologie Spatiale.

818

819 **Funding:** This work was supported by grants from the CNRS and from the European (ESA)  
820 and French (CNES) Space Agencies. AD and RD are supported by graduate fellowships from  
821 CNES.

822

823 **Clinical trial registration:** NCT03313869

824

825 **Disclosure summary**

826 Michel Prost, director of Spiral company developed the cocktail XXS-2A to market it if  
827 efficient.

828

829 **Figure 1: Design of the study**

830

831 **Figure 2: Plasma TG, insulin and glucose concentrations during the OGTT**

832 Evolution of plasma TG (A), insulin (B) and glucose (C) concentrations during an oral  
833 glucose tolerance test in the Control (left) (n=10) and Supplemented (right) (n=10) groups  
834 before the intervention, after 10 days of reduced physical activity and after 10 more days of  
835 physical inactivity coupled with fructose/glucose overnutrition. Values are means  $\pm$  SEM.

836

837 **Figure 3: Carbohydrate metabolism**

838 Area under the curve calculated over each OGTT for plasmatic insulin (A), glucose  
839 concentration (B), Matsuda index (C) and U<sup>13</sup>C-Fructose oxidation (E) over 20 days of  
840 reduced physical activity in Control (n=10) and Supplemented (n=10) groups. Area under the  
841 curve calculated over each OGTT for Glucose oxidation (D) and metabolic flexibility (F)  
842 over 20 days of reduced physical activity in Control (n=10) and Supplemented (n=8) groups.  
843 Reported data have been adjusted for baseline. Oxidation data are adjusted on fat mass (FM)  
844 and fat-free mass (FFM).

845 Results of the linear mixed model analysis are displayed on the figure with p-value for  
846 intervention (Interv.), cocktail supplementation (Suppl.) and the supplementation-by-  
847 intervention interaction (Interv. x Suppl.). Between-group differences were assessed by using  
848 post-hoc tests at each time point and are represented as follow \* p<0.05, \*\* p<0.01, \*\*\*  
849 p<0.001. Values are means  $\pm$  SEM.

850

851 **Figure 4: Lipid metabolism and free radicals defense**

852 Area under the curve calculated over each OGTT for plasmatic triglycerides (A), VLDL-TG  
853 (B), palmitate VLDL-TG (C), 1-<sup>13</sup>C Palmitate VLDL-TG (D), and KRL (F) over 20 days of  
854 reduced physical activity and fructose overfeeding in Control (n=10) and Supplemented  
855 (n=10) groups. Area under the curve calculated over each OGTT for lipid oxidation (E) over

856 20 days of reduced physical activity and fructose overfeeding in Control (n=10) and  
857 Supplemented (n=8) groups. Reported data have been adjusted for baseline. Oxidation data  
858 are adjusted on fat mass (FM) and fat-free mass (FFM).  
859 Results of the linear mixed model analysis are displayed on the figure with p-value for  
860 intervention (Interv.), cocktail supplementation (Suppl.) and the supplementation-by-  
861 intervention interaction (Interv. x Suppl.). Between-group differences were assessed by using  
862 post-hoc tests at each time point and are represented as follow \* p<0.05, \*\* p<0.01, \*\*\*  
863 p<0.001. #p<0.05, ##p<0.01, ###p<0.001 vs baseline. Values are means ± SEM.  
864

865 **Table 1: Anthropometry and physical activity data**

866 Means are  $\pm$  SEM. <sup>1</sup>Mixed models are adjusted on baseline.

867

868 **Table 2: Fasting metabolites and hormones**

869 Means are  $\pm$  SEM. <sup>1</sup>Mixed models are adjusted on baseline.

870 Post-hoc tests: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs control. # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$

871 vs baseline.

872 HOMA index: Homeostasis model assessment of insulin resistance; TG: Triglycerides; HDL:

873 High Density Lipoprotein; LDL: Low Density Lipoprotein; HMW: High Molecular Weight

874

875 **Table 3: Fasting oxidative stress and damages**

876 Means are  $\pm$  SEM. <sup>1</sup>Mixed models are adjusted on baseline.

877 Post-hoc tests: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs control.

878 KRL: Total antiradical defense potential (in French, Kit Radicaux Libres); Reseda (in French,

879 REServes Défenses Antioxydantes) 1, 2 and 3: antiradical defense reserves unmasked by

880 treatment of serum with respectively glucosides, sulphates or glucuronides. GSH: Reduced

881 Glutathione; GSSG: Oxidized Glutathione.

882

883 **Table 4: Fasting energy & substrate oxidation**

884 <sup>1</sup>Mixed models are adjusted on baseline, fat mass and fat-free mass. Data are presented as

885 LSMeans  $\pm$  SEM.

886 Post-hoc tests: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs control.

887 RMR: Resting metabolic rate; NPRQ: Non-protein respiratory quotient

888 **Table 5: Vastus lateralis western blots and enzyme activity**

889 Means are  $\pm$  SEM. <sup>1</sup>Mixed models are adjusted on baseline.

890 Post-hoc tests: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs control. # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$   
891 vs baseline.

892 Muscle fiber type : CSA: Cross Sectional Area; MHC: Myosin Heavy Chain; Western blots:

893 pAKT: phosphorylated Protein Kinase B; pGSK3: phosphorylated Glycogen Synthase Kinase

894 3; MURF-1: Muscle-Ring-finger protein 1; HSP90: Heat Shock Protein 90; NRF2: Nuclear

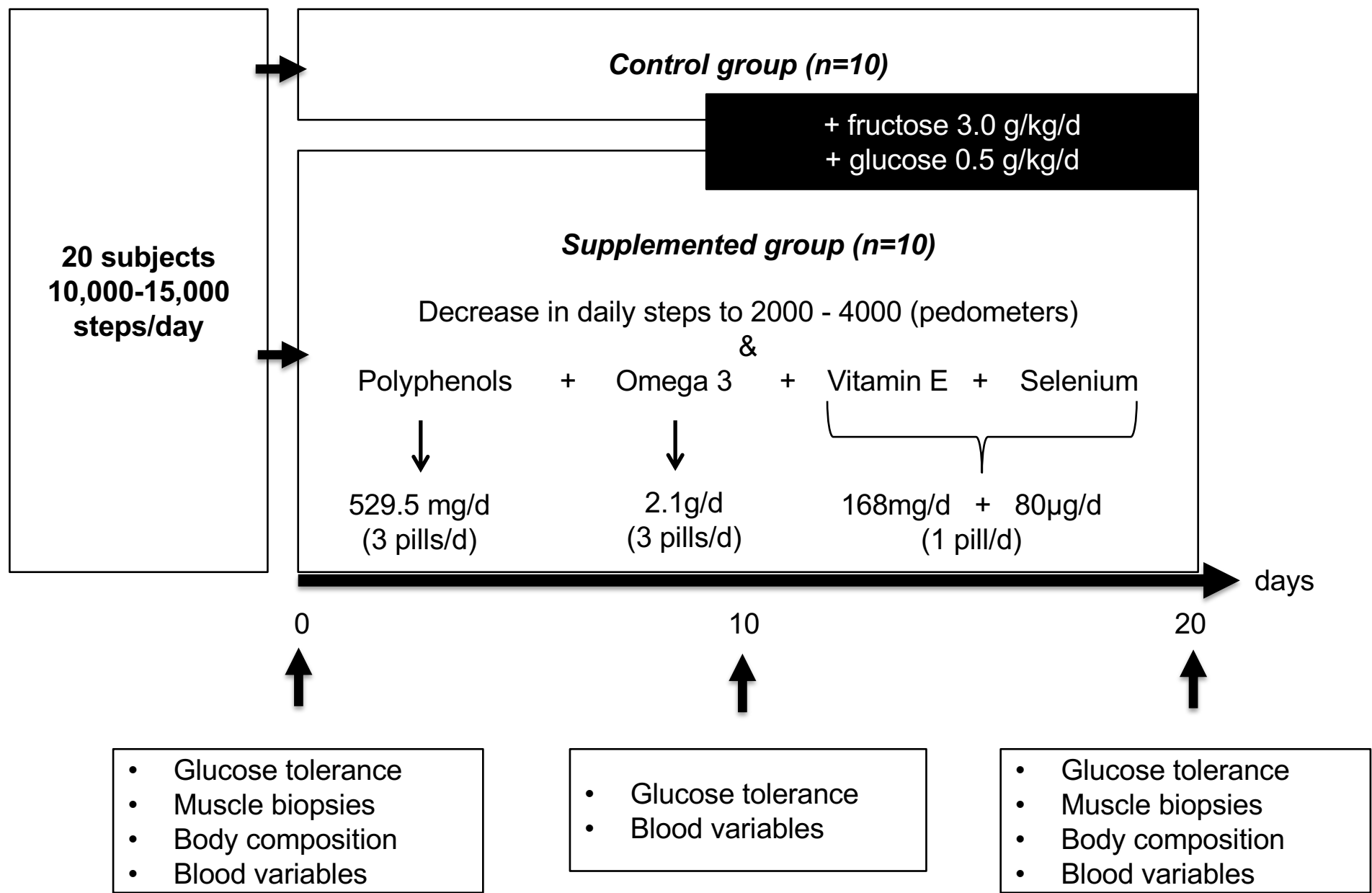
895 Factor erythroid-derived 2 like 2; CBR1: Carbonyl Reductase 1; AKR7A2: Aldo-Keto

896 Reductase Family 7 Member A2; TNF $\alpha$ : Tumor Necrosis Factor  $\alpha$ ; PGC1 $\alpha$ : Peroxisome

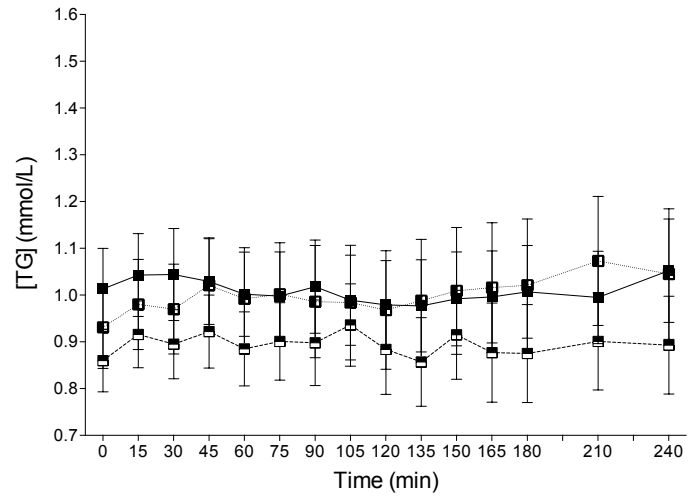
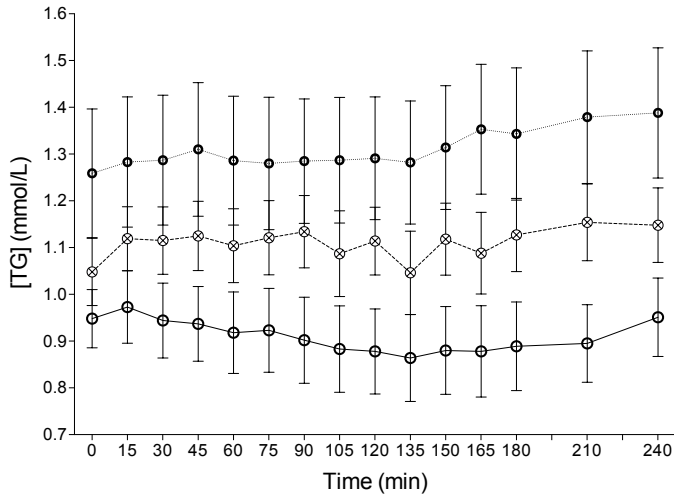
897 proliferator-activated receptor gamma coactivator 1 $\alpha$ ; FATP1: Fatty Acid Transport Protein

898 1. Enzyme activity: citrate synthase.

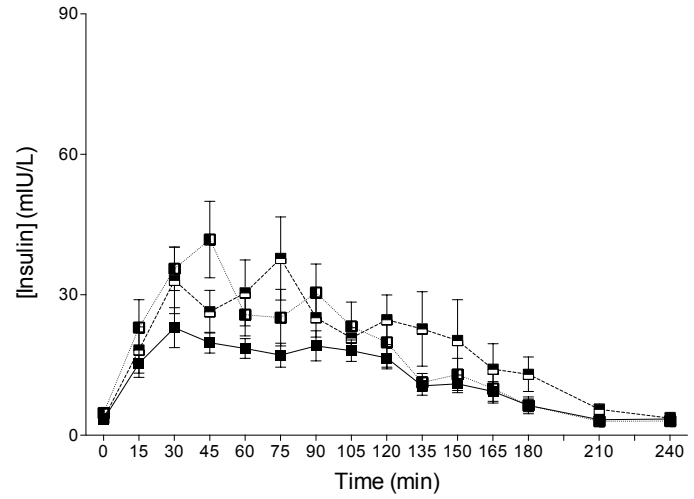
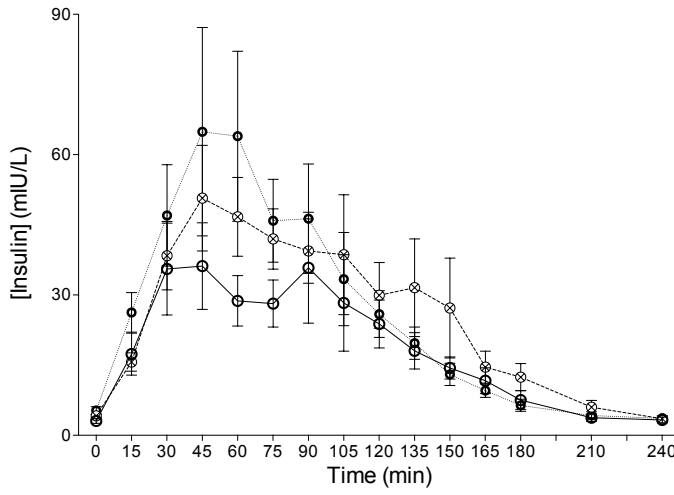




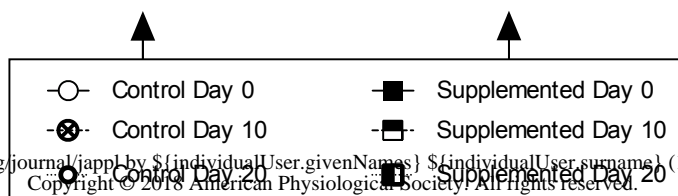
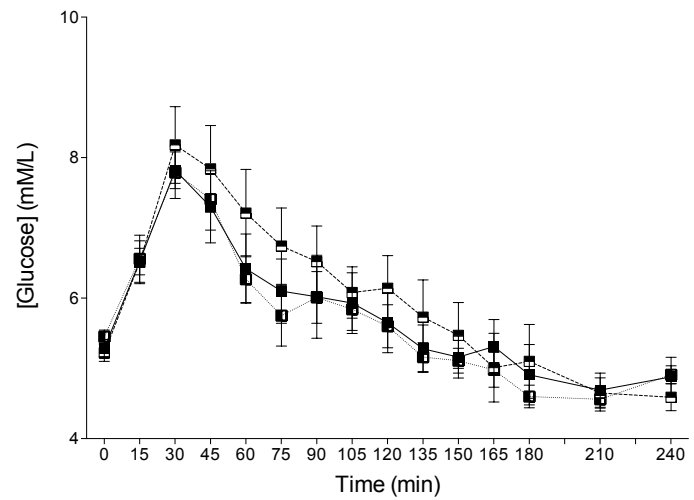
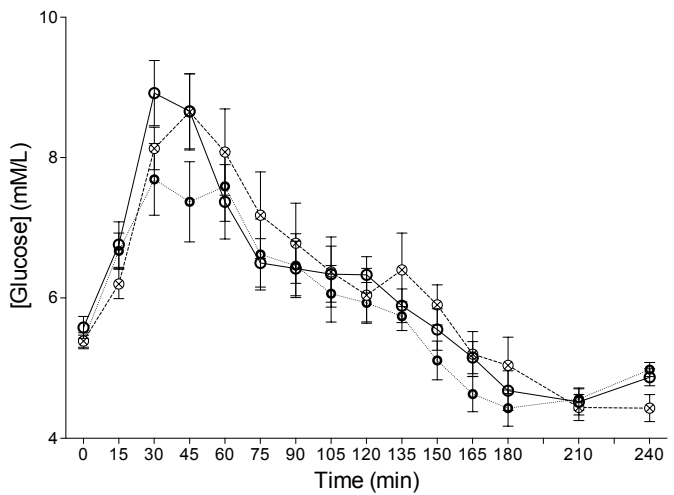
### A. TG



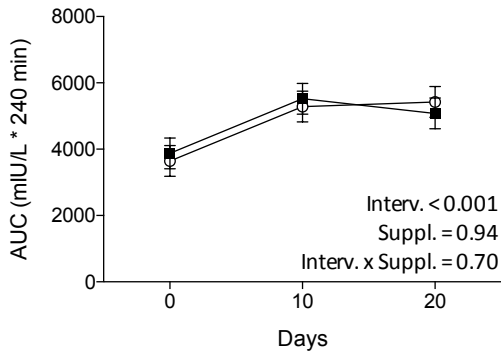
### B. Insulin



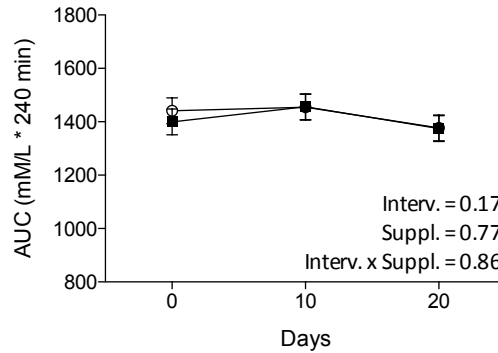
### C. Glucose



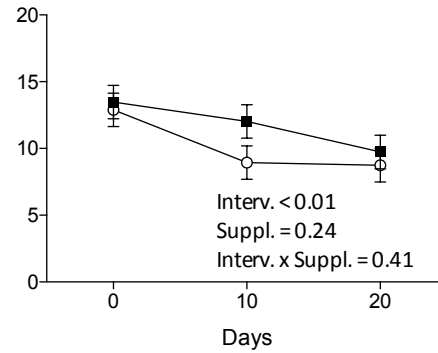
### A. Insulin



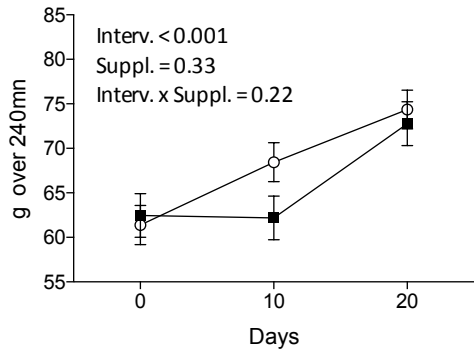
### B. Glucose



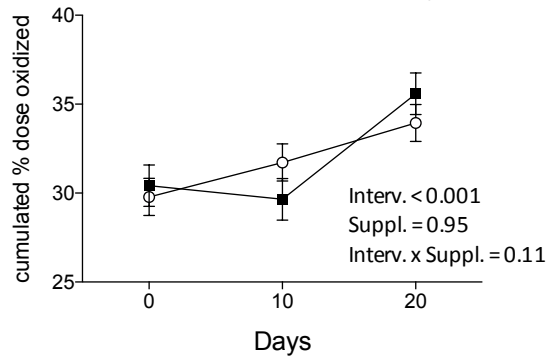
### C. Matsuda Index



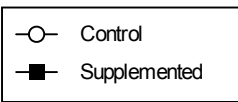
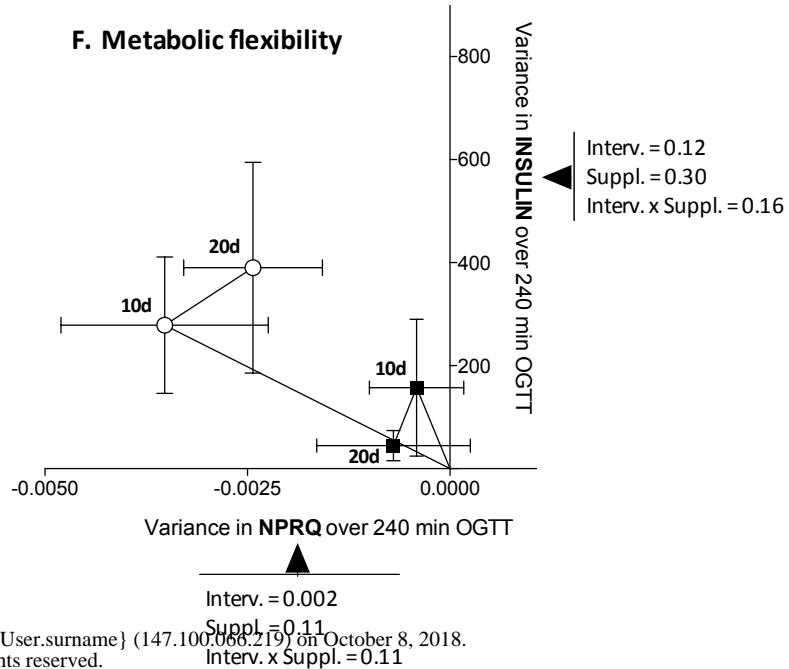
### D. Glucose oxidation<sub>LBM/FM</sub>



### E. U<sup>13</sup>C-Fructose oxidation<sub>LBM/FM</sub>



### F. Metabolic flexibility



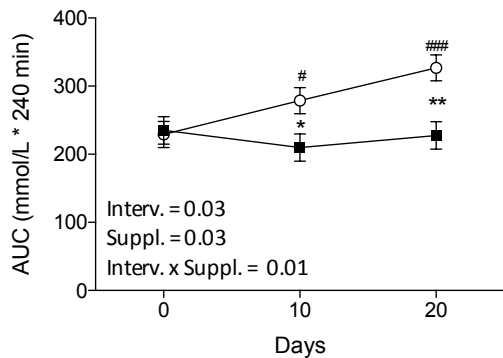
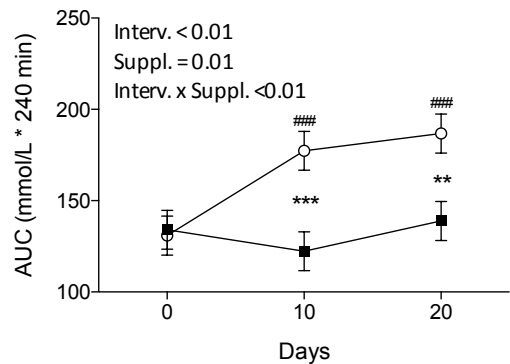
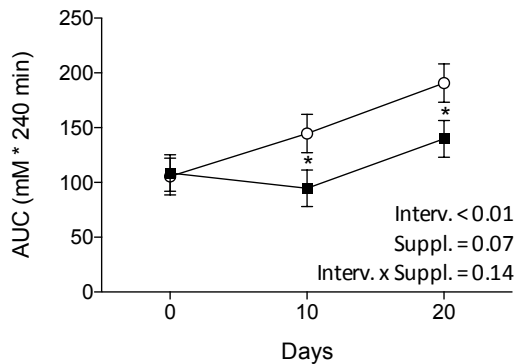
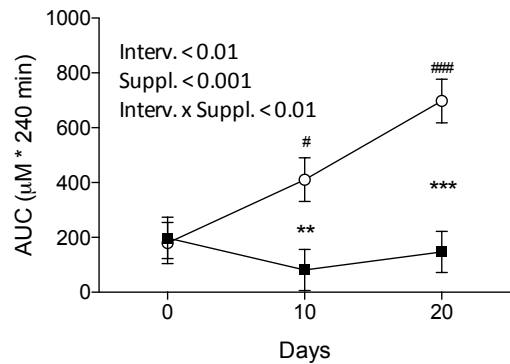
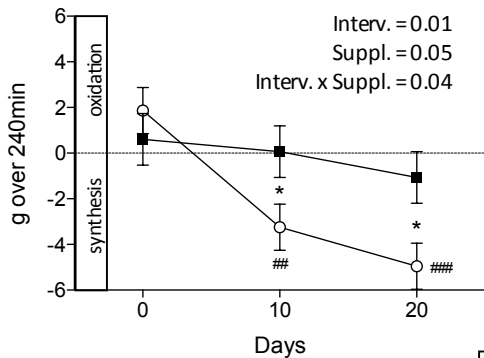
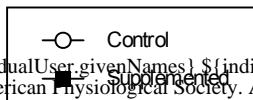
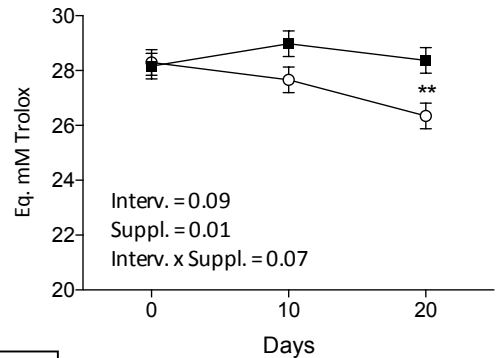
**A. TG****B. VLDL-TG****C. Palmitate VLDL-TG****D. 1-<sup>13</sup>C Palmitate VLDL-TG****E. Lipid oxidation<sub>LBM/FM</sub>****F. KRL post OGTT**

Table 1. Anthropometry and physical activity data

Variables	Baseline		day 10		day 20		Mixed model <sup>1</sup>		
	Control	Supplemented	Control	Supplemented	Control	Supplemented	Supplementation	Intervention	Interaction
	n=10	n=10	n=10	n=10	n=10	n=10			
Weight (kg)	77.5 ± 2.3	76.5 ± 2.2	77.6 ± 2.2	76.7 ± 2.3	77.8 ± 2.1	76.9 ± 2.4	0.89	0.28	0.87
Fat mass (g)	16.9 ± 1.4	17.8 ± 1.8	17.2 ± 1.4	18.1 ± 1.7	17.4 ± 1.3	18.3 ± 1.7	0.44	<0.001	0.75
Fat mass (%)	22.0 ± 1.4	23.4 ± 2.0	22.3 ± 1.4	23.8 ± 2.0	22.6 ± 1.3	24.1 ± 2.0	0.58	<0.001	0.89
Lean Body mass (kg)	56.7 ± 1.5	54.7 ± 1.7	56.5 ± 1.4	54.5 ± 1.8	56.4 ± 1.4	54.4 ± 1.9	0.87	0.32	0.92
Daily steps	13032.6 ± 875.1	14951.7 ± 1720.1	2511.8 ± 235.3	2663.8 ± 359.7	2644.9 ± 330.7	3008.9 ± 297.8	0.69	<0.001	0.40
Time spent sitting during waking time (min.day <sup>-1</sup> )	445.9 ± 37.7	461.7 ± 29.4	477.7 ± 43.3	528.2 ± 39.7	552.0 ± 42.2	527.6 ± 41.6	0.98	<0.01	0.22
Time spent in light-intensity activity (min.day <sup>-1</sup> )	213.8 ± 22.5	206.0 ± 21.9	114.5 ± 20.2	119.2 ± 23.4	125.8 ± 26.7	130.9 ± 23.8	0.58	<0.05	0.74
Time spent in moderate to vigorous intensity activity (min.day <sup>-1</sup> )	104.5 ± 10.5	134.3 ± 17.7	18.2 ± 4.8	21.7 ± 7.7	17.6 ± 3.2	29.1 ± 9.2	0.67	<0.001	0.69

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Table 2. Fasting metabolites and hormones

Variables	Baseline		day 10		day 20		<sup>1</sup> Mixed model		
	Control n=10	Supplemented n=10	Control n=10	Supplemented n=10	Control n=10	Supplemented n=10	Supplementation	Intervention	Interaction
<b>Metabolites</b>									
Glucose (mM)	5.58 ± 0.16	5.29 ± 0.12	5.39 ± 0.09	5.22 ± 0.12	5.39 ± 0.11	5.45 ± 0.10	0.83	0.28	0.18
HOMA index	0.76 ± 0.13	0.80 ± 0.13	1.00 ± 0.07	0.84 ± 0.12	1.30 ± 0.21	1.16 ± 0.21	0.15	<0.001	0.54
TG (mmol/L)	0.95 ± 0.06	1.01 ± 0.09	1.05 ± 0.07	0.86 ± 0.07 <sup>#</sup>	1.26 ± 0.14 <sup>##</sup>	0.93 ± 0.09 <sup>***</sup>	0.01	0.04	0.01
HDL (mmol/L)	1.31 ± 0.04	1.23 ± 0.06	1.18 ± 0.04	1.29 ± 0.08 <sup>***</sup>	1.20 ± 0.06	1.30 ± 0.07 <sup>***</sup>	<.0001	0.25	<.0001
LDL (mmol/L)	2.90 ± 0.22	2.69 ± 0.19	2.88 ± 0.21	2.94 ± 0.18	3.08 ± 0.26	3.05 ± 0.25	0.18	<0.01	0.18
<b>Hormones</b>									
Insulin (mU/L)	3.05 ± 0.49	3.40 ± 0.52	4.16 ± 0.29	3.57 ± 0.49	5.33 ± 0.77	4.74 ± 0.85	0.07	<0.001	0.35
Total adiponectin (µg/mL)	5.19 ± 0.68	6.99 ± 1.54	5.36 ± 0.94	7.27 ± 1.04	6.73 ± 1.05	9.22 ± 1.33	0.29	<0.001	0.75
HMW adiponectin (µg/mL)	2.93 ± 0.43	4.18 ± 0.74	3.08 ± 0.41	4.60 ± 0.66	3.99 ± 0.64 <sup>##</sup>	6.27 ± 0.85 <sup>####</sup>	0.15	<.0001	0.05

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Table 3. Fasting oxidative stress

Variables	Baseline		day 10		day 20		<sup>1</sup> Mixed model		
	Control	Supplemented	Control	Supplemented	Control	Supplemented	Supplementation	Intervention	Interaction
	n=10	n=10	n=10	n=10	n=10	n=10			
Malondialdehyde (µg/mL)	0.78 ± 0.05	0.70 ± 0.07	0.67 ± 0.05	0.81 ± 0.05	0.69 ± 0.08	0.74 ± 0.05	0.27	0.79	0.22
KRL (mEq Trolox)	24.16 ± 1.16	23.38 ± 0.89	23.09 ± 0.84	23.70 ± 0.81	22.72 ± 1.00	22.26 ± 0.64	0.54	0.13	0.48
Reseda 1 (Glucosides, mEq. Trolox)	3.34 ± 0.18	3.52 ± 0.19	3.75 ± 0.23	4.02 ± 0.22	3.54 ± 0.21	3.32 ± 0.16	0.81	0.10	0.56
Reseda 2 (Sulphates, mEq. Trolox)	2.65 ± 0.16	2.65 ± 0.19	2.83 ± 1.13	3.10 ± 0.22	2.69 ± 0.12	2.78 ± 0.15	0.78	0.43	0.77
Reseda 3 (Glucuronides, mEq. Trolox)	4.95 ± 0.20	5.26 ± 0.25	5.95 ± 0.28	5.64 ± 0.30	5.00 ± 0.19	4.99 ± 0.21	0.13	<0.01	0.11
GSH	143.0 ± 19.7	227.5 ± 51.2	146.2 ± 15.0	229.9 ± 21.6	227.7 ± 71.9	292.2 ± 74.2	0.54	0.17	0.99
GSSG	334.9 ± 22.3	313.5 ± 28.9	329.0 ± 10.85	339.4 ± 25.6	331.4 ± 14.5	343.1 ± 26.9	0.21	0.79	0.27
GSH/GSSG ratio	0.42 ± 0.05	0.86 ± 0.28	0.45 ± 0.05	0.72 ± 0.10	0.64 ± 0.16	0.85 ± 0.18	0.52	0.27	0.77

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Table 5. Vastus lateralis western blots and enzyme activity

Variables	Baseline		day 20		Mixed model		
	Control n=8	Supplemented n=10	Control n=10	Supplemented n=9	Supplementation	Intervention	Interaction
<b>Muscle fiber type</b>							
CSA MHC1 ( $\mu\text{m}^2$ )	5330.6 $\pm$ 399.0	4968.8 $\pm$ 314.3	3951.5 $\pm$ 267.6	4453.4 $\pm$ 349.2	0.27	<0.01	0.18
CSA MHC2A ( $\mu\text{m}^2$ )	5477.8 $\pm$ 478.3	5184.0 $\pm$ 329.4	4303.4 $\pm$ 230.3 <sup>###</sup>	4990.3 $\pm$ 261.7 <sup>***</sup>	0.001	<0.01	<0.01
CSA MHC hybride ( $\mu\text{m}^2$ )	0.0 $\pm$ 0.0	582.3 $\pm$ 582.3	2763.8 $\pm$ 759.8	2514.2 $\pm$ 756.6	0.35	<0.001	0.25
MHC1 (%)	67.8 $\pm$ 5.8	62.3 $\pm$ 4.2	52.7 $\pm$ 3.4	46.7 $\pm$ 4.2	0.38	0.001	0.85
MHC2A (%)	32.2 $\pm$ 5.8	37.7 $\pm$ 4.2	46.0 $\pm$ 2.9	51.4 $\pm$ 4.1	0.37	<0.01	0.89
Hybrids (%)	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	1.3 $\pm$ 0.7	1.9 $\pm$ 1.0	0.79	0.02	0.79
<b>Insulin/glucose metabolism</b>							
pAKT serine 473/AKTtotal	15.00 $\pm$ 8.04	15.03 $\pm$ 4.56	8.77 $\pm$ 2.69	17.71 $\pm$ 5.56	0.08	0.61	0.21
pGSK3 $\alpha^3$	2.91 $\pm$ 1.28	2.36 $\pm$ 0.92	1.74 $\pm$ 0.50	2.88 $\pm$ 1.13	0.14	0.61	0.19
pGSK3 $\beta$	2.00 $\pm$ 1.09	1.44 $\pm$ 0.79	1.19 $\pm$ 0.48	1.13 $\pm$ 0.45	0.57	0.22	0.58
<b>Protein synthesis/degradation</b>							
atrogin-1	0.17 $\pm$ 0.08	0.20 $\pm$ 0.09	0.25 $\pm$ 0.10	0.25 $\pm$ 0.12	0.55	<0.05	0.62
MURF-1	0.017 $\pm$ 0.004	0.016 $\pm$ 0.004	0.016 $\pm$ 0.003	0.015 $\pm$ 0.002	0.94	0.71	0.87
Ubiquitination	0.64 $\pm$ 0.06	0.36 $\pm$ 0.04	0.83 $\pm$ 0.08	0.36 $\pm$ 0.05 <sup>**</sup>	0.01	0.1	0.09
HSP90	4.59 $\pm$ 0.26	4.26 $\pm$ 0.41	4.26 $\pm$ 0.34	4.41 $\pm$ 0.70	0.35	0.77	0.40
<b>Oxidative stress</b>							
NRF2	6.52 $\pm$ 1.55	4.39 $\pm$ 0.62	4.99 $\pm$ 0.76	5.28 $\pm$ 0.63	0.68	0.50	0.45
Catalase	2.00 $\pm$ 0.84	1.20 $\pm$ 0.41	1.14 $\pm$ 0.28	1.43 $\pm$ 0.38	0.55	0.68	0.59
CBR1	4.13 $\pm$ 0.60	4.61 $\pm$ 0.78	4.55 $\pm$ 0.50	4.30 $\pm$ 0.79	0.52	0.97	0.41
AKR7A2	8.41 $\pm$ 0.59	8.51 $\pm$ 0.79	8.92 $\pm$ 0.63	8.17 $\pm$ 0.83	0.33	0.86	0.33
TNF $\alpha$	3.55 $\pm$ 0.59	3.39 $\pm$ 0.61	2.97 $\pm$ 0.50	3.60 $\pm$ 0.76	0.21	0.52	0.19
<b>Mitochondria markers</b>							
mitofusine	5.98 $\pm$ 0.77	6.14 $\pm$ 1.51	4.18 $\pm$ 0.96	4.36 $\pm$ 1.15	0.95	<0.01	0.99
PGC-1 $\alpha$	0.37 $\pm$ 0.04	0.25 $\pm$ 0.02	0.38 $\pm$ 0.09	0.20 $\pm$ 0.03	0.21	0.65	0.64
Citrate synthase activity ( $\mu\text{mol}/\text{min}/\text{mg}$ protein)	0.17 $\pm$ 0.03	0.14 $\pm$ 0.02	0.14 $\pm$ 0.03	0.15 $\pm$ 0.03	0.44	0.81	0.28
FATP1	3.50 $\pm$ 0.33	2.71 $\pm$ 0.39	3.34 $\pm$ 0.34	3.40 $\pm$ 0.70	0.07	0.45	0.07

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