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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

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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-¹³C) 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 α : 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 (ω -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 ω -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, ω -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 ω -3 & green tea extracts (49), ω -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, ω -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.

211 **METHODS**

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+TM, 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).

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 ω -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 μ 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 μ g and
263 400 μ g per day. The selected dose provided a daily supplement that set the daily intake at 135 μ 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-¹³C-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 4th 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}C in expired
308 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 Na₃VO₄, 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

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 ω -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 ω -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 ω -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 ω -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 ω -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 ω -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 ω -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 ω -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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- 814

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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 U¹³C-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), 1-¹³C 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. ¹Mixed models are adjusted on baseline.

867

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

869 Means are \pm SEM. ¹Mixed 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. ¹Mixed 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 ¹Mixed 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. ¹Mixed 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

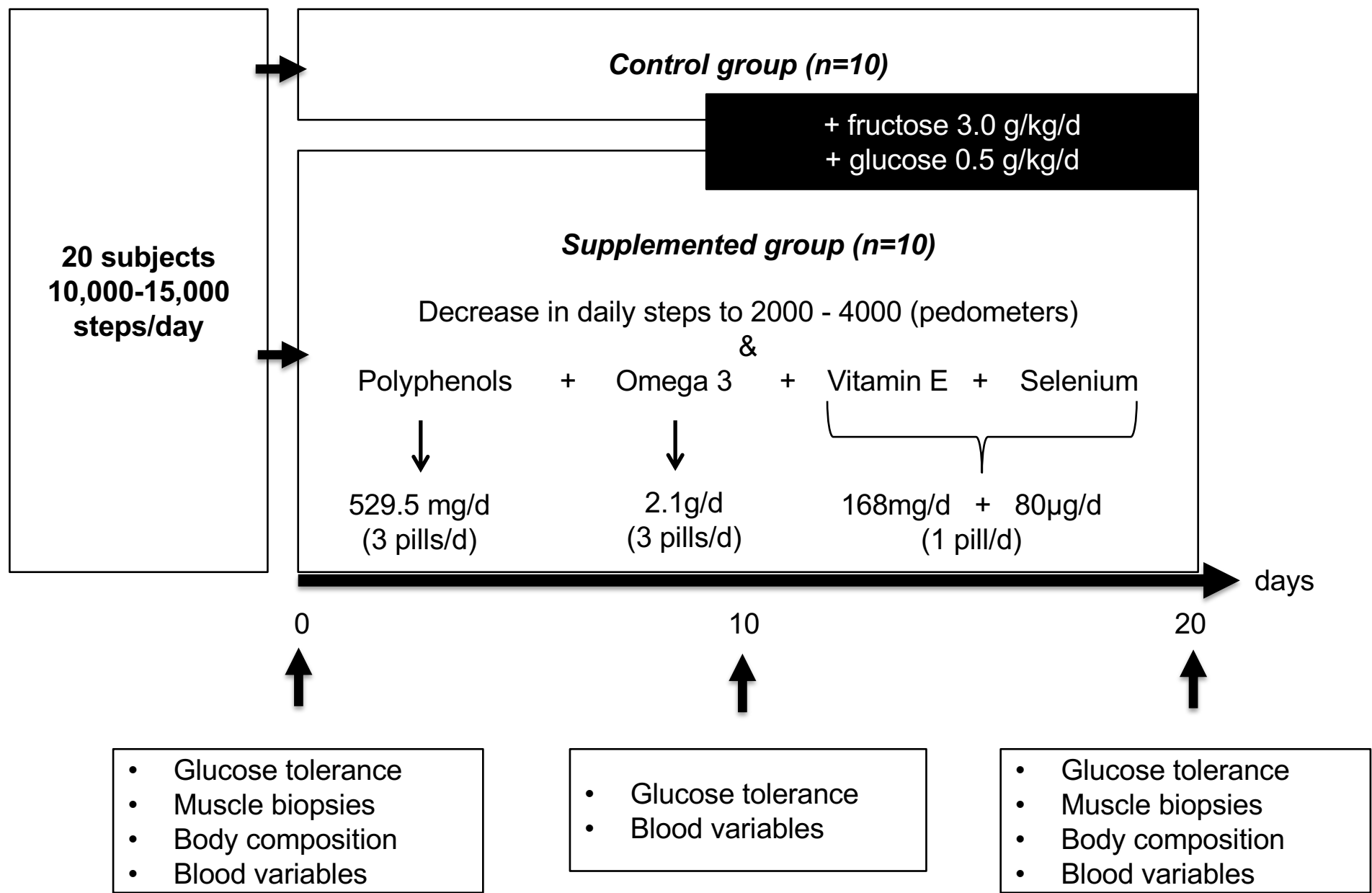
894 3; MURF-1: Muscle-Ring-finger protein 1; HSP90: Heat Shock Protein 90; NRF2: Nuclear

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

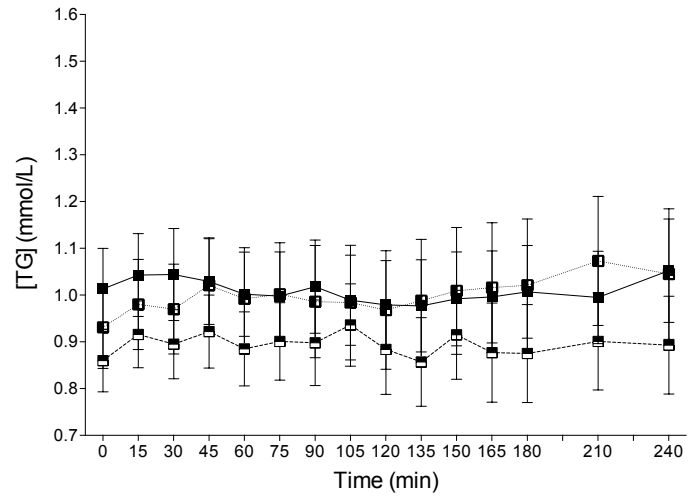
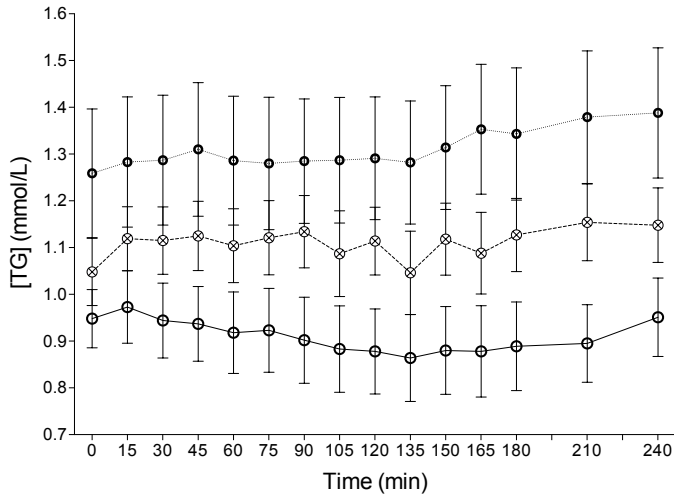
896 Reductase Family 7 Member A2; TNF α : Tumor Necrosis Factor α ; PGC1 α : Peroxisome

897 proliferator-activated receptor gamma coactivator 1 α ; FATP1: Fatty Acid Transport Protein

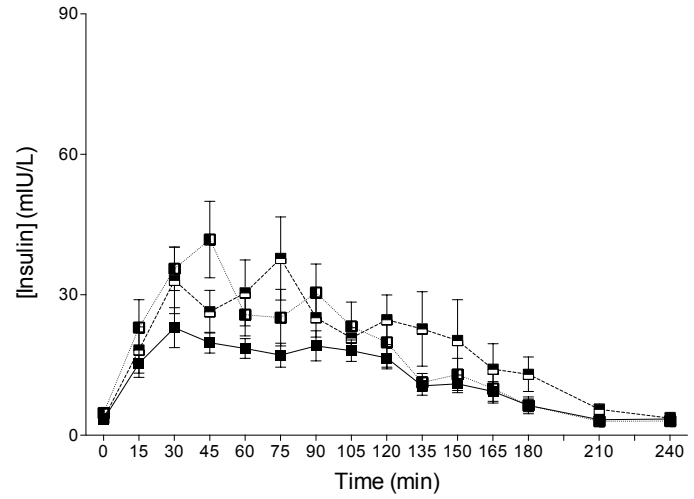
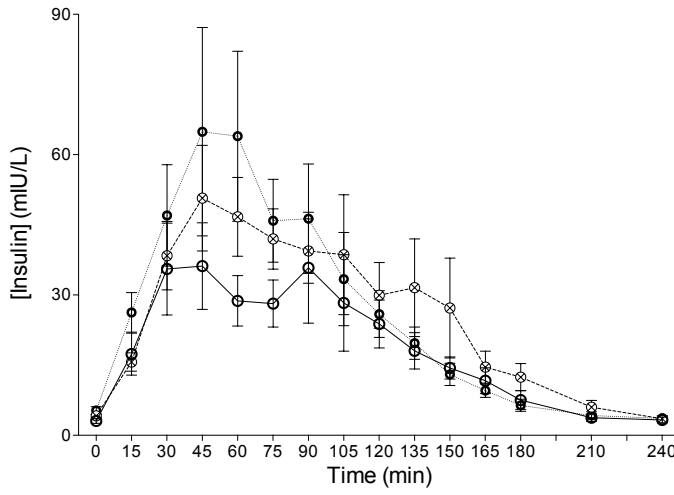
898 1. Enzyme activity: citrate synthase.



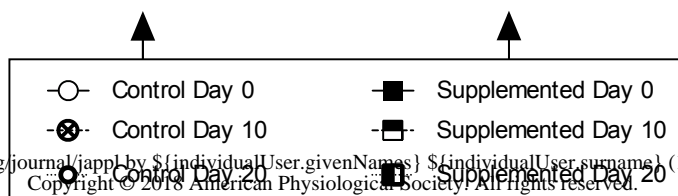
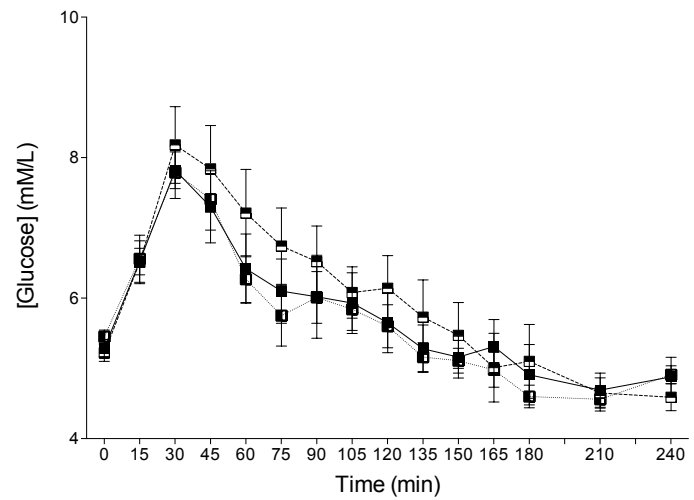
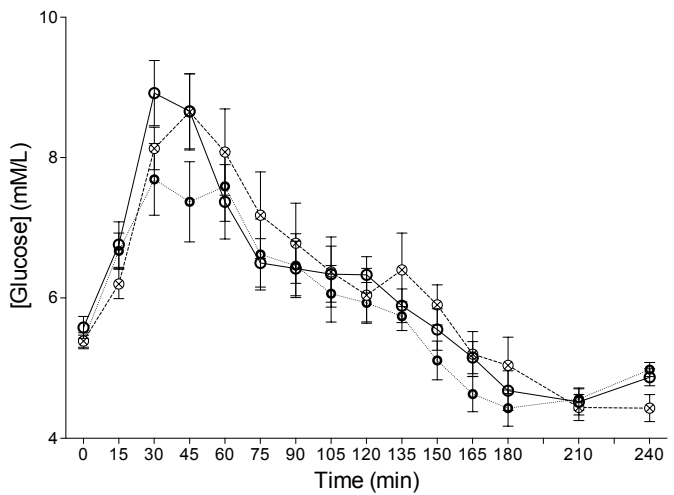
A. TG



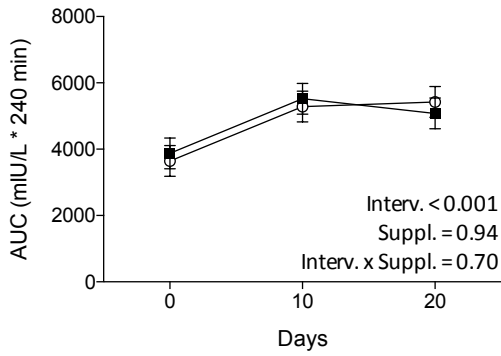
B. Insulin



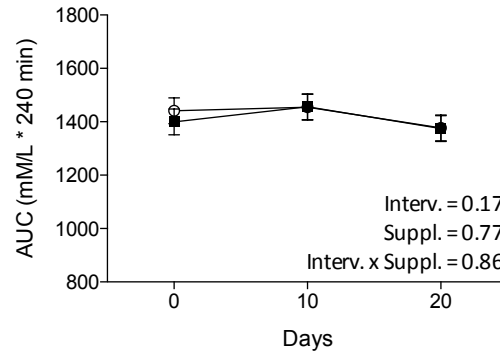
C. Glucose



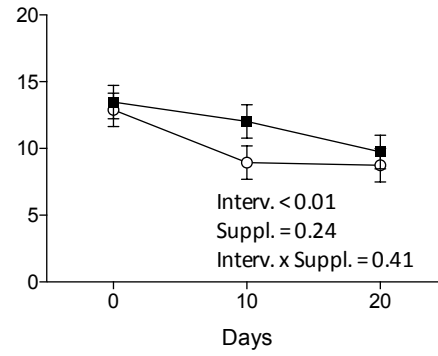
A. Insulin



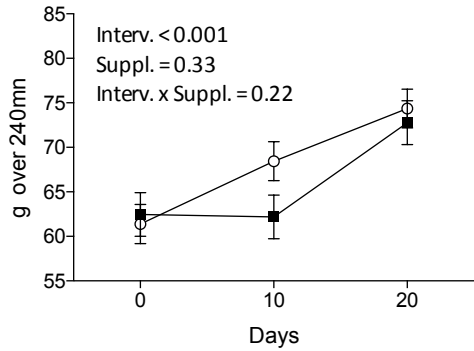
B. Glucose



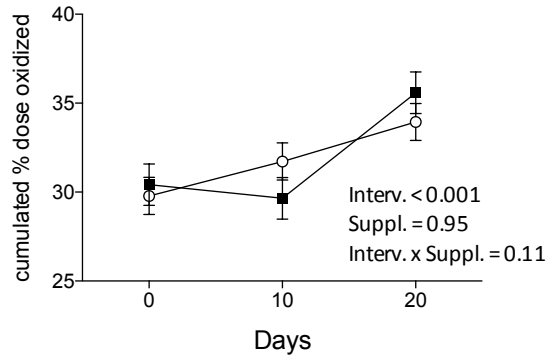
C. Matsuda Index



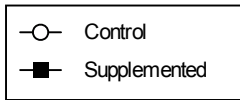
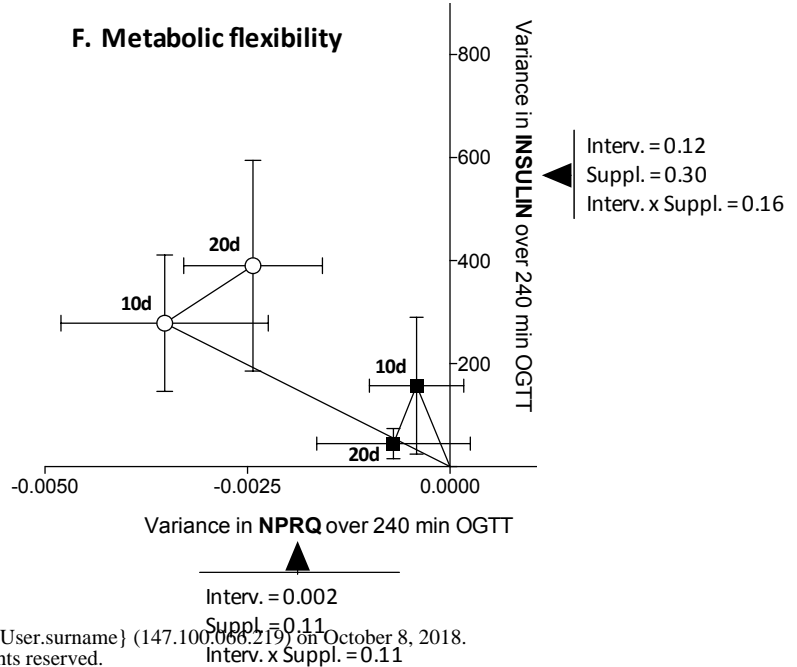
D. Glucose oxidation_{LBM/FM}



E. U¹³C-Fructose oxidation_{LBM/FM}



F. Metabolic flexibility



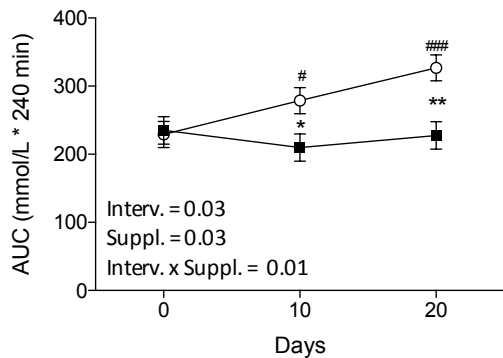
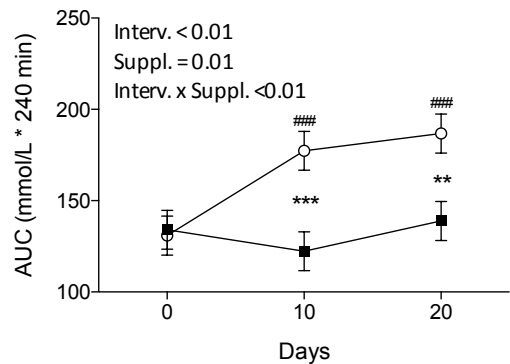
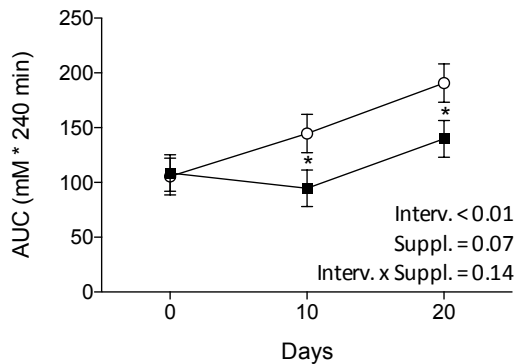
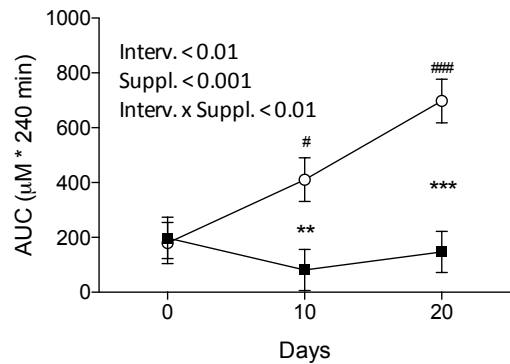
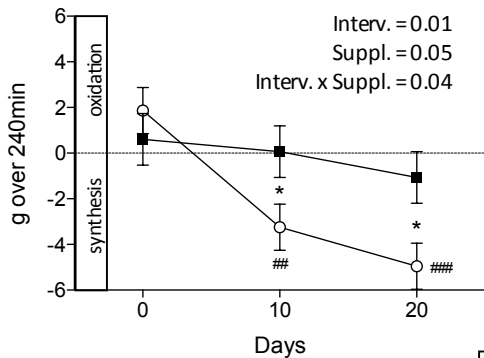
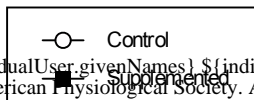
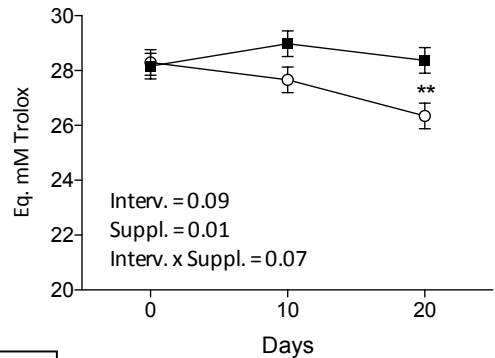
A. TG**B. VLDL-TG****C. Palmitate VLDL-TG****D. 1-¹³C Palmitate VLDL-TG****E. Lipid oxidation_{LBM/FM}****F. KRL post OGTT**

Table 1. Anthropometry and physical activity data

Variables	Baseline		day 10		day 20		Mixed model ¹		
	Control n=10	Supplemented n=10	Control n=10	Supplemented n=10	Control n=10	Supplemented n=10	Supplementation	Intervention	Interaction
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 ⁻¹)	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 ⁻¹)	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 ⁻¹)	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		¹ Mixed model		
	Control n=10	Supplemented n=10	Control n=10	Supplemented n=10	Control n=10	Supplemented n=10	Supplementation	Intervention	Interaction
Metabolites									
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 ^{##}	1.26 ± 0.14 ^{##}	0.93 ± 0.09 ^{***}	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 ^{***}	1.20 ± 0.06	1.30 ± 0.07 ^{***}	<.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
Hormones									
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 ^{##}	6.27 ± 0.85 ^{####}	0.15	<.0001	0.05

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Beraouiqnan, A., O'Gorman, D. (2019). A nutrient cocktail prevents lipid metabolism alterations

Table 3. Fasting oxidative stress

Variables	Baseline		day 10		day 20		¹ 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
Muscle fiber type							
CSA MHC1 (μm^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 (μm^2)	5477.8 \pm 478.3	5184.0 \pm 329.4	4303.4 \pm 230.3 ^{###}	4990.3 \pm 261.7 ^{***}	0.001	<0.01	<0.01
CSA MHC hybride (μm^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
Insulin/glucose metabolism							
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 α^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 β	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
Protein synthesis/degradation							
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 ^{**}	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
Oxidative stress							
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 α	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
Mitochondria markers							
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 α	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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